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(54)【発明の名称】プロキネチシンポリペプチド、関連組成物および方法

(57)【要約】
本発明は、胃腸管の平滑筋収縮を刺激する単離されたポリペプチドを提供し、これには、ヒトプロキネチシン１ポリペプチドおよびヒトプロキネチシン２ポリペプチドならびにそれらの機能的フラグメントおよび改変物が挙げられる。また、有効量のプロキネチシンポリペプチドを哺乳動物に投与することによって、哺乳動物において胃腸管の平滑筋収縮を刺激する方法が提供される。本発明はまた、プロキネチシンポリペプチドをコードする核酸分子、およびプロキネチシンポリペプチドに選択的に結合する抗体を提供する。さらに、プロキネチシンレセプターのリガンド、アゴニストまたはアンタゴニストを同定する方法が提供される。

【特許請求の範囲】
【請求項１】
胃腸管の平滑筋収縮を刺激する単離されたポリペプチドであって、該ポリペプチドは、ヒトプロキネチシン１（配列番号３）の配列と少なくとも８０％同一なアミノ酸配列を含み、該配列は、配列番号３のＮ末端の６つのアミノ酸、配列番号３の１０つの保存されたシステイン残基および配列番号３のＣ末端の９つのアミノ酸うちの０～９つを含む、単離されたポリペプチド。
【請求項２】
配列番号３における残基とは異なるアミノ酸残基が、その保存的置換である、請求項１に記載の単離されたポリペプチド。
【請求項３】
配列番号３における残基とは異なるアミノ酸残基が、配列番号６由来の対応する残基から構成される、請求項１に記載の単離されたポリペプチド。
【請求項４】
配列番号１３を含む、請求項３に記載の単離されたポリペプチド。
【請求項５】
配列番号３のアミノ酸１～７７を含む、請求項１に記載の単離されたポリペプチド。
【請求項６】
配列番号３を含む、請求項１に記載の単離されたポリペプチド。
【請求項７】
６ＸＨｉｓタグを含む、請求項１に記載の単離されたポリペプチド。
【請求項８】
検出可能に標識される、請求項１に記載の単離されたポリペプチド。
【請求項９】
配列番号３の、少なくとも１０つ連続したアミノ酸を含む単離されたペプチドであって、該ペプチドが免疫原性である、単離されたペプチド。
【請求項１０】
請求項１に記載の単離されたポリペプチドおよび薬学的に受容可能なキャリアを含む、薬学的組成物。
【請求項１１】
哺乳動物において、胃腸管の平滑筋収縮を刺激する方法であって、該方法は、有効量の請求項１に記載のポリペプチドを、該哺乳動物に投与する工程を包含する、方法。
【請求項１２】
請求項１に記載のポリペプチドをコードする、核酸分子。
【請求項１３】
遺伝子発現のプロモーターに作動的に連結された請求項１２に記載の核酸分子を含む、発現ベクター。
【請求項１４】
請求項１３に記載の発現ベクターを含む、宿主細胞。
【請求項１５】
請求項１に記載の単離されたポリペプチドを調製する方法であって、該方法は、該ポリペプチドを発現させるために、請求項１４に記載の宿主細胞を培養する工程、該ポリペプチドを実質的に精製する工程、および該ポリペプチドを再折り畳みする工程を包含する、方法。
【請求項１６】
請求項１に記載のポリペプチドに選択的に結合する、抗体。
【請求項１７】
胃腸管の平滑筋収縮を刺激する単離されたポリペプチドであって、該ポリペプチドは、ヒトプロキネチシン２（配列番号６）の配列と少なくとも８０％同一なアミノ酸配列を含み、該配列は、配列番号６のＮ末端の６つのアミノ酸、配列番号６の１０つの保存されたシ

ステイン残基および配列番号６のＣ末端の４つのアミノ酸うちの０～４つを含む、単離されたポリペプチド。
【請求項１８】
配列番号６における残基とは異なるアミノ酸残基が、その保存的置換である、請求項１７に記載の単離されたポリペプチド。
【請求項１９】
配列番号６における残基とは異なるアミノ酸残基が、配列番号３由来の対応する残基から構成される、請求項１７に記載の単離されたポリペプチド。
【請求項２０】
配列番号１４を含む、請求項１９に記載の単離されたポリペプチド。
【請求項２１】
配列番号６のアミノ酸１～７７を含む、請求項１７に記載の単離されたポリペプチド。
【請求項２２】
配列番号６を含む、請求項１７に記載の単離されたポリペプチド。
【請求項２３】
６ＸＨｉｓタグを含む、請求項１７に記載の単離されたポリペプチド。
【請求項２４】
検出可能に標識される、請求項１７に記載の単離されたポリペプチド。
【請求項２５】
配列番号６の、少なくとも１０つの連続したアミノ酸を含む単離されたペプチドであって、該ペプチドが免疫原性である、単離されたペプチド。
【請求項２６】
請求項１７に記載の単離されたポリペプチドおよび薬学的に受容可能なキャリアを含む、薬学的組成物。
【請求項２７】
哺乳動物において、胃腸管の平滑筋収縮を刺激する方法であって、該方法は、有効量の請求項１７に記載のポリペプチドを、該哺乳動物に投与する工程を包含する、方法。
【請求項２８】
請求項１７に記載のポリペプチドをコードする、核酸分子。
【請求項２９】
遺伝子発現のプロモーターに作動的に連結された請求項１７に記載の核酸分子を含む、発現ベクター。
【請求項３０】
請求項２９に記載の発現ベクターを含む、宿主細胞。
【請求項３１】
請求項１７に記載の単離されたポリペプチドを調製する方法であって、該方法は、該ポリペプチドを発現させるために、請求項３０に記載の宿主細胞を培養する工程、該ポリペプチドを実質的に精製する工程、および該ポリペプチドを再折り畳みする工程を包含する、方法。
【請求項３２】
請求項１７に記載のポリペプチドに選択的に結合する、抗体。
【請求項３３】
プロキネチシンレセプターのリガンドを同定する方法であって、該方法は、プロキネチシンレセプターを含む調製物と、１つ以上の候補化合物とを接触させる工程、および該レセプターに特異的に結合する化合物を同定する工程を包含し、該化合物がプロキネチシンレセプターのリガンドとして特徴付けられる、方法。
【請求項３４】
前記調整物が、腸の平滑筋調製物またはその膜調製物である、請求項３３に記載の方法。
【請求項３５】
前記調製物が細胞株またはその膜調製物である、請求項３３に記載の方法。

【請求項３６】
前記細胞株がＭ２Ａ７（ＡＴＣＣ　ＣＲＬ－２５００）である、請求項３５に記載の方法。
【請求項３７】
プロキネチシンレセプターシグナル伝達を選択的にアゴナイズするか、またはアンタゴナイズする前記リガンドの能力がさらに決定される、請求項３３に記載の方法。
【請求項３８】
前記シグナル伝達が細胞株において決定される、請求項３７に記載の方法。
【請求項３９】
前記細胞株がＭ２Ａ７（ＡＴＣＣ　ＣＲＬ－２５００）である、請求項３８に記載の方法。
【請求項４０】
前記シグナル伝達が、カルシウム動員をモニタリングすることによって決定される、請求項３７に記載の方法。
【請求項４１】
平滑筋収縮性を調節する前記リガンドの能力がさらに決定される、請求項３３に記載の方法。
【請求項４２】
プロキネチシンレセプターのアゴニストを同定する方法であって、該方法は、プロキネチシンレセプターを含む調製物と、１つ以上の候補化合物とを接触させる工程、およびプロキネチシンレセプターシグナルの産生を選択的に促進する化合物を同定する工程を包含し、該化合物がプロキネチシンレセプターのアゴニストとして特徴付けられる、方法。
【請求項４３】
前記調製物が細胞株である、請求項４２に記載の方法。
【請求項４４】
前記細胞株がＭ２Ａ７（ＡＴＣＣ　ＣＲＬ－２５００）である、請求項４３に記載の方法。
【請求項４５】
前記シグナル伝達が、カルシウム動員をモニタリングすることによって決定される、請求項４２に記載の方法。
【請求項４６】
平滑筋収縮性を調節するための前記アゴニストの能力がさらに決定される、請求項４２に記載の方法。
【請求項４７】
プロキネチシンレセプターのアンタゴニストを同定する方法であって、該方法は、プロキネチシンレセプターを含む調製物と、プロキネチシンの存在下で１つ以上の候補化合物とを接触させる工程、およびプロキネチシンレセプターシグナルの産生を選択的に阻害する化合物を同定する工程を包含し、該化合物がプロキネチシンレセプターのアンタゴニストとして特徴付けられる、方法。
【請求項４８】
前記プロキネチシンが、配列番号３のアミノ酸１～７７および配列番号６のアミノ酸１～７７からなる群より選択されるアミノ酸配列を含む、請求項４７に記載の方法。
【請求項４９】
前記調製物が細胞株である、請求項４７に記載の方法。
【請求項５０】
前記細胞株がＭ２Ａ７（ＡＴＣＣ　ＣＲＬ－２５００）である、請求項４９に記載の方法。
【請求項５１】
前記シグナル伝達が、カルシウム動員をモニタリングすることによって決定される、請求項４７に記載の方法。

【請求項５２】
平滑筋収縮性を調節する前記アンタゴニストの能力がさらに決定される、請求項４７に記載の方法。
【発明の詳細な説明】
【０００１】
（発明の背景）
胃腸（ＧＩ）平滑筋の主要な機能は、内腔内内容物を混合し、そして推進することで、この胃腸平滑筋は、効率的な食物の消化、栄養素の吸収の促進、および残留成分の最終的な除去を可能にする。ＧＩ平滑筋の活性は、内因性および外因性の神経シグナル（古典的な神経伝達物質、共存する神経ペプチド、および循環ペプチドホルモンを含む）によって、調節される。さらに、非神経性ＧＩ細胞によって生成される多数の体液因子（ヒスタミン、セロトニン、およびアデノシンを含む）もまた、平滑筋細胞の活性に影響を与える。
【０００２】
多くの臨床的状態が改変されたＧＩ運動に関連し、その状態としては以下が挙げられる：過敏性腸症候群、糖尿病性胃不全麻痺、術後の腸閉塞、慢性便秘、胃小腸反射疾患、慢性下痢、感染症、吸収不良障害、炎症性腸障害、および腸癌。胃腸運動性の調節因子の同定は、胃腸運動性減損または胃腸運動性増強に関する障害のための、新規な治療の開発を促進する。
【０００３】
近年、胃腸運動の２つの潜在的な調節因子が同定された。マンバ腸管毒素（Ｍａｍｂａ　ｉｎｔｅｓｔｉｎａｌ　ｔｏｘｉｎ）（ＭＩＴｌ）（モルモット回腸の収縮を強力に刺激する低分子タンパク質）は、マンバヘビ毒から精製された（Ｓｃｈｗｅｉｔｚら、Ｔｏｘｉｃｏｎ　２８：８４７～８５６（１９９０）およびＳｃｈｗｅｉｔｚら、ＦＥＢＳ　Ｌｅｔｔｅｒｓ　４６１：１８３～１８８（１９９９））。近年、同様の大きさで、かつＭＩＴｌと４０％よりも高い同一性を有するタンパク質（全１０個の保存性システインを含む）が、カエルの皮膚の分泌物から精製された（Ｍｏｌｌａｙら、Ｅｕｒ．Ｊ．Ｐｈａｒｍａｃｏｌ．３７４：１８９～１９６（１９９９））。カエルのタンパク質（Ｂｖ８と命名された）もまた、ＧＩ平滑筋の収縮を強力に刺激することが、認められた。
【０００４】
これらのヘビポリペプチドおよびカエルポリペプチドを組換え的に調製する方法、または１０個のシステインを含む他のポリペプチドを組換え的に調製する方法は、以前に記載されておらず、これら調節因子の有用性を治療的使用に限定していない。さらに、ヘビポリペプチドおよびカエルポリペプチドは、これらポリペプチドの治療としての有効性が減少する可能性がある哺乳動物に投与される場合、抗体を誘発し得る。
【０００５】
したがって、胃腸運動を刺激するかまたは阻害する内因性ヒトポリヌクレオチドを同定すること、および治療としてのこれら化合物を組換え的に調製する方法を開発することが、必要である。治療上使用され得る内因性胃腸調節因子の低分子アゴニストおよび低分子アンタゴニストを同定することもまた、必要である。本発明は、この必要性を満たし、そして、さらに、関連した利用を提供する。
【０００６】
（発明の要約）
本発明は、胃腸平滑筋の収縮を刺激する単離されたポリペプチドを提供する。１つの実施形態において、このポリペプチドは、ヒトプロキネチシン（ｐｒｏｋｉｎｅｔｉｃｉｎ）１（配列番号３）の配列と少なくとも８０％同一なアミノ酸配列を含み、ここで、この配列は、配列番号３のＮ末端の６個のアミノ酸、配列番号３の１０個の保存性システイン残基、および配列番号３の９個のＣ末端アミノ酸残基のうちの０個～９個を含む。別の実施形態において、このポリペプチドは、ヒトプロキネチシン２（配列番号６）の配列と少なくとも８０％同一なアミノ酸残基を含み、ここで、この配列は、配列番号６のＮ末端の６個のアミノ酸、配列番号６の１０個の保存性システイン残基、および配列番号６の４個の

Ｃ末端アミノ酸残基のうちの０個～４個を含む。
【０００７】
有効量のプロキネチシンポリヌクレオチドを哺乳動物へ投与することによって、哺乳動物中の胃腸平滑筋収縮を刺激する方法もまた、提供される。
【０００８】
本発明はまた、プロキネチシンポリペプチドをコードする核酸分子を提供する。
【０００９】
さらに、プロキネチシンポリペプチドに選択的に結合する抗体が提供される。
【００１０】
本発明はまた、プロキネチシンレセプターを含む調製物と１つ以上の候補化合物を接触させることによって、プロキネチシンレセプターリガンドを同定する方法、およびこのレセプターに特異的に結合する化合物を同定する方法を提供する。このような化合物は、プロキネチシンレセプターリガンドとして特徴付けられる。
【００１１】
プロキネチシンレセプターを含む調製物と１つ以上の候補化合物を接触させることによって、プロキネチシンレセプターアゴニストを同定する方法、およびプロキネチシンレセプターシグナルの生成を選択的に促進する化合物を同定する方法もまた、提供される。このような化合物は、プロキネチシンレセプターアゴニストとして特徴付けられる。
【００１２】
プロキネチシンレセプターを含む調製物と１つ以上の候補化合物を、プロキネチシン存在下で接触させることによって、プロキネチシンレセプターアンタゴニストを同定する方法、およびプロキネチシンレセプターシグナルの生成を選択的に阻害する化合物を同定する方法がさらに、提供される。このような化合物は、プロキネチシンレセプターアンタゴニストとして特徴付けられる。
【００１３】
（発明の詳細な説明）
本発明は、胃腸（ＧＩ）平滑筋収縮を刺激し得る、単離されたプロキネチシンポリペプチドを提供する。本発明のプロキネチシンポリペプチドは、例えば、障害性のＧＩ運動に関する障害を処置するための治療法において、使用され得る。このようなポリペプチドはまた、例えば、レセプターアゴニストおよびレセプターアンタゴニストを含むプロキネチシンレセプターリガンドを同定するためのスクリーニング法において使用され得、これは、障害性のＧＩ運動性または増強されたＧＩ運動性に関する障害を処置するために、治療的に使用され得る。
【００１４】
本明細書中で使用する場合、用語「プロキネチシンポリペプチド」は、図１Ａ（配列番号３）中で下線を引いていない配列として示されるヒトプロキネチシン１のアミノ酸配列を含むポリペプチド、または図１Ｂ（配列番号６）中で下線を引いていない配列として示されるヒトプロキネチシン２のアミノ酸配列を含むポリペプチド；および、ＧＩ平滑筋収縮活性を有する配列番号３または配列番号６に対する、少数の改変を含むポリペプチド；ならびに、ＧＩ平滑筋収縮活性を有する参照ポリペプチドのフラグメントを、いう。
【００１５】
本明細書中で使用する場合、用語「包含する」「有する」「コードする」および「含む」、ならびにこれらの用語の派生語は、開放型であることが意図される。用語「からなる」は、閉鎖型であることが意図される。
【００１６】
本明細書中で使用する場合、配列番号３または配列番号６と命名された配列に対する、用語「少数の改変」は、列挙されたアミノ酸配列と比較して、１つ以上の付加、欠失もしくは置換；そのポリペプチドに対する１つ以上の化学修飾もしくは酵素修飾；または、対応するＤ－型アミノ酸による１つ以上のＬ－型アミノ酸の置換を、いう。このような改変は、例えば、そのポリペプチドの安定性、発現、生理活性もしくはレセプター親和性の増強

において、またはそのポリペプチドの同定もしくは精製を容易にするために、有利であり得る。

【００１７】

改変されたポリペプチドのＧＩ平滑筋収縮活性は、その改変されたポリペプチドがＧＩ平滑筋収縮活性を有することを確認するための当該分野において公知であるエキソビボ法またはインビボ法（例えば、実施例中に記載されるエキソビボおよびインビボのモルモット回腸バイオアッセイ）によって決定され得る。ＧＩ平滑筋収縮活性を決定するための適切なアッセイが、プロキネチシン１またはプロキネチシン２に応答する他のＧＩ平滑筋組織（例えば、胃底部の筋肉ストリップ（ｆｕｎｄｉｃ　ｍｕｓｃｌｅ　ｓｔｒｉｐ）または近位結腸（実施例を参照のこと））を使用して、代わりに実施され得る。同様に、適切なアッセイが、他の哺乳動物（例えば、マウス、ラット、ネコ、イヌ、ヒツジ、ヤギ、ブタ、雌ウシおよび霊長類を含む）を使用して、実施され得る。

【００１８】

ＧＩ平滑筋収縮活性を誘発する改変されたプロキネチシンポリペプチドは、同一の条件下で、ヒトプロキネチシン１またはヒトプロキネチシン２の最大ＧＩ平滑筋収縮の少なくとも１０％、２５％、５０％、７５％、１００％またはそれ以上を誘発し得る。ＧＩ平滑筋収縮活性を誘発する改変されたプロキネチシンポリペプチドは、同一条件下で、ヒトプロキネチシン１またはヒトプロキネチシン２よりも弱い能力、類似の能力またはより強い能力であり得る。例えば、改変されたポリペプチドは、ヒトプロキネチシン１またはヒトプロキネチシン２についてのEC_{50}よりも５倍、１０倍、５０倍または１００倍高いか、またはその１／５、１／１０、１／５０、１／１００であるEC_{50}を有し得る。ＧＩ平滑筋収縮活性を誘発する改変されたプロキネチシンポリペプチドはまた、同一条件下で、ヒトプロキネチシン１もしくはプロキネチシン２と同じ期間、またはヒトプロキネチシン１もしくはプロキネチシン２よりもより長い期間もしくはより短い期間、収縮を誘発し得る。

【００１９】

プロキネチシン１のエクソン１およびエクソン２、ならびプロキネチシン２のエクソン３によってコードされるキメラポリペプチド（キメラ１２（配列番号１３）と命名された）（図６を参照のこと）は、プロキネチシン１またはプロキネチシン２と類似の能力で回腸収縮を誘発する、改変されたプロキネチシンの例である（図７Ａを参照のこと）、しかし、このキメラポリペプチドは、プロキネチシン１またはプロキネチシン２と比較して、長い収縮を生じる（図７Ｂを参照のこと）。

【００２０】

プロキネチシン２のエクソン１およびエクソン２、ならびにプロキネチシン１のエクソン３によってコードされるキメラポリペプチド（キメラ２１（配列番号１３）と命名された）（図６を参照のこと）は、プロキネチシン１またはプロキネチシン２の１／８の能力で回腸収縮を誘発する、改変されたプロキネチシンの例であり（図７Ａを参照のこと）、そして、このキメラポリペプチドは、プロキネチシン１またはプロキネチシン２と比較して、長い収縮を生じる（図７Ｂを参照のこと）。

【００２１】

配列番号３または配列番号６と命名されたアミノ酸配列に対する改変は、配列番号３または配列番号６をコードする核酸分子中に、無作為に生成され得る（例えば、ヌクレオチドの無作為な挿入、欠失、または置換によって）。あるいは、改変は、指向され得る（例えば、配列番号３または配列番号６をコードする核酸分子の部位特異的変異によって）。

【００２２】

当該分野において公知のコンピュータープログラムは、ポリペプチドの機能を消失することなしにアミノ酸残基が改変され得ることを予測することにおいて、指針を提供し得る（例えば、Ｅｒｏｓｈｋｉｎら、Ｃｏｍｐｕｔ．Ａｐｐｌ．Ｂｉｏｓｃｉ．９：４９１～４９７（１９９３）を参照のこと）。

【００２３】

さらに、活性を保持しながら配列番号３または配列番号６のアミノ酸残基を改変することにおける指針は、配列番号３または配列番号６とそれらの哺乳動物のホモログの配列（例えば、非ヒト霊長類、マウス、ラット、ウサギ、ウシ、ブタ、ヒツジ、イヌまたはネコ種のホモログ）ならびに非哺乳動物の脊椎動物におけるそれらのホモログの配列（カエルＢｖ８ポリペプチド（配列番号１１）およびヘビＭＩＴ１ポリペプチド（配列番号１２）を含む）（図１を参照のこと）を比較することによって、提供され得る。進化的に保存されたアミノ酸残基およびドメインは、あまり保存されていない残基およびドメインよりも、生物学的活性を維持するためにより重要である可能性があるということは、当該分野において周知である。従って、哺乳動物のプロキネチシン、カエルＢｖ８ポリペプチドおよびヘビＭＩＴ１ポリペプチドの間で高度に保存される残基（例えば、Ｎ末端配列または１０個のシステインのいずれか）を置換することは、活性に悪影響をもたらしそうである一方で、あまり高度に保存されていない残基（例えば、Ｃ末端残基）の置換は許容されそうである、ということが予想される。

【００２４】

実施例において記載されるように、付加、欠失または置換なしにプロキネチシンの６個のＮ末端残基（ＡＶＩＴＧＡ）を保持することは、平滑筋収縮活性を維持するために、明らかに必要である（表１を参照のこと）。しかし、ＡＶＩＴＧＡ配列の改変は、ＧＩ平滑筋収縮活性を示さないが野生型プロキネチシンの平滑筋収縮活性を拮抗するポリペプチドを、生じ得る。配列番号１６および配列番号１８と命名されたＮ末端変異体は、拮抗的活性を有する改変されたプロキネチシンポリペプチドの例である。

【００２５】

プロキネチシンのＮ末端ドメインは、ＧＩ平滑筋収縮活性のために明らかに必要とされる一方で、ＧＩ平滑筋収縮活性に十分でない。特に、プロキネチシンＮ末端ペプチド（配列番号１９）または、コリパーゼもしくはｄｉｃｃｋｏｐｆ　４のいずれかのシステインリッチなドメインを用いて置換されたプロキネチシンのシステインリッチなドメインを有するポリペプチドは、平滑筋収縮活性を示さなかった。

【００２６】

プロキネチシンのシステインリッチドメインもまた、ＧＩ平滑筋収縮活性のために必要とされることが示された（表１を参照のこと）。詳細には、２つのシステインいずれかでの置換が、活性を無効にする場合、活性を保持するために正確なシステイン対形成が必要とされることが示された。

【００２７】

位置に挿入された６ＸＨｉｓタグを有するプロキネチシンポリペプチドのＧＩ平滑筋収縮活性が、ことによって明らかとされるように、最後のシステインに対するＣ末端の配列は、恐らく必要とされない。さらに、プロキネチシン１および２の類似の活性によって明らかとされるように、４４％のみが同一であるにも関わらず、Ｎ末端および保存されたシステイン残基以外の位置でのアミノ酸配列の置換が、十分に許容される。

【００２８】

配列番号３または６と命名されたアミノ酸配列への置換は、保存的かもしれないし、保存的でないかもしれない。保存的アミノ酸置換としては、無極性のアミノ酸と別の無極性のアミノ酸との置換（例えば、ロイシンと、イソロイシン、バリン、アラニン、プロリン、トリプトファン、フェニルアラニン、またはメチオニンとの置換）；荷電したアミノ酸と同様に荷電したアミノ酸との置換（例えば、グルタミン酸とアスパラギン酸との置換、またはアルギニンとリジンもしくはヒスチジンとの置換）；非荷電極性アミノ酸と別の非荷電極性アミノ酸との置換（例えば、セリンとグリシン、スレオニン、チロシン、システイン、アスパラギンもしくはグルタミンとの置換）；あるいはある残基と類似のサイズおよび形の残基を有する異なる官能基との置換（例えば、セリンとアラニンとの置換；アルギニンとメチオニンとの置換；もしくはチロシンとフェニルアラニンとの置換）が挙げられるが、これらに限定されない。

【００２９】

配列番号３および６と命名されたアミノ酸配列に対して特に意図される置換としては、野生型プロキネチシン１由来の残基とプロキネチシン２由来の残基との置換、およびその逆が挙げられる。この置換は、単一の残基、ポリペプチドに渡った複数の残基または、複数の連続する残基であり得る。配列番号３と６との間のキメラ、すなわち配列番号１３および１４が、野生型プロキネチシンとの比較において延長された収縮活性を示すことが実証されるため、置換されたプロキネチシンが、インビボでの強力な治療剤であり得ることが考えられる。

【００３０】

配列番号３または６と命名されたアミノ酸配列への付加は、「タグ」配列の付加を含むがこれに限定されず、これは、好ましくはＣ末端に加えられる。このようなタグ配列としては、例えば、エピトープタグ、ヒスチジンタグ、グルタチオン－Ｓ－転移酵素（ＧＳＴ）など、または選別配列が挙げられる。このようなさらなる配列は、例えば、組換え発現を容易にするため、プロキネチシンの精製または特徴づけのために使用され得る。配列番号３または６と命名された配列に対する付加を含む例示的なポリペプチドは、Ｃ末端システインの後に６ＸＨｉｓタグを挿入することによって、実施例に記載されるように調製される活性プロキネチシンである。

【００３１】

配列番号３または６と命名されたアミノ酸の欠失としては、図１に示される高い保存性の活性ポリペプチドではないＣ末端の１つ以上の残基の欠失が挙げられるが、これに限定されない。欠失された配列は、必要に応じて、前記のようにタグ配列によって置換され得る。

【００３２】

配列番号３または６と命名されたアミノ酸配列を含むポリペプチドに対する化学的修飾および酵素的修飾としては、以下が挙げられるがそれらに限定されない：アルキル基、アシル基またはアミノ基による水素の置換；適切なアルキル部分またはアリール部分とのカルボキシル基のエステル化；エーテル誘導体を形成するヒドロキシル基のアルキル化；セリン残基、スレオニン残基またはチロシン残基のリン酸化または脱リン酸化；あるいはＮ結合もしくはＯ結合グリコシル化。

【００３３】

本明細書中で使用される場合、用語「単離された」は、分子が、その自然環境においてどのように発見されたものからヒトの手助けによって変化されることを示す。好ましくは、「単離された」プロキネチシンポリペプチドは、「実質的に精製された」分子であり得、これは、少なくとも６０％、７０％、８０％、９０％または９５％、自然に関連する細胞の成分を含まない。単離されたポリペプチドは、任意の形態（例えば、緩衝溶液、懸濁物、凍結乾燥した粉末中）であり得、異種細胞において組み換え的に発現されるか、レセプターに結合されるか、または固体支持に付着される。

【００３４】

本発明は、胃腸の平滑筋収縮を刺激する単離ポリペプチドを提供する。１つの実施形態において、このポリペプチドは、ヒトプロキネチシン１（配列番号３）の配列に少なくとも５０％同一であるアミノ酸配列を含み、そして配列番号３のＮ末端の６個のアミノ酸、配列番号３の１０個の保存されたシステイン残基、および配列番号３の０～９個の９つのＣ末端アミノ酸を含む。従って、このコードされるポリペプチドは、少なくとも６０％、６５％、７０％、７５％同一性を有し得、配列番号３に対して少なくとも８０％、８５％、９０％、９５％、９６％、９８％、９９％以上の同一性を含む。例示的なポリペプチドは、配列番号３と命名されたアミノ酸配列、またはこれらのアミノ酸１～７７を含む。

【００３５】

１つの実施形態において、単離されたポリペプチドは、アミノ酸配列ＮＮＦＧＮＧ］ＲＱＥＲＲＫＲＫＲＳＫＲＫＫＥ（配列番号７）を含まない。別の実施形態において、単離されたポリペプチドは、アミノ酸配列ＳＨＶＡＮＧＲＱＥＲＲＲＡＫＲＲＫＲＫＫＥ（配列番号８）を含まない。

【００３６】
別の実施形態において、このポリペプチドは、ヒトプロキネチシン２（配列番号６）の配列に少なくとも５０％同一であるアミノ酸配列を含み、そして配列番号６のＮ末端６個のアミノ酸、配列番号６の１０個の保存されたシステイン残基、および配列番号６の４個のＣ末端アミノ酸の０～４を含む。従って、コードされるポリペプチドは、少なくとも６０％、６５％、７０％、７５％の同一性を有し得、配列番号６に対して少なくとも８０％、８５％、９０％、９５％、９６％、９８％、９９％以上の同一性を含む。例示的なポリペプチドは、配列番号６と呼ばれるアミノ酸配列またはこれらのアミノ酸１～７７を含む。
【００３７】
本明細書中で使用される場合、２つの分子に関する用語「％同一性」は、２つの配列の整列された部分間の同一のヌクレオチドまたはアミノ酸残基の数をいうことを意図され、最適化された操作アライメントまたはコンピュータアライメント（例えば、ＢＬＡＳＴ　２．０アライメント（Ｔａｔｕｓｏｖａら、ＦＥＭＳ　Ｍｉｃｒｏｂｉｏｌ　Ｌｅｔｔ．１７４：２４７－２５０（１９９９））を用いて全体の配列を比較することによって決定されるような、整列された残基の総数のパーセンテージとして発現される。
【００３８】
特定の適用について、本明細書中で開示されるようなスクリーニング方法において、プロキネチシンポリペプチドは、検出可能な部分（例えば、放射線標識、蛍光色素、強磁性体の物質、発光タグまたは検出可能な結合剤（例えば、ビオチン））を用いて標識され得る。他の適切な標識化部分は、当該分野において周知である。
【００３９】
本発明はまた、組換えプロキネチシンポリペプチドを発現するために宿主細胞を培養し、そしてタンパク質の凝集を最小化する条件下でポリペプチドを再折り畳みすることによって（以下に記載される）、ＧＩ平滑筋収縮を刺激し得る、単離されたプロキネチシンポリペプチドを調製するための方法を提供する。
【００４０】
複数のシステイン残基を含むポリペプチドの組替え発現は、しばしば分子間および分子内のジスルフィド結合の不正確な形成を生じ、これは、不活性の、凝集された細菌のタンパク質の産生を導く。本明細書中で開示されるように、これらの問題は、発現されるポリペプチドの再折り畳みの間、タンパク質の凝集を最小化する条件を用いて克服され得る。タンパク質の凝集を最小化する例示的な条件は、実施例に記載され、そして１つ以上の以下の再折り畳みの条件を含むことにより、組換えタンパク質を調製するための従来の条件とは異なる：１）タンパク質濃度を低く保つ工程（例えば、約１００μｇ／ｍｌ）；２）変性剤を除くためにペプチドを希釈するよりもむしろ透析する工程；３）緩衝液から酸化剤を除く工程；４）全ての緩衝液中の尿素の高い濃度を維持する工程；５）緩衝液中のグリセロールの高い濃度を維持する工程（例えば、少なくとも約１０％）；ならびに６）ペプチドおよび緩衝液を低い温度に保つ工程（例えば、約４℃）。これらの条件において、低いタンパク質濃度（すなわち、約２５０μｇ／ｍｌ未満、好ましくは２００μｇ／ｍｌ、１５０μｇ／ｍｌ、１００μｇ／ｍｌ、もしくは５０μｇ／ｍｌ未満）および高い尿素濃度（例えば、少なくとも約１．５Ｍ（例えば、約２Ｍ、４Ｍ、６Ｍ、８Ｍ、より高い）が、活性プロキネチシンの首尾よい再折り畳みにおいて最も重要な因子であることが企図される。
【００４１】
本明細書中で記載される条件と同じまたは類似の条件が、複数のシステインを含む他のポリペプチドを組換え的に発現および再折り畳みするために用いられ得、生物学的に活性なポリペプチドを単離するためにｄｉｃｋｋｏｐｆ、コリパーゼ、ＭＩＴ－１およびＢｖ８を含むことが、予想される。
【００４２】
ＧＩ平滑筋収縮を刺激し得る単離プロキネチシンポリペプチドを調製するための好ましい方法において、プロキネチシンポリペプチドは、タグ（例えば、６ＸＨｉｓタグ）を含む

融合タンパク質（例えば、ＧＳＴ融合物として）として細菌において組み換え的に発現され、そして親和性単離（例えば、ニッケルカラム上で）によって部分的に精製される。次いで、融合ポリペプチドは、異種タンパク質（例えば、ＧＳＴとプロキネチシンとの間のプロテアーゼ因子Ｘａ切断を用いて）、およびタンパク質の凝集を最小化する上記の条件下で再折り畳みされるプロキネチシンポリペプチドを除くために切断される。より高度に精製されたポリペプチドを得るために、このポリペプチドは、さらにカラムクロマトグラフィー（例えば、逆相ＨＰＬＣ）によって精製され得る。当業者は、活性プロキネチシンポリペプチドを組み換え的に発現、再折り畳みおよび精製するためのこれらの好ましい方法に対する改変が、容易に決定され得る（例えば、代替の異種の配列、切断可能な配列、タグ、宿主細胞および緩衝液の条件を用いて）ことを認識する。

【００４３】

あるいは、単離されたプロキネチシンポリペプチドは、生化学的手順によって調製され得る。本明細書中で開示されるように、プロキネチシン１および２が、種々のヒト組織において発現される（実施例、および特に図２を参照のこと）。それ故、単離されたプロキネチシンポリペプチドは、膜画分化、クロマトグラフィー、電気泳動およびリガンド親和性方法を含む当該分野において慣用的に使用される生化学的手順によってか、または本明細書粒に記載されるプロキネチシン抗体を用いる免疫親和性方法を用いて、これらのポリペプチドを通常発現する組織または細胞から単離され得る。生化学的単離後、不活性プロキネチシンは、活性を回復する上記の方法によって再折り畳みされ得る。

【００４４】

同様に、単離されたプロキネチシンポリペプチドは、当該分野において公知の化学合成手順によって調製され得る。化学合成後、不活性なプロキネチシンは、活性を回復する本明細書中に記載される方法によって再折り畳みされ得る。

【００４５】

その機能的活性、選択性、安定性またはバイオアベイラビリティーを最適化するように、所望される場合、化学的に合成されたポリペプチドは、Ｄ－立体異性体、天然に存在しないアミノ酸、ならびにアミノ酸アナログおよびアミノ酸模倣物を含むように改変され得る。改変されたアミノ酸およびそれらの使用の例が、Ｓａｗｙｅｒ，Ｐｅｐｔｉｄｅ Ｂａｓｅｄ Ｄｒｕｇ Ｄｅｓｉｇｎ，ＡＣＳ，Ｗａｓｈｉｎｇｔｏｎ（１９９５）、ならびにＧｒｏｓｓおよびＭｅｉｅｎｈｏｆｅｒ，Ｔｈｅ Ｐｅｐｔｉｄｅｓ：Ａｎａｌｙｓｉｓ，Ｓｙｎｔｈｅｓｉｓ，Ｂｉｏｌｏｇｙ，Ａｃａｄｅｍｉｃ ｐｒｅｓｓ，Ｉｎｃ．，Ｎｅｗ Ｙｏｒｋ（１９８３）に示される。特定の適用について、１つ以上の検出可能に標識されたアミノ酸（例えば、放射線標識されたアミノ酸または蛍光標識されたアミノ酸）を、化学合成されたポリペプチドまたはペプチドに組み込むこともまた有用であり得る。

【００４６】

本発明はまた、胃腸の（ＧＩ）平滑筋収縮を刺激し得る（必ずしも必要ではない）配列番号３または６と呼ばれるアミノ酸配列の少なくとも１０個連続するアミノ酸を含むか、またはそれからなる単離されたペプチドを提供する。このような単離されたペプチドは、例えば、本発明のプロキネチシン抗体を調製および精製する際に、有用である。このようなペプチドはまた、プロキネチシンレセプターを介するシグナル伝達をブロックするアンタゴニストとして作用し得、従って、治療的方法およびスクリーニング方法において用いられ得る。従って、単離されたプロキネチシンペプチドは、配列番号３または６の少なくとも１２、１５、２０，２５以上連続するアミノ酸を含み得るか、またはそれからなり得、少なくとも３０、４０、５０、６０、７０、８０、８１または８６あるいはそれ以下の連続するアミノ酸を含む。

【００４７】

１つの実施形態において、単離されたプロキネチシンペプチドは、配列番号３の６および４８のアミノ酸残基内に由来する少なくとも１０個連続する残基を含むか、またはそれからなる。別の実施形態において、単離されたプロキネチシンペプチドは、配列番号６の６

および４８のアミノ酸残基内に由来する少なくとも１０個連続する残基を含むか、またはそれからなる。

【００４８】

配列番号３または６の少なくとも１０個連続するアミノ酸を含む単離されたペプチドは、免疫原性であり得る。本明細書中で使用される場合、用語「免疫原性」は、プロキネチシン特異的抗体を誘導し得るか、またはプロキネチシンに結合するためのプロキネチシン特異的抗体と競合し得るかのいずれかのペプチドをいう。免疫原性である傾向があるペプチドは、当該分野において公知のおよび例えば、ＩｒｎａｔｅｎらによるＰｒｏｔｅｉｎ Ｅｎｇ．１１：９４９－９５５（１９９８）、およびＳａｖｏｉｅら、Ｐａｃ．Ｓｙｍｐ．Ｂｉｏｃｏｍｐｕｔ．１９９９：１８２－１８９（１９９９）に記載される方法およびアルゴリズムを用いて予想され得る。本発明のペプチドの免疫原性は、当該分野において公知の方法によって確認され得る。

【００４９】

本発明の単離されたプロキネチシンポリペプチドおよびペプチドは、その免疫原性を増強する標準的な速結技術を用いて、キャリア（例えば、ＫＬＨ、血清アルブミン、破傷風トキソイドなど）に必要に応じて結合体化され得る。さらに、またはあるいは、単離されたポリペプチドおよびペプチドは、当該分野において公知のアジュバント（例えば、フロイント完全アジュバントまたはフロイント不完全アジュバント）を用いて処方され得る。

【００５０】

少なくとも１０個連続する残基の単離されたプロキネチシンペプチドは、化学的合成、または上記のように調製されたより長いペプチドの化学的消化もしくは酵素的消化によって、都合よく調製され得る。少なくとも１０個連続する残基の単離されたプロキネチシンペプチドもまた、組み換え的（例えば、タンパク質タグに融合される）に調製され得る。当業者は、そのサイズ、配列および意図される適用に依存して、単離されたプロキネチシンペプチドを調製する適切な方法を決定し得る。

【００５１】

本発明はまた、ＧＩ平滑筋収縮を刺激し得るプロキネチシンポリペプチドをコードする単離された核酸分子を提供する。本発明の核酸分子は、種々のスクリーニング適用、治療的適用および診断的適用に適切である。例えば、本発明の核酸分子は、インビトロで発現され得、そしてコードされるプロキネチシンポリペプチドが、単離され得る。本発明の核酸分子はまた、患者における正常なプロキネチシン活性を回復するためにインビボで発現し得るか、またはこれらを必要とする患者におけるプロキネチシンの発現をブロックするアンチセンス配向で発現され得る。さらに、本発明の核酸分子は、他の種由来のプロキネチシンをコードする核酸分子を同定および単離するか、または構造的に関連する分子を同定するプローブまたはプライマーとして使用され得る。このようなプローブおよびプライマーはまた、ヒト組織におけるプロキネチシンの正常な発現および異常な発現を測定するために診断的に有用であり、従って、変更されたプロキネチシン発現に関連する条件に対する感受性を予想する。

【００５２】

本明細書中で使用される場合、用語「単離された核酸分子」は、核酸分子が、自然環境において発見されるものから人の手によって、変更されることを意味することが意図される。例えば、単離された核酸分子は、外因性の核酸配列に作動可能に連結される分子であり得る。単離された核酸分子はまた、その通常隣接する核酸配列のいくつかまたは全てから除かれる分子であり得る。

【００５３】

単離された分子は、あるいは、またはさらに「実質的に純粋な」分子であり得る。この場合、この分子は、細胞成分の少なくとも６０％、７０％、８０％、９０％または９５％が無く、ここでこれは天然に関連する。単離された核酸分子は、任意の形態（例えば、緩衝溶液中、懸濁物、凍結乾燥粉末、個体支持（例えば、ＤＮＡアレイの構成成分として）に付着される、または細胞中）であり得る。

【００５４】
本明細書中で使用される場合、用語「核酸分子」は、一本鎖または二本鎖であり得、ゲノムＤＮＡ、ｃＤＮＡまたはＲＮＡに対応し得、そしてセンス鎖もしくはアンチセンス鎖のいずれか、または両方を示し得る、天然または合成起源のポリヌクレオチドをいう。
【００５５】
用語「核酸分子」は、１つ以上の非天然ヌクレオチド（例えば、塩基、糖、またはリン酸部分への改変を有するヌクレオチド、または１つ以上の非天然の連結（例えば、ホスホチオエート連結）を有するヌクレオチド）を含む核酸分子を含むことが意図される。このような改変は、特にハイブリダイゼーションの適用において使用される場合、核酸分子の安定性を増加するのに有利であり得る。
【００５６】
さらに、用語「核酸分子」とは、検出可能な部分（例えば、放射標識、蛍光色素、強磁性物質、発光性タグまたはビオチンのような検出可能な結合因子）を含むように修飾された核酸分子を含有することを意図する。このような部分を含有する核酸分子は、プロキネチシン（ｐｒｏｋｉｎｅｔｉｃｉｎ）核酸分子の存在または発現を検出するためのプローブとして有用である。
【００５７】
ＧＩ平滑筋収縮を刺激し得るプロキネチシンポリペプチドは、上記に記載されている。従って、そのようなポリペプチドをコードする単離された核酸分子を調製することは、当業者にとって慣用的である。ＧＩ平滑筋収縮を刺激し得るプロキネチシンポリペプチドをコードする、例示的に単離された核酸分子は、以下を含むか、または以下から構成される：ａ）配列番号１もしくは配列番号４で示されるヌクレオチド配列；ｂ）配列番号３もしくは配列番号６をコードする配列番号１もしくは配列番号４で示されるヌクレオチド配列の一部（すなわち、配列番号１で示されるヌクレオチド配列のヌクレオチド５５～３７０、および配列番号４で示されるヌクレオチド配列のヌクレオチド１０～３３４）；ｃ）配列番号３もしくは配列番号６の活性な改変もしくは活性なフラグメントをコードするヌクレオチド配列；およびｄ）ａ）、ｂ）もしくはｃ）のいずれかに関して縮重する配列。
【００５８】
１つの実施形態において、単離された核酸分子は、アミノ酸配列ＮＮＦＧＮＧＲＱＥＲＲＫＲＫＲＳＫＲＫＫＥ（配列番号７）をコードしない。別の実施形態において、単離された核酸分子は、アミノ酸配列ＳＨＶＡＮＧＲＱＥＲＲＲＡＫＲＲＫＲＫＫＥ（配列番号８）をコードしない。なお別の実施形態において、プロキネチシンポリペプチドをコードする単離された核酸分子は、図１Ａおよび図１Ｂに示されるアミノ酸配列の下線を引かれた部分（ＭＲＧＡＴＲＶＳＩＭＬＬＬＶＴＶＳＤＣ（配列番号９）およびＭＲＳＬＣＣＡＰＬＬＬＬＬＬＬＰＬＬＬＴＰＰＡＧＤＡ（配列番号１０））をコードする核酸分子のような天然に存在するシグナルポリペプチドを除外する。
【００５９】
特定の実施形態において、プロキネチシンポリペプチドをコードする単離された核酸分子は、一般に入手可能なデータベースに編集される（ｃｏｍｐｉｌｅ）配列（例えば、ＧｅｎＢａｎｋ登録番号ＡＩ２７７３４９、ＡＡ８８３７６０、ＡＱ４２６３８６、ＡＣ０６８５１９、ＡＣ０２６９７３、ＡＬ３５８２１５およびＡＬ３９０７９７またはＧｅｎＢａｎｋ登録番号ＡＦ１８２０６６、ＡＦ１８２０６４、ＡＦ１８２０６９およびＡＦ１８２０６５を有するアミノ酸配列をコードする配列）のゲノムフラグメントＥＳＴならびにｃＤＮＡのまさにその配列を有する核酸分子を特に除外する。
【００６０】
１つの実施形態において、プロキネチシンポリペプチドをコードする単離された核酸分子は、配列番号３および配列番号６をコードしない配列を含むＧｅｎＢａｎｋデータベース中に存在する哺乳動物の配列（例えば、第１染色体もしくは第３染色体上に存在する、５’非翻訳領域および３’非翻訳領域、イントロンまたは他のエキソンをコードする核酸分子）を除く。

【００６１】
本発明はさらに、ＧＩ平滑筋収縮を刺激し得るプロキネチシンポリペプチドをコードする単離された核酸分子を提供し、ここでこの核酸分子は、遺伝子発現のプロモーターに作動可能に連結される。本明細書中で使用される場合、用語「作動可能に連結される」とは、プロモーターが鋳型として核酸分子を使用してＲＮＡの転写を指示するような様式で、核酸分子が内因性のプロモーター、または異種性のプロモーターのいずれかに関して位置付けられることを意味することを意図する。
【００６２】
核酸分子を異種性のプロモーターに作動可能に連結するための方法は、当該分野において周知であり、例えば、核酸を所望のプロモーターを含むベクター中にクローニングすること、またはＰＣＲを使用してプロモーターを核酸配列に付けることが挙げられる。ＲＮＡ転写のプロモーターに作動可能に連結される核酸分子は、所望の宿主細胞またはインビトロの転写－翻訳系において、プロキネチシンの転写物およびポリペプチドを発現させるために使用され得る。
【００６３】
本発明の核酸分子に作動可能に連結されるプロモーターの選択は、意図される用途に依存し、そして当業者によって決定され得る。例えば、特定の遺伝子産物が特定の宿主細胞に対して有害であり得る場合、遺伝子発現が作動または停止され得るように本発明の核酸分子を、調節されたプロモーターに連結することが所望され得る。あるいは、弱いかまたは強いかのいずれかの構成的なプロモーターによって作動される発現を有することが好まれ得る。哺乳動物細胞系に対して適した例示的なプロモーターとしては、例えば、ＳＶ４０初期プロモーター、サイトメガロウイルス（ＣＭＶ）プロモーター、マウス乳腺腫瘍ウイルス（ＭＭＴＶ）ステロイド誘導性プロモーター、およびモロニーマウス白血病ウイルス（ＭＭＬＶ）プロモーターが挙げられる。細菌細胞系に対して適した例示的なプロモーターとしては、例えば、Ｔ７、Ｔ３、ＳＰ６、ｌａｃおよびｔｒｐプロモーターが挙げられる。
【００６４】
本発明はさらに、プロキネチシンポリペプチドをコードする単離された核酸分子を含むベクターを提供する。例示的なベクターとしては、ウイルス（例えば、バクテリオファージ、バキュロウイルスまたはレトロウイルス）に由来するベクター、ならびに細菌に由来するベクター、または細菌の配列および他の生物からの配列の組み合わせ（例えば、コスミドまたはプラスミド）が挙げられる。本発明のベクターは、一般的に、企図される宿主細胞に適合性の複製起点；転写終結およびＲＮＡプロセシングのシグナル；企図される宿主細胞に適合性の１つ以上の選択マーカー；ならびに１つ以上の多重クローニング部位のような要素を含む。必要に応じて、このベクターはさらに、コードされたポリペプチドの発現および精製を容易にする、タグ配列（例えば、ＧＳＴタグ）、および／またはプロテアーゼ切断部位（例えば、Ｘａ因子部位）をコードする配列を含有する。
【００６５】
ベクター中に含むべき特定の要素の選択は、企図される宿主細胞；挿入物サイズ；挿入された配列の発現が所望されるか否か；所望されるコピー数のベクター；所望される選択系などといった因子に依存する。異なる用途に対する宿主細胞とベクターとの間の適合性を確実にすることに関わる因子は、当該分野において周知である。
【００６６】
ベクターがコードされるポリペプチドの組換え発現のために使用されるような用途において、単離された核酸分子は、一般的に、上記に記載されるように、ベクターまたは挿入された核酸分子内に存在され得る、遺伝子発現のプロモーターに作動可能に連結される。細菌細胞における融合タンパク質の発現のために適切な例示的なベクターは、ｐＧＥＸ－３Ｘベクター（Ａｍｅｒｓｈａｍ　Ｐｈａｒｍａｃｉａ　Ｂｉｏｔｅｃｈ，Ｐｉｓｃａｔａｗａｙ，ＮＪ）である。
【００６７】

プロキネチシンポリペプチドをコードする単離された核酸分子を含有する細胞もまた、提供される。単離された核酸分子は、一般的に、ベクター中に含まれる。単離された核酸分子は、エピソーム的に維持され得るか、または宿主細胞ゲノム中に組込まれ得る。

【００６８】

本発明の細胞は、例えば、単離された核酸分子の伸長、サブクローニングまたは改変のような分子生物学の用途のために使用され得る。このような用途において、細菌細胞（例えば、実験室株のＥ．ｃｏｌｉ）は有用であり、そしてコードされるポリペプチドの発現は要求されない。

【００６９】

本発明の細胞はまた、コードされるポリペプチドを組換え的に発現および単離するために有利に使用され得る。このような用途のために、以下が挙げられる：細菌細胞（例えば、Ｅ．ｃｏｌｉ）、昆虫細胞（例えば、Ｄｒｏｓｏｐｈｉｌａ）、酵母細胞（例えば、Ｓ．ｃｅｒｅｖｉｓｉａｅ、Ｓ．ｐｏｍｂｅ、またはＰｉｃｈｉａ ｐａｓｔｏｒｉｓ）、および脊椎動物細胞（例えば、哺乳動物の初代細胞および樹立された細胞株）；ならびに両生類細胞（例えば、Ｘｅｎｏｐｕｓの胚および卵母細胞）。プロキネチシンポリペプチドを組換え的に発現するために適した例示的な細胞は、Ｅ．ｃｏｌｉ ＢＬ２１細胞である。

【００７０】

本発明はさらに、配列番号１もしくは配列番号４（例えば、配列番号２、配列番号３、配列番号５もしくは配列番号６をコードする配列番号１もしくは配列番号４の一部分）に由来するか、またはそれらの相補体に由来する、少なくとも２０個連続するヌクレオチドを含む、単離されたポリヌクレオチドを提供する。従って、本発明のポリヌクレオチドは、プロキネチシンポリペプチドをコードする核酸分子を検出または単離するための配列決定プライマー、ＰＣＲプライマーおよびハイブリダイゼーションプローブとして使用するために十分な長さであり、そしてまた、プロキネチシンの発現を阻害するための治療的アンチセンス試薬として有用である。本発明のポリヌクレオチドは、必要ではないが、ＧＩ平滑筋収縮を刺激し得るプロキネチシンポリペプチドをコードし得る。当業者は、特定の用途のための本発明のポリヌクレオチドの適切な長さおよび配列を決定し得る。

【００７１】

本明細書中で使用される場合、用語「ポリヌクレオチド」とは、参照配列から少なくとも２０個連続するヌクレオチドを含み、必要ではないが、機能的ポリペプチドをコードし得る核酸分子をいう。従って、本発明のポリヌクレオチドは、配列番号１または配列番号４由来か、あるいはそれらの相補体由来の、少なくとも、２０個、２２個または２５個連続するヌクレオチド、例えば、少なくとも、または多くとも、３０個、４０個、５０個、６０個、７０個、８０個、９０個、１００個、１２５個、１５０個、１７５個、２００個、２５０個、もしくは３００個連続するヌクレオチドを含有し得る。本発明のポリヌクレオチドは、一般に入手可能なデータベース（ＧｅｎＢａｎｋ登録番号ＡＩ２７７３４９、ＡＡ８８３７６０、ＡＱ４２６３８６、ＡＣ０６８５１９、ＡＣ０２６９７３、ＡＬ３５８２１５、およびＡＬ３９０７９７によって示される配列、またはＧｅｎＢａｎｋ登録番号ＡＦ１８２０６６、ＡＦ１８２０６４、ＡＦ１８２０６９およびＡＦ１８２０６５を有するアミノ酸配列をコードする配列を含む）に存在するＥＳＴのまさにその配列から構成されない。

【００７２】

特定の用途のために（例えば、サンプル中のプロキネチシンの発現を検出するために）、プロキネチシンをコードする核酸分子に特異的にハイブリダイズする、本発明の単離されたポリヌクレオチド分子を使用することが所望される。用語「特異的にハイブリダイズする」とは、以下に記載されるようなストリンジェントなハイブリダイゼーション条件下で、プロキネチシンをコードする核酸分子に対してハイブリダイズし、同一の条件下で、プロキネチシンをコードしない核酸分子（例えば、プロキネチシンと同一の短い領域を偶然に含む無関係な分子）と実質的な程度ハイブリダイズしない核酸分子の能力をいう。従っ

て、「特異的にハイブリダイズする」核酸分子とは、十分な長さの核酸分子であり、そして組織（ｔｉｓｓｕｅ）ブロットおよびノザンブロットのような発現分析における使用に対してプロキネチシンを十分に識別する配列を含む（図２を参照のこと）。

【００７３】

本明細書中で使用される場合、用語「ストリンジェントな条件」とは、フィルターに結合した核酸分子を、４２℃で５０％ホルムアミド、５×デンハルト溶液、５×ＳＳＣ、０．２％　ＳＤＳを含有する溶液中の核酸分子にハイブリダイゼーションし、続いて６５℃で３０分間０．１×ＳＳＣおよび０．１％　ＳＤＳで２回フィルターを洗浄することと等価な条件をいう。上記に記載されるストリンジェントな条件に等価な条件は、当該分野において周知であり、そして例えば、Ｓａｍｂｒｏｏｋら、Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ、Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｂｏｒ　Ｌａｂｏｒａｔｏｒｙ、Ｎｅｗ　Ｙｏｒｋ（１９９２）中に記載される。

【００７４】

本発明はさらに、単一の容器または別々の容器のいずれかに一緒に包装された本発明の一組のポリヌクレオチドを含有するキットを提供する。一組のポリヌクレオチドは、好ましくは、ポリメラーゼ連鎖反応（ＰＣＲ）の用途での使用のために適切である。従って、この一組のポリヌクレオチドは、プロキネチシンをコードする核酸分子の正常または異常な発現を検出または定量化するために使用され得る。この一組のポリヌクレオチドはまた、配列決定、サブクローニングのためか、または配列改変体を調製するために、プロキネチシンまたはその任意の一部分をコードする核酸分子を増幅するために使用され得る。このキットはさらに、ＰＣＲの用途における一組のポリヌクレオチド、またはこのような用途に対して適切な溶液および緩衝液の使用のための書面の使用説明書を含み得る。

【００７５】

本発明の単離されたプロキネチシン核酸分子は、当該分野において公知の方法によって調製され得る。単離されたプロキネチシン核酸分子を調製するための例示的な方法は、プロキネチシンに特異的なプライマーおよびポリメラーゼ連鎖反応（ＰＣＲ）を使用する核酸分子の増幅に関わる。ＰＣＲを使用して、任意の所望される境界（ｂｏｕｎｄａｒｙ）を有するプロキネチシン核酸分子が、単一の細胞由来のようなほんのわずかなＤＮＡ分子またはＲＮＡ分子から開始して指数関数的に増幅され得る。縮重したプライマーを使用する他の種における所定の核酸分子のホモログを単離する方法を包含するＰＣＲ法は、当該分野において周知である。

【００７６】

あるいは、単離されたプロキネチシン核酸分子は、検出可能なプロキネチシン核酸分子または抗体を有する、ゲノムライブラリー、ｃＤＮＡライブラリーまたは発現ライブラリーのような、ライブラリーをスクリーニングすることによって調製され得る。ヒトのライブラリー、および多種多様な哺乳動物種由来のライブラリーは、市販であるか、または目的の種もしくは細胞から作製され得る。プロキネチシン核酸分子を含有すると同定されたライブラリークローンは、慣用的な方法によって、単離、サブクローニングまたは配列決定され得る。

【００７７】

さらに、単離されたプロキネチシン核酸分子は、直接的な合成方法によって調製され得る。例えば、一本鎖核酸分子は、当該分野において公知の自動化された合成方法によって、１つの小片またはいくつかの小片に化学的に合成され得る。相補鎖は、同様に１つ以上の小片に合成され得、そして二本鎖分子が相補鎖をアニーリングすることによって作製され得る。直接的な合成は、比較的短い分子（例えば、プローブおよびプライマー）を作製するため、そしてまた、改変されたヌクレオチドまたは連結を含有する核酸分子を作製するために特に有利である。

【００７８】

本発明はまた、プロキネチシンポリペプチドまたはプロキネチシンペプチドに対して特異的な抗体（例えば、配列番号３または配列番号６のアミノ酸配列を有するポリペプチドに

対して特異的な抗体）を提供する。配列番号３または配列番号６の少なくとも１０個連続するアミノ酸を含有する、単離された免疫原性のペプチドに対して特異的な抗体もさらに提供される。

【００７９】

本発明の抗体は、例えば、研究および診断的適用におけるプロキネチシンの発現を検出するために使用され得る。このような抗体はまた、哺乳動物の発現ライブラリー中に存在するプロキネチシンポリペプチドをコードする核酸分子を同定するため、および免疫親和性法によってプロキネチシンポリペプチドを精製するために有用である。さらに、このような抗体は、ＧＩ平滑筋収縮を阻害することが所望される適用におけるような、プロキネチシンに結合して、そしてプロキネチシンの活性をブロックするために治療的に投与され得る。

【００８０】

本明細書中で使用される場合、用語「抗体」とは、少なくとも$1\times10^{5}M^{-1}$、好ましくは少なくとも$1\times10^{7}M^{-1}$、さらに好ましくは少なくとも$1\times10^{9}M^{-1}$で、プロキネチシンペプチドまたはプロキネチシンポリペプチドに対して特異的な結合活性を有する分子を含むことが意図される。用語「抗体」は、ポリクローナル抗体およびモノクローナル抗体の両方、ならびにそのような抗体の抗原結合フラグメント（例えば、Ｆａｂフラグメント、$F(ab')_2$フラグメント、ＦｄフラグメントおよびＦｖフラグメントなど）を含む。さらに、用語「抗体」は、例えば、単鎖抗体、キメラ抗体、二官能性抗体、ＣＤＲ移植抗体およびヒト化抗体、ならびにそれらの抗原結合フラグメントを含む、天然に存在しない抗体を包含することを意図される。

【００８１】

ペプチド免疫原およびポリペプチド免疫原を使用して、ポリクローナル抗体およびモノクローナル抗体を含む、抗体を調製および単離する方法は、当該分野において周知であり、そして例えば、ＨａｒｌｏｗおよびＬａｎｅ、Ａｎｔｉｂｏｄｉｅｓ：Ａ Ｌａｂｏｒａｔｏｒｙ Ｍａｎｕａｌ、Ｃｏｌｄ Ｓｐｒｉｎｇ Ｈａｒｂｏｒ Ｌａｂｏｒａｔｏｒｙ Ｐｒｅｓｓ（１９８８）中に記載される。天然に存在しない抗体は、固相ペプチド合成を使用して構築され得るか、組換え的に作製され得るか、または例えば、可変重鎖および可変軽鎖から構成されるコンビナトリアルライブラリーをスクリーニングすることによって入手され得る。このような方法は、例えば、Ｈｕｓｅら、Ｓｃｉｅｎｃｅ、２４６：１２７５－１２８１（１９８９）；ＷｉｎｔｅｒおよびＨａｒｒｉｓ、Ｉｍｍｕｎｏｌ．Ｔｏｄａｙ、１４：２４３－２４６（１９９３）；Ｗａｒｄら、Ｎａｔｕｒｅ、３４１：５４４－５４６（１９８９）；Ｈｉｌｙａｒｄら、Ｐｒｏｔｅｉｎ Ｅｎｇｉｎｅｅｒｉｎｇ：Ａ ｐｒａｃｔｉｃａｌ ａｐｐｒｏａｃｈ（ＩＲＬ Ｐｒｅｓｓ １９９２）；およびＢｏｒｒａｂｅｃｋ、Ａｎｔｉｂｏｄｙ Ｅｎｇｉｎｅｅｒｉｎｇ、第２版（Ｏｘｆｏｒｄ Ｕｎｉｖｅｒｓｉｔｙ Ｐｒｅｓｓ １９９５）中に記載される。

【００８２】

本発明は、プロキネチシンレセプターリガンドを同定する方法を提供する。この方法は、プロキネチシンレセプターを含有する調製物を１つ以上の候補化合物と接触させること、およびレセプターに特異的に結合する候補化合物を同定することによって実施される。このような化合物は、プロキネチシンレセプターリガントとして特徴付けられる。

【００８３】

本明細書中で使用される場合、用語「リガンド」は、プロキネチシンと同一または異なる部位で、プロキネチシンレセプターに結合する化合物を含む。

【００８４】

本明細書中で使用される場合、用語「候補化合物」とは、任意の生物学的または化学的な化合物をいう。例えば、候補化合物は、ポリペプチド、核酸、炭水化物、リピド、またはそれらのいずれかの組み合わせのような天然に存在する高分子であり得る。候補化合物はまた、このような高分子の部分的もしくは完全に合成的な誘導体、アナログもしくは模倣物、またはコンビナトリアル化学の方法によって調製された有機低分子であり得る。特定

のアッセイの形式において所望される場合、候補化合物は、検出可能に標識化され得るか、または固体支持体に結合され得る。

【００８５】

単純な有機分子、複雑な有機分子、金属含有化合物、炭水化物、ペプチド、タンパク質、ペプチド模倣物、糖タンパク質、リポタンパク質、核酸、抗体などを含む化合物の巨大なライブラリーを調製するための方法は、当該分野で周知であり、例えば、Ｈｕｓｅの、米国特許第５，２６４，５６３号；Ｆｒａｎｃｉｓら、Ｃｕｒｒ．Ｏｐｉｎ．Ｃｈｅｍ．Ｂｉｏｌ．２：４２２－４２８（１９９８）；Ｔｉｅｔｚｅら、Ｃｕｒｒ．Ｂｉｏｌ．，２：３６３－３７１（１９９８）；Ｓｏｆｉａ，Ｍｏｌ．Ｄｉｖｅｒｓ．３：７５－９４（１９９８）；Ｅｉｃｈｌｅｒら、Ｍｅｄ．Ｒｅｓ．Ｒｅｖ．１５：４８１－４９６（１９９５）；などに記載される。多数の天然化合物および合成化合物を含むライブラリーもまた、市販の供給源から入手し得る。

【００８６】

本発明の方法における試験のための異なる候補化合物の数は、この方法の適用に依存する。例えば、手で行うスクリーニング手順において、またはいくつかの予測されるリガンド、アゴニストまたはアンタゴニストの中で効力を比較することが望まれる場合、１つまたは少数の候補化合物が有利である。しかしながら、候補化合物が多くなるほど、スクリーニングアッセイにおいて、所望の活性を有する化合物を同定する可能性が高くなることが理解される。さらに、多数の化合物は、自動化されたハイスループットのスクリーニングアッセイにおいて処理され得る。従って、「１つ以上の候補化合物」は、例えば、２つ以上、例えば５以上、１０以上、１５以上、２０以上、５０以上または１００以上あるいはそれより多くの異なる化合物、例えば、約10^3、10^5または10^7の異なる化合物であり得る。

【００８７】

プロキネチシン（ｐｒｏｋｉｎｅｔｉｃｉｎ）レセプターリガンドを同定するための適切な調製物は、この調製物がＧＩ平滑筋組織上で発現されるプロキネチシンレセプターと類似の親和性および特異性を有するプロキネチシンに結合するための適切なコンフォメーションの、プロキネチシンレセプターを含む場合に限り、組織、細胞、細胞膜、または精製されたプロキネチシンレセプターを使用する。

【００８８】

１つの実施形態において、調製物は、腸管平滑筋調製物、例えば哺乳動物の回腸、胃底部の（ｆｕｎｄｉｃ）筋肉または近位結腸調製物、あるいはそれらの膜調製物である。適切な腸管平滑筋調製物は、本実施例に記載の方法により調製される、モルモット回腸調製物である。

【００８９】

別の実施形態において、調製物は、プロキネチシンレセプターを発現する細胞株かまたはその膜調製物である。プロキネチシンレセプターを発現する細胞株は、実施例に記載される競合結合アッセイのような、当該分野で公知の方法によって同定され得る。プロキネチシンレセプターを発現する例示的な細胞株は、黒色腫細胞株Ｍ２Ａ７である（ＡＴＣＣ ＣＲＬ－２５００としてＡｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎから入手可能である）。プロキネチシンレセプターを発現する他の細胞株としては、Ｍ２黒色腫細胞（Ｃｕｎｎｉｎｇｈａｍら、Ｓｃｉｅｎｃｅ ２５５；３２５－３２７（１９９２））およびＲＣ－４Ｂ／Ｃ下垂体腫瘍細胞（ＡＴＣＣ ＣＲＬ－１９０３）が挙げられる。

【００９０】

プロキネチシンレセプターを発現しない適切なコントロール細胞株は、ＨＥＫ２９３である（ＣＲＬ－１５７３としてＡｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎから入手可能である）。他のコントロール細胞としては、ＣＯＳ－７、ＣＯＳ－１、Ｌｔｋ－、ＮＩＨ３Ｔ３、Ｃ６、ＮＳ１０ＹおよびＨＴ－２９細胞が挙げられる。

【００９１】

レセプターリガンドを同定するための適切なアッセイは、当該分野で公知である。このようなアッセイは、候補化合物のレセプター調製物への結合を直接決定する工程を包含し得る。プロキネチシンレセプターを含まない適切なコントロール調製物が利用可能である場合、直接的なアッセイが、適切である。このようなアッセイはまた、レセプター調製物への結合についてプロキネチシンポリペプチドと競合する候補化合物の能力を決定する工程を包含し得る。競合アッセイは、候補化合物を検出可能に標識化し、そして標識化されないプロキネチシンポリペプチドとこの化合物とを競合させるか、または検出可能に標識化されたプロキネチシンポリペプチドと標識化されない候補化合物とを競合させることによって実施され得る。

【００９２】

本明細書中で使用される場合、用語「検出可能に標識化された」とは、任意の分析用手段によって検出可能な部分での誘導またはこの部分との結合のことをいう。例示的な検出可能な部分は、放射性同位元素（例えば、^{14}Ｃ、^{131}Ｉ、^{32}Ｐまたは^{3}Ｈ）、蛍光色素（例えば、フルオレセイン、グリーン蛍光タンパク質）、強磁性体、または発光物質である。このような部分を用いて有機化合物および無機化合物を検出可能に標識化する方法は、当該分野において周知である。

【００９３】

プロキネチシンレセプターリガンドを検出するために適切である例示的な競合結合アッセイが、以下の実施例に記載される。ハイスループットアッセイを含む他の適切なレセプター結合アッセイは、例えば、Ｍｅｌｌｅｎｔｉｎ－Ｍｉｃｅｌｏｔｔｉら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．２７２：Ｐ１８２－１９０（１９９９）；Ｚｕｃｋら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ ９６：１１１２２－１１１２７（１９９９）；およびＺｈａｎｇら、Ａｎａｌ．Ｂｉｏｃｈｅｍ．２６８；１３４－１４２（１９９９）に記載される。

【００９４】

結合を検出するための他の適切なアッセイとして、例えば、シンチレーション近接アッセイ（ｓｃｉｎｔｉｌｌａｔｉｏｎ ｐｒｏｘｉｍｉｔｙ ａｓｓａｙ）（ＳＰＡ）（Ａｌｏｕａｎｉ、Ｍｅｔｈｏｄｓ Ｍｏｌ．Ｂｉｏｌ．１３８：１３５－４１（２０００））、ＵＶまたは化学的架橋（Ｆａｎｃｙ、Ｃｕｒｒ．Ｏｐｉｎ．Ｃｈｅｍ．Ｂｉｏｌ．４：２８－３３（２０００））、競合結合アッセイ（Ｙａｍａｍｕｒａら、Ｍｅｔｈｏｄｓ ｉｎ Ｎｅｕｒｏｔｒａｎｓｍｉｔｔｅｒ Ｒｅｃｅｐｔｏｒ Ａｎａｌｙｓｉｓ、Ｒａｖｅｎ Ｐｒｅｓｓ、Ｎｅｗ Ｙｏｒｋ、１９９０）、表面プラズモン共鳴（ＳＰＲ）（Ｗｅｉｎｂｅｒｇｅｒら、Ｐｈａｒｍａｃｏｇｅｎｏｍｉｃｓ １：３９５－４１６（２０００））のような生体分子相互作用分析（ＢＩＡ）、質量分析法（ＭＳ）（ＭｃＬａｆｆｅｒｔｙら、Ｓｃｉｅｎｃｅ ２８４：１２８９－１２９０（１９９９）およびＤｅｇｔｅｒｅｖら、Ｎａｔｕｒｅ Ｃｅｌｌ Ｂｉｏｌｏｇｙ ３：１７３－１８２（２００１））、核磁気共鳴法（ＮＭＲ）（Ｓｈｕｋｅｒら、Ｓｃｉｅｎｃｅ ２７４：１５３１－１５３４（１９９６）、Ｈａｊｄｕｋら、Ｊ． Ｍｅｄ． Ｃｈｅｍ．４２：２３１５－２３１７（１９９９）、およびＣｈｅｎおよびＳｈａｐｉｒｏ、Ａｎａｌ．Ｃｈｅｍ．７１：６６９Ａ－６７５Ａ（１９９９））および蛍光偏光アッセイ（ＦＰＡ）（Ｄｅｇｔｅｒｅｖら、上述、２００１）が挙げられる。適切な結合アッセイは、レセプター調製物の性質および純度ならびに候補化合物の数および性質に依存して選択され得る。

【００９５】

プロキネチシンレセプターリガンドであると決定された化合物は、それがプロキネチシンレセプターのアゴニストかまたはアンタゴニストかどうかを決定するために、さらに試験され得る。同様に、プロキネチシンレセプターリガンドであると決定された化合物は、その化合物がＧＩ平滑筋収縮能をポジティブに変調するかまたはネガティブに変調するかどうかを決定するために、本明細書中に記載されるアッセイのような当該分野で公知のインビトロまたはインビボアッセイを使用して、さらに試験され得る。

【００９６】

本発明はさらに、プロキネチシンレセプターアゴニストを同定する方法を提供する。この方法は、プロキネチシンレセプターを含有する調製物を１つ以上の候補化合物と接触させることにより実施され、プロキネチシンレセプターシグナルの生成を選択的に促進する化合物を同定する。このような化合物は、プロキネチシンレセプターアゴニストとして特徴付けられる。

【００９７】

本発明はまた、プロキネチシンレセプターアンタゴニストを同定する方法を提供する。この方法は、プロキネチシンレセプターを含有する調製物を、プロキネチシンの存在下で１つ以上の候補化合物と接触させることにより実施され、プロキネチシンレセプターシグナルの生成を選択的に阻害する化合物を同定する。このような化合物は、プロキネチシンレセプターアンタゴニストとして特徴付けられる。本発明の方法を使用して、配列番号：１６および１８として示されたプロキネチシン変異体は、プロキネチシンレセプターアンタゴニストであると同定された。

【００９８】

本発明の方法は、適切な濃度のプロキネチシンの存在下（例えば、プロキネチシンのＥＣ$_{50}$の１０倍以内）で、実施され得る。従って、プロキネチシンレセプターを介するシグナル伝達についてプロキネチシンと競合するアゴニスト、またはプロキネチシンのシグナル伝達活性を間接的に増強するアゴニストは、容易に同定され得る。同様に、プロキネチシンのプロキネチシンレセプターへの結合を阻害するアンタゴニスト、またはプロキネチシンのシグナル伝達活性を間接的に減少させるアンタゴニストもまた、同定され得る。

【００９９】

本明細書中で使用される場合、用語「プロキネチシンレセプターアゴニスト」とは、プロキネチシンレセプターを介する正常なシグナル伝達を、選択的に活性化または増強させる分子のことをいう。本明細書中で使用される場合、用語「プロキネチシンレセプターアンタゴニスト」とは、プロキネチシンレセプターを介する正常なシグナル伝達を、選択的に阻害または減少させる化合物のことをいう。

【０１００】

治療適用のために、プロキネチシンレセプターアゴニストは好ましくは、約10^{-7}Ｍ未満、例えば10^{-8}Ｍ未満、そして好ましくは10^{-9}または10^{-10}Ｍ未満のＥＣ$_{50}$を有し、そしてプロキネチシンレセプターアンタゴニストは、好ましくは約10^{-7}Ｍ未満、例えば10^{-8}Ｍ未満、そして好ましくは10^{-9}または10^{-10}Ｍ未満のＩＣ$_{50}$を有する。しかしながら、化合物の安定性、選択性および毒性にも依存して、高いＥＣ$_{50}$を有するプロキネチシンレセプターアゴニスト、または高いＩＣ$_{50}$を有するプロキネチシンレセプターアンタゴニストはまた、治療上有用であり得る。

【０１０１】

本明細書中に記載される場合、内因性のプロキネチシンレセプターは、Ｇタンパク質共役レセプターである。プロキネチシンレセプターを介するシグナル伝達は、細胞内カルシウムイオン動員を促進し、これは、プロキネチシンレセプターが、Ｇαｑ含有Ｇタンパク質と通常結合していることを示唆する。従って、プロキネチシンレセプターを介するシグナル伝達は、細胞内カルシウムイオン動員を検出する、当該分野において公知の任意のアッセイによって検出され得る。このようなアッセイは、プロキネチシンの存在下または非存在下でにおいて、実施され得る。

【０１０２】

カルシウムイオン動員を検出するための適切な調製物は、腸管平滑筋調製物のような、プロキネチシンレセプターを発現する組織もしくは細胞株またはＭ２Ａ７細胞株であり得る。

【０１０３】

カルシウムイオン動員は、蛍光標識指示薬または放射性標識指示薬のような、検出可能に標識化されたCa^{2+}イオン指示薬をおよび適切な検出システムを使用して簡便に測定され得る。例示的なCa^{2+}イオン指示薬として、ＦＬＵＯ－３　ＡＭ、ＦＬＵＯ－４　Ａ

M、FURA－2、INDO－1、FURA RED、CALCIUM GREEN、CALCIUM ORANGE、CALCIUM CRIMSON、BTCおよびOREGON GREEN BAPTAが挙げられる（例えば、Grynkiewitzら、J. Biol. Chem. 260：3440－3450（1985）；Sullivanら、Calcium Signal Protocol, Methods in Molecular Biology 114：125－133、David G. Lambert編、Human Press、Totowa、New Jersey（1999）；Miyawakiら、Proc. Natl. Acad. Sci. USA 96：2135－2140（1999）；およびCowardら、Analyt. Biochem. 270：242－248（1999）を参照のこと）。カルシウムイオン動員をモニタリングするための適切な検出システムは、Molecular Devicesから入手可能なFLIPR（Fluorometric Imaging Plate Reader）システムである。

【0104】

細胞表面レセプターに対するGαサブユニットの特異性は、GαのC末端の5つのアミノ酸により決定される。従って、種々のシグナル伝達経路は、既知のGαのC末端の5残基を含有するかまたはADP－グルコースレセプターと結合すると予測されるGαキメラ（例えばGαqまたはGα16のような乱交雑（promiscuous）のGα）を組換え発現することによって、プロキネチシンレセプターによるGタンパク質共役シグナルの変換を決定するためにアッセイされ得、タンパク質の残部は、アッセイされるシグナル伝達経路と結合するGαに対応する（例えば、cAMP生成の増加をアッセイするためのGαs、または細胞内Ca^{2+}動員をアッセイするためのGαq）。Gαサブユニットの既知の配列に基づいて、キメラGαをコードする核酸分子が、構築され得、そして当該分野で公知の方法によって発現され得、これらは例えば、Conklinら、Nature 363：274－276（1993）、およびKomatsuzakiら、FEBS Letters 406：165－170（1995）に記載される。

【0105】

従って、アッセイシステムにおいて内因的にまたは組換えにより発現されるGαサブユニットに依存しては、決定され得るプロキネチシンレセプターシグナルとして、カルシウムイオン動員；アラキドン酸、アセチルコリン、ジアシルグリセロール、cGMP、cAMP、イノシトールリン酸およびそのイオンの産生または遊離の増加または減少；細胞膜電位の変化；GTP加水分解；アミノ酸の流入または流出；細胞内タンパク質のリン酸化の増加または減少；および内因性遺伝子の転写の活性化または上記に記載される任意のセカンドメッセンジャー経路の下流のプロモーター－レポーター構築物の活性化が挙げられる。

【0106】

Gタンパク質共役レセプターのアゴニスト活性およびアンタゴニスト活性を検出するための適切なアッセイとして、ハイスループットシグナル伝達アッセイが挙げられ、これは当該分野で周知であり、かつ概観され、例えば、総説として、例えば、Tateら、Trends in Biotech. 14：426－430（1996）がある。

【0107】

プロキネチシンレセプターに選択的に結合する化合物またはプロキネチシンレセプターを介するシグナル伝達を変調する化合物（例えば、リガンド、アゴニストおよびアンタゴニスト）を同定するためのアッセイ方法は、一般的にコントロールとの比較を包含する。「コントロール」の型の1つは、コントロールが候補化合物に曝露されないことを除いて、試験調製物と同様に処理される調製物である。別の型の「コントロール」は、コントロール調製物がレセプターを発現しないか、またはプロキネチシンに対して選択的に応答しないように改変されていることを除いて、試験調製物と類似する調製物である。このような状況の下、試験調製物の候補化合物に対する応答は、実質的に同一の反応条件の下で、コントロール調製物の同一の化合物に対する応答（または応答の欠如）と比較される。

【０１０８】
プロキネチシンレセプターアゴニストまたはアンタゴニストであると決定される化合物は、それがＧＩ平滑筋収縮能をポジティブに変調するかまたはネガティブに変調するかどうかを決定するために、本明細書中に記載されるアッセイのような当該分野で公知のインビトロまたはインビボアッセイを使用して、さらに試験され得る。
【０１０９】
本発明はまた、プロキネチシンリガンド、アゴニスト、アンタゴニストを同定するためのアッセイにおいて使用するための、適切な組成物を提供する。適切な組成物は、プロキネチシンレセプターおよびプロキネチシンポリペプチド（これらは必要に応じて検出可能に標識され得る）を含有する細胞または組織を含む。例示的な組成物は、回腸平滑筋調製物のようなＧＩ平滑筋調製物を含む。さらなる例示的な組成物は、Ｍ２Ａ７のような細胞株を含む。
【０１１０】
本明細書中に記載されるプロキネチシンポリペプチド、ならびに記載されるスクリーニング方法によって同定された、プロキネチシンリガンド、アゴニストおよびアンタゴニストは、潜在的な治療用の化合物であり、これらは、胃腸の異常な運動性に関連する状態を有する個体、あるいはプロキネチシンまたはそのレセプターの改変された発現または活性に関連する他の状態を有する個体に投与され得る。本発明の化合物は、処置される状態；個体の体重、性、年齢および健康状態；特定の化合物の生物学的性質、生体活性、バイオアベイラビリティ、および副作用に適切な様式および適切な量で；併用処置レジメンと両立し得る様式で処方および投与され得る。ヒトにおける特定の治療適用のための適切な量および処方は、本明細書中に記載されるインビトロでの結合アッセイおよびシグナル伝達アッセイにおける化合物の活性に基づいてか、または特定の障害について認められている動物モデルから推定され得る。
【０１１１】
治療用化合物の総量は、単回の用量としてかまたは比較的短期間にわたる注入によって投与され得、あるいはより長い期間にわたる複数回投与の用量を用いて投与され得る。さらに、この化合物は、徐放性（ｓｌｏｗ－ｒｅｌｅａｓｅ）マトリクスで投与され得、これは全身的な送達のために標的組織部位またはその近くに移植され得る。治療用化合物の制御された放出のために有用な検討されるマトリクスは、当該分野で周知であり、そして例えば、ＤｅｐｏＦｏａｍ（登録商標）、生体高分子、マイクロポンプなどの基質が挙げられる。
【０１１２】
治療用化合物は、以下のような当該分野で公知の経路、例えば：静脈内に、筋肉内に、皮下に、眼窩内に、関節内に、腹腔内に、槽内に（ｉｎｔｒａｃｉｓｔｅｒｎａｌｌｙ）、関節腔内に、大脳内に、経口的に、膣内に、直腸内に、局所的に、鼻腔内にまたは経皮的に、哺乳動物に投与され得る。ヒトに投与するための好ましい経路は、経口投与および静脈内投与であり、経口経路は特に好ましい。
【０１１３】
好ましくは、治療用化合物は、化合物および薬学的に受容可能なキャリアを含む、薬学的組成物として哺乳動物に投与される。薬学的に受容可能なキャリアの選択は、化合物の投与経路ならびにその特定の物理学的性質および化学的性質に依存する。薬学的に受容可能なキャリアは、当該分野において周知であり、そして生理学的に緩衝化した生理的食塩水のような無菌の水性溶媒、およびグリコール、グリセロール、油（例えば、オリーブ油）および注射可能な有機エステルのような他の溶媒またはビヒクルが挙げられる。薬学的に受容可能なキャリアは、さらに、化合物を安定化し、化合物の溶解性を増加させ、または化合物の吸収を増大させる、生理学的に受容可能な化合物を含む。このような、生理学的に受容可能な化合物としては、グルコース、ショ糖またはデキストランのような炭水化物；アスコルビン酸またはグルタチオンのような抗酸化薬；キレート剤；および低分子量のタンパク質が挙げられる。

【０１１４】
血液脳関門を通過するため、または細胞膜を透過するために、化合物および組成物を必要とする用途については、化合物の親油性を増加する処方物が、特に望ましい。例えば、本発明の化合物は、リポソームに取り込まれ得る（Ｇｒｅｇｏｒｉａｄｉｓ、Ｌｉｐｏｓｏｍｅ　Ｔｅｃｈｎｏｌｏｇｙ、Ｉ～ＩＩＩ巻、第２版（ＣＲＣ　Ｐｒｅｓｓ、Ｂｏｃａ　Ｒａｔｏｎ　ＦＬ（１９９３））。リン脂質または他の脂質からなるリポソームは、無毒で、生理学的に受容可能な、そして代謝可能な、比較的作製および投与の容易なキャリアである。
【０１１５】
ひとつの実施形態において、プロキネチシンポリペプチドまたはプロキネチシンアゴニストを含有する薬学的組成物は、胃腸運動を刺激するのに有効な量で哺乳動物に投与される。障害されたＧＩ運動は、過敏性腸症候群、糖尿病性胃不全麻痺、術後の腸閉塞、慢性便秘症、および胃腸の逆流疾患を含む種々の障害の共通する臨床的な症状であり、従って、本発明の組成物は、そのような障害の症状を回復させるのに使用され得る。
【０１１６】
別の実施形態において、プロキネチシンアンタゴニストを含有する薬学的組成物は、胃腸運動を阻害するのに有効な量で哺乳動物に投与される。過剰なＧＩ運動は、下痢に関連し、感染症、吸収不全性障害、炎症性腸障害、および腸癌の共通する症状である。従って、本発明の拮抗性の組成物は、そのような障害の症状を回復させるのに使用され得る。
【０１１７】
ラットの脳室へのＢｖ８またはＭＩＴ１の注射は、痛覚過敏を引き起こす（Ｍｏｌｌａｙら、Ｅｕｒ　Ｊ　Ｐｈａｒｍａｃｏｌ．３７４：１８９－１９６（１９９９））。従って、プロキネチシンアンタゴニスト（例えば、プロキネチシン抗体、配列番号１６または１８を含有する変異ポリペプチド、および本明細書中に記載される方法によって決定される他の化合物）が、鎮痛剤（ペインキラー（ｐａｉｎ　ｋｉｌｌｅｒ））として作用するのに有効な量で哺乳動物に投与され得る。
【０１１８】
当業者は、本発明の薬学的組成物を投与するのに適切な他の条件を決定し得、そして治療の安全性および有効性をモニターし得る。
【０１１９】
好ましくは、本発明の薬学的組成物を投与される哺乳動物は、ヒトであるが、特定の適用に関しては、その動物は、代わりに獣医学的動物または研究用動物であり得る。例えば、前臨床試験において、本発明の方法は、ヒト疾患の確実なモデルとして役立つ動物（例えば、非ヒト霊長類、ブタ、イヌ、ネコ、およびゲッ歯類（例えば、ラット、マウスおよびモルモット））を用いて実施され得る。当業者は、これらの動物が、目的のヒト疾患の適切なモデルとして役立つことを理解する。
【０１２０】
以下の実施例は、例示の目的であって、本発明を制限するものではないことが意図される。
【０１２１】
（実施例Ｉ）
（プロキネチシン１および２の同定、調製および特徴づけ）
この実施例は、ヒトプロキネチシン１および２、ならびにそれらの改変クローニング、組換え発現、精製および生物学的活性、を示す。
【０１２２】
（材料および方法）
（ＲＮＡブロット）
ポリＡ　ＲＮＡの規格化されたサンプルを、含有するヒト多重組織ＲＮＡブロットを、製造業者の説明書（Ｃｌｏｎｔｅｃｈ）に記載されるように使用した。無作為なプライマー標識プローブ（プロキネチシン１　ｃＤＮＡおよびプロキネチシン２　ｃＤＮＡのヌクレ

オチド１～５５０および１～１１７８）を用いてブロットをプローブし、シグナルを、Ｋｏｄａｋ　ＸＡＲフィルムに感光させることで可視化した。

【０１２３】

（組換えプロキネチシンの生成、再折り畳みおよび精製）

成熟プロキネチシンのコード配列を、原核細胞（ｐｒｏｋａｃｙｏｔｉｃ）発現ベクターｐＧＥＸ－３Ｘ（Ｐｈａｒｍａｃｉａ）中にクローン化した。ＧＳＴ（グルタチオン－ｓ－トランスフェラーゼ）ｔａｇの第Ｘａ因子プロテアーゼ消化部位と成熟プロキネチシンとの間の延長ヌクレオチドを、部位特異的変異誘発によって除去し、配列決定によって確認した。タンパク質精製を促進するために、６×Ｈｉｓ－ｔａｇを、Ｃ末端に付加し、その結果、融合タンパク質を、Ｎｉ－ＮＴＡアフィニティークロマトグラフィー（Ｑｉａｇｅｎ）で精製し得た。

【０１２４】

融合タンパク質の生成方法は、以下のようである。Ｅ．ｃｏｌｉ細胞（ＢＬ２１）を、ＯＤ０．８まで増殖し、そして６００ｎＭのＩＰＴＧを用いて３７℃で２時間誘導した。次いで、その細胞をペレット状にし、洗浄し、そして緩衝液Ａ（６Ｍのグアニジン塩酸塩、１００ｍＭのNaH_2PO_4および１０ｍＭのＴｒｉｓ、ｐＨ８．０）で溶解した。融合タンパク質をＮｉ－ＮＴＡビーズに結合させ、次いで緩衝液Ｃ（８Ｍの尿素、１００ｍＭのNaH_2PO_4、および１０ｍＭのＴｒｉｓ、ｐＨ６．３）および緩衝液Ｄ（８Ｍの尿素、１００ｍＭのNaH_2PO_4、および１０ｍＭのＴｒｉｓ、ｐＨ５．９）で広範囲に洗浄した。融合タンパク質結合ビーズを第Ｘａ因子消化緩衝液（５０ｍＭのＴｒｉｓ、１５０ｎＭのＮａＣｌ、および１ｍＭの$CaCl_2$、ｐＨ７．５）で平衡状態にした。第Ｘａ因子消化を、１０ｎｇ／μの融合タンパク質を用いて室温で一晩実施した。次いで、切断されたＧＳＴタグを緩衝液Ｄで洗い流した。次いで、成熟プロキネチシンを緩衝液Ｅ（８Ｍの尿素、１００ｍＭのNaH_2PO_4、および１０ｍＭのＴｒｉｓ、ｐＨ４．５）で溶出した。画分をＳＤＳ－ＰＡＧＥで分析した。次いで、プールされた組換えプロキネチシンを以下のように再び折り畳んだ。タンパク質を緩衝液Ｅで１００μｇ／ｍｌまで希釈し、そして再生緩衝液（４Ｍの尿素、５ｍＭのシステイン、０．０２％のＴｗｅｅｎ－２０、１０％のグリセロール、１０ｍＭのＴｒｉｓ、１５０ｍＭのＮａＣｌ、１００ｍＭのNaH_2PO_4、ｐＨ８．３）に対して透析した。次いで、新しい再生緩衝液（２Ｍの尿素以外は同じ成分）を加え、そして少なくともあと１度再生緩衝液を換えて、あと４日間透析し続けた。次いで、再び折り畳まれたタンパク質をスピンカラム（Ｑｕｉａｇｅｎ）で脱塩し、レセプター結合アッセイまたはバイオアッセイによって分析した。最終的な精製を逆相ＨＰＬＣ（ＬＫＢ）で実施した。機能性タンパク質を０．０８％のトリフルオロ酢酸および１０～５０％のアセトニトリル勾配で溶出した。このタンパク質の溶出を２０６ｎｍでモニターした。次いで、トリフルオロ酢酸およびアセトニトリルを凍結乾燥によってエバポレートした。

【０１２５】

（質量分析法）

エレクトロスプレーイオン化質量分析を６．５Ｔ　ＨｉＲｅｓＥＳＩ　フーリエ変換質量分析器（ＩｏｎＳｐｅｃ、Ｉｒｖｉｎｅ、ＣＡ）で、前に記載したように実施した（Ｌｉら、Ａｎａｌ．Ｃｈｅｍ．６６：２０７７－２０８３（１９９４））。ＲＰ－ＨＰＬＣから溶出されたタンパク質を凍結乾燥し、そしてナノ純水（ｎａｎｏｐｕｒｅ　ｗａｔｅｒ）に溶解し、次いでメタノール－水－酢酸（４９．５％：４９．５％：１％（ｖ／ｖ／ｖ））で１ｎＭの濃度まで希釈した。１００μｌのサンプルを注入した。

【０１２６】

（単離された器官標本中の平滑筋収縮の測定）

モルモットをCO_2で安楽死させ、そして盲腸に対して約１０ｃｍ吻側の回腸切片（２～３ｃｍ）を取り出した。その回腸をクレブスリンガー炭酸水素塩（ＫＲＢ）緩衝液（１２４ｍＭのＮａＣｌ、５ｍＭのＫＣｌ、１．３ｍＭの$MgSO_4$、２６ｎＭの$NaHCO_3$、１．２ｍＭのKH_2PO_4、１．８ｍＭの$CaCl_2$、および１０ｍＭのグルコース）

できれいに洗浄し、ＫＲＢ緩衝液を含有する器官バスに縦方向に取りつけた。等長性収縮を強制置換トランスデューサーおよびポリグラフで前に記載したように測定した（Ｔｈｏｍａｓら、Ｂｉｏｃｈｅｍ．Ｐｈａｒｍａｃｏｌ．５１：７７９－７８８（１９９３））。この回腸を１時間インキュベートし、次いで、ムスカリンアゴニスト、オキソトレモリン－Ｍの３回の試験投与を、その収縮が再現可能であり、十分な大きさであることを確実にするために加えた。その回腸を洗浄し、そして各試験投与間は５分おいておいた。その縦方向の底のストリップおよびジグザグ気管の調製物を前記（１５）のように調製した。単離された結腸（近位のおよび遠位の）をＳａｗｙｅｒら、Ｊ．Ｐｈａｒｍａｃｏｌ．Ｅｘｐ．Ｔｈｅｒ．２８４：２６９－２７７（１９９８）に記載されるように調製した。大動脈および大腿動脈を成体ラットから取り出した。１０ｍｌのバスを大動脈および大腿動脈実験に使用した。張力を腸組織および気管調製物に対しては０．５ｇ、ならびに大動脈および大腿動脈に対しては２ｇの初期負荷でＧｒａｓｓポリグラフに記録した。

【０１２７】

（ヨウ素化）

プロキネチシン１をＦｒａｋｅｒおよびＳｐｅｃｋ、Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍｕｎ．８０：８４９－８５７（１９７８）に記載されるようなヨウ素化法（ｉｏｄｏｇｅｎ　ｍｅｔｈｏｄ）によってヨウ素化した。手短に言えば、再び折り畳まれたプロキネチシン１（７．５μｇ）を５０μＬの０．５Ｍ　ＰＢＳ緩衝液（ｐＨ７．２）中の５０μｇのヨウ素と共に、室温で１５分間、インキュベートした。その反応をヨード管から混合物を除去することによって停止させ、１ｍＭのＮａＩを含有する１００μＬのＰＢＳを含むミクロチューブ中に配置した。１ｍＭのＮａＩおよび０．１％のＢＳＡを含む１００μＬのＰＢＳを加えることに続いて、遊離ヨウ素をＢｉｏ－Ｇｅｌ　Ｐ２に対するゲル濾過によって除去し、そして放射能をカウントした。全ての放射能が、回収された（回収率８０％）６．０μｇのプロキネチシン１に取り込まれると仮定すると、比放射能は、８１９ｃｐｍ／ｆｍｏｌまたは３７２Ｃｉ／ｍｏｌｅとして計算された。

【０１２８】

（レセプター結合）

膜を、バックグラウンド結合を減少するのに適用した差次的な遠心分離（８００ｇ、１０，０００ｇ、１００，０００ｇ、４℃、各２０分）のさらなる工程を除いて、Ｌｉら、Ｍｏｌ．Ｐｈａｒｍａｃｏｌ．５７：４４６－４５２（２０００）に記載されるようにモルモットの回腸から調製した。インキュベーションを室温で、０．１％のＢＳＡを含有する４ｍｌの緩衝液（２０ｍＭのＴｒｉｓ－ＨＣｌ　ｐＨ７．４）中で実施した。飽和結合のために、１．５～２００ｐＭの標識されたプロキネチシン１を使用した。非特異性結合を２０ｎＭの標識されていないプロキネチシン１の存在下で規定した。置換実験のために、標識されていないタンパク質を３ｍｌの総反応体積中で１時間、膜と共にプレインキュベートし、次いで^{125}Ｉ－プロキネチシン１（２０ｐＭ）を加えた。その膜をさらに３時間、室温でインキュベートした。この結合混合物をＧＦ－Ｃガラスフィルターを通して濾過し、そして１０ｍｌの２０ｍＭ　Ｔｒｉｓ－ＨＣｌ（ｐＨ７．４）で洗浄した。フィルター上に保持された放射能をγカウンターでカウントした。このデータをＬＩＧＡＮＤプログラムで分析した。

【０１２９】

（結果）

（２つの哺乳動物ホモログ（カエル　Ｂｖ８およびヘビ　ＭＩＴ１）の同定および分析）

カエル　Ｂｖ８およびヘビ　ＭＩＴ１の哺乳動物ホモログを同定する試みにおいて、複数データベース（ＥＳＴおよびＨＧＴＳ）を、クエリーとしてこれらのタンパク質配列を用いてＢＬＡＳＴ２．１アルゴリズム（Ａｌｔｓｃｈｕｌら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２５：３３８９－３４００（１９９７））を使用して検索した。このＥＳＴデータベースの検索によって２つのヒトＥＳＴ配列（ａｉ２７７３４９およびａａ８８３７６０）の存在が明らかとなった。これらの２つのＥＳＴクローンの配列分析によって、ａａ８８３７６０が、１９アミノ酸のシグナルペプチドおよび８６アミノ酸の成熟タンパク

質を有する推定タンパク質（Ｈｅｉｊｎｅ　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．１４：４６８３－４６９０（１９８６））をコードすることが明らかとなった。クローンａｉ２７７３４９は、部分的なｃＤＮＡであることが明らかとなった。ＥＳＴクローンａｉ２７７３４９の全長配列は、鋳型としてヒトの脳のｃＤＮＡと５’ＲＡＣＥによってクローン化され、２７アミノ酸のシグナルペプチドおよび８１アミノ酸の成熟タンパク質を含有することが明らかとなった（図１）。これらのタンパク質をプロキネチシン１およびプロキネチシン２とそれぞれ名付けた（以下参照）。

【０１３０】

配列分析によって、プロキネチシン１および２が、約４４％のアミノ酸同一性を有し、１０個の保存されたシステインを含有することが明らかとなった。両方のプロキネチシンはカエル　Ｂｖ８およびヘビ　ＭＩＴ１と約４３％の同一性を示した。興味深いことに、第１のシステイン（ＡＶＩＴＧＡ）より前のＮ末端配列は、全ての種の間で完全に保存され（図１）、この領域の機能的な意義を示唆した。マウスのプロキネチシン１遺伝子の予備的な分析は、このＮ末端配列ＡＶＩＴＧが、システイン富化された配列が他のエキソン由来であるにも拘わらず、第１エキソンから誘導され、またシグナルペプチド配列を含有することを示す。

【０１３１】

（プロキネチシンは、種々の成体組織および胚性組織において発現される）

プロキネチシン発現の最初の調査のように、ヒトマスターブロット（ｍａｓｔｅｒｒ　ｂｌｏｔ）を、ヒトプロキネチシンｃＤＮＡのフラグメントを使用してプローブした。両方のプロキネチシンは種々の成体組織に広範囲に発現しており、プロキネチシン１は、プロキネチシン２と比較して一般的により高い発現レベルであった（図２Ａ、２Ｂ）。プロキネチシン２の発現は、プロキネチシン１の発現に匹敵するようであるにも拘わらず、例外がＧＩ路、肝臓および脾臓中で見出された。最も高いレベルのプロキネチシン１発現は、精巣および胎盤中で見出された。ヒト胎児組織の間では、その全てが同様のレベルの発現を示し、やはりプロキネチシン１の発現レベルは、プロキネチシン２よりも高かった。

【０１３２】

ヒト脳におけるプロキネチシンの発現を、ノーザンブロット分析によってさらに試験した。図２Ｄは、プロキネチシン１　ｍＲＮＡサイズが、約１．５ｋｂであり、被殻、視床、側頭葉、および脳梁において最も高く発現することを示した。ヒト脳におけるプロキネチシン２発現は、検出不可能であった（データ示さず）。

【０１３３】

（ヒトプロキネチシンの生成、再折り畳みおよび精製）

Ｎ末端配列が完全に保存されていたので（図１）、確証的なＮ末端残基を伴う組換えタンパク質を、まずＧＳＴ融合タンパク質として生成し、続いてプロテアーゼ第Ｘａ因子で消化して、ＧＳＴタグを除去した。図３は、正確な分子量を有するタンパク質がＸａ因子消化によって生成されたことを示す。

【０１３４】

モルモット回腸調製物でのバイオアッセイによって、折り畳まれていない組換えタンパク質が不活性であることが明らかとなった。ＮＭＲ試験は、ＭＩＴ１の１０個のシステインが、５つのジスルフィド結合に形成され（Ｂｏｉｓｂｏｕｖｉｅｒら、Ｊ．Ｍｏｌ．Ｂｉｏｌ．２８３：２０５－２１９（１９９８））、そしてこれらの１０個のシステインが、全てヒトプロキネチシンに保存されることを示し、このことは、これらのジスルフィド結合が、おそらくタンパク質生物活性に必須であると考えられた。従って、かなりの試みが、適切なジスルフィド結合形成（９４５の可能な組合せのうち）を保証するために充てられた。

【０１３５】

ほとんど全ての組換えタンパク質が沈殿したので、再折り畳み緩衝液への単一の希釈中での最初の再折り畳みは、失敗であった。これはおそらく分子間のジスルフィド結合の形成に起因する。次いで、タンパク質凝集を制御し、ジスルフィド結合生成を遅くする改変を

採用した。これらの改変は以下：１）分子間ではなく分子内のジスルフィド結合を好ましく形成するために、１００μｇ／ｍｌ以下までタンパク質濃度を減少させること；２）直接的な希釈の代わりに透析方法によってタンパク質を再び折り畳むこと；３）全ての透析緩衝液において、より高いレベルの尿素（４Ｍおよび続いて２Ｍ）を使用すること；４）レドックス対から酸化剤システインまたは酸化されたグルタチオン省略し、５ｍＭのシステインまたは３ｍＭの還元グルタチオンのみを残すこと；５）タンパク質凝集をさらに減少するためにグリセロールを加えること；６）再折りたたみ工程を始める前に、４℃までタンパク質および緩衝液を冷却すること、を包含する。これらの慎重に制御された工程は、最小のタンパク質凝集を伴う組換えプロキネチシンの再折り畳みを成功させる。

【０１３６】

最後に、再折り畳みされたタンパク質を、ＲＰ－ＨＰＬＣによって精製した（図３Ａ、レーン５）。質量分析によって、再折り畳みされた組換えプロキネチシン１における５つのジスルフィド結合の生成を確認した。６×Ｈｉｓでタグされたプロキネチシン１の分子量は、フーリエ変換質量分析器で決定され、１０４８０．３０Ｄａであることが明らかとなった（図３Ｃ）。還元された形態中に存在する１０のシステイン全てを有する計算された分子量が、１０４９０．２０であったので、５つの対のジスルフィド結合が、明らかに形成された。

【０１３７】

（再折り畳みされた組換えプロキネチシンは胃腸の平滑筋を強く収縮させる）

次いで、再折り畳みされた組換えプロキネチシンを、単離された平滑筋調製物上で試験した。図４は、組換えプロキネチシン１とプロキネチシン２の両方が、モルモットの回腸縦走筋の収縮を強力に刺激するＥＤ５０値は、それぞれ約０．４６ｎＭおよび０．９０ｎＭであることを示す。プロキネチシン１（５ｎＭ）はまた、底部の筋肉片および近位の結腸の収縮を刺激するが、遠位の結腸においては影響はない（２５ｎＭ、データ示さず）。組換えプロキネチシン１（２５ｎＭ）はまた、大動脈および大腿動脈、気管および胆嚢を含む他の平滑筋組織にも影響を及ぼさない。従って、プロキネチシンの収縮性の影響は、ＧＩ平滑筋に対して特異的であるようである。

【０１３８】

プロキネチシンの可能なシグナル伝達機構をプローブするために、多くのキナーゼおよびイオンチャネルインヒビターを試験した。テトロドトキシン（ＴＴＸ）は、神経の活動電位の伝達をブロックすることが公知であり、プロキネチシン１で刺激された回腸縦走筋収縮に影響を及ぼさず（図４Ｂ）、プロキネチシン１が平滑筋に直接的に作用することを示す。プロキネチシンの収縮機構は、多くの化合物でさらに調査され、その化合物としては以下が挙げられる：プロテインキナーゼＣインヒビターカルフォスチンＣ（１μＭ）、ホスホリパーゼＡ２インヒビター７、７－ジメチル－（５Ｚ，８Ｚ）－エイコサジエン酸（１０μＭ）、チロシンキナーゼインヒビターゲニステイン（５μＭ）、ＭＥＫインヒビターＰＤ０９８０５９（１０μＭ）およびＬ型カルシウムチャネルブロッカーベラパミル。ベラパミルのみが有効であり、１μＭで２ｎＭのプロキネチシン１の収縮性の影響を完全に阻害する（図４Ｃ）。同じ濃度のベラパミルはまた、１００ｎＭのオキソトレモリン－Ｍの収縮作用を完全にブロックした（図４Ｆ）。この結果は、ムスカリン性Ｍ３レセプター類が、回腸の収縮を媒介する（Ｅｇｌｅｎら、Ｐｈａｒｍａｃｏｌ．Ｒｅｖ．４８：５３１－５６５（１９９６）およびＥｈｌｅｒｔら、Ｍｕｓｃｕｒｉｎｉｃ Ｒｅｃｅｐｔｏｒｓ ａｎｄ Ｇａｓｔｒｏｉｎｔｅｓｔｉｎａｌ Ｓｍｏｏｔｈ Ｍｕｓｃｌｅ、Ｅｇｌｅｎ（編）、ＣＲＣ Ｐｒｅｓｓ、９２－１４７頁（１９９７））のと同様に、電位開口型のカルシウムチャネルを介して入るカルシウムは、プロキネチシンシグナル伝達に必須の要素であることを示す。

【０１３９】

（膜レセプターによって媒介されるプロキネチシンの生物学的活性）

モルモットのＧＩ平滑筋に対する組換えプロキネチシンの強力な収縮作用およびカルシウムチャネルブロッカーベラパミルの阻害影響は、プロキネチシンのＲセプター－媒介機構

を示唆する。プロキネチシンが選択的な膜レセプター、組換えプロキネチシンと相互作用するという直接的な証拠を提供するために、組換えプロキネチシンを^{125}Ｉで標識し、そしてレセプター結合実験を実施した。

【０１４０】

スキャッチャード解析は、プロキネチシン１の特異的結合が２つの部位モデルに最良適合であることを示した（Ｆ＝３８．７８、１つの部位モデルに対してＰ＜０．００１；図５Ａ）。高親和性定数および低親和性定数（K_d）は、それぞれ、５．０±０．８ｐＭおよび２２７±６３ｐＭ（ｎ＝３）であった。高親和性部位および低親和性部位についてのB_{max}は、それぞれ、７．８±１．２および２６．４±８．４ｆｍｏｌ／ｍｇのタンパク質であった（ｎ＝３）。競合実験は、特異的結合が組換え型プロキネチシン１によって置換されることを示した。この置換曲線はまた、２つの部位モデルに最良適合であった（高親和性部位および低親和性部位について、それぞれ、８．０±３．９ｐＭおよび１．５０±０．９ｎＭのK_i、ｎ＝３）（図５Ｂ）。図５Ｂはまた、プロキネチシン２が、標識されたプロキネチシン１と同様の親和性で置換されることを示す（２回の実験の平均で、高親和性についてのK_iは４．２ｐＭ、そして低親和性についてのK_iは１．２２ｎＭ）。

【０１４１】

多くのＧタンパク質共役レセプターに結合するアゴニストは、ＧＴＰによって阻害されるので、ＧＴＰγＳが、特定の^{125}Ｉ－標識されたプロキネチシン１結合性の任意の効果を有するかどうかを調べた。図５Ｂに示すように、ＧＴＰγＳは、^{125}Ｉ－プロキネチシン１結合性の濃度依存的阻害を引き起こした。最も高濃度（１０μＭ）で試験した場合、ＧＴＰγＳは、回腸膜に結合する特異的プロキネチシンの８５％を置換した。これらの結果は、プロキネチシンレセプターが、Ｇタンパク質共役レセプターファミリーに属することを示唆する。

【０１４２】

（プロキネチシンの安定性）

プロキネチシンの半減期を定量するための実験もまた、行った。静脈内に注射された、ヨウ素標識されたヒトプロキネチシン１の半減期は、モチリンについての１０分と比較して、約３時間であった。このモチリンもまた、ＧＩの運動性を増加させる小さなポリペプチドである。血液循環における適度に長い半減期は、治療効果を得るために欠かせない。従って、プロキネチシンは、治療法として有効であると考えられる。

【０１４３】

（プロキネチシンの構造／活性相関研究）

配列解析は、プロキネチシンが２つの機能ドメイン（すなわち、短いＮ末端およびシステインリッチなＣ末端）を含み得ることを示した。第１のシステインの前にあるこのＮ末端配列は、プロキネチシン内に完全に保存されているので（図１）、この領域は機能的に重要であることが予測された。

【０１４４】

プロキネチシンに加え、１０個のシステインモチーフはまた、コリパーゼ、腸管の脂質消化酵素リパーゼについてのコファクター、およびｄｉｃｋｋｏｐｆ（初期胚発生において重要な役割を有するタンパク質のファミリー）を含む多数の分泌されたタンパク質中においても見出される。

【０１４５】

多数のＮ末端置換変異、欠失変異、および挿入変異が構築され、そして組換えられて、再折り畳みされたタンパク質が産生される。回腸の平滑筋調製物を用いたバイオアッセイは、２５０ｎＭまでの濃度のこれらの変異タンパク質が、収縮を誘発し得ないことを示した（表１）。しかし、Ｎ末端欠失変異体（配列番号１６）およびＮ末端挿入変異体（配列番号１８）は、プロキネチシン１の収縮効果を弱く拮抗し得る。従って、プロキネチシンのＮ末端改変体（例えば、配列番号１６および１８）は、ＧＩ収縮性を阻害するための潜在的な治療法である。

【０１４６】

【表１】

表　1

	ポリペプチド	収縮活性	拮抗活性
野生型 (SEQ ID NO:3)	AVITGA[プロキネチシン　1]	+	-
挿入 (SEQ ID NO:15)	GILAVITGA[プロキネチシン　1]	-	ND
欠失 (SEQ ID NO:16)	VITGA[プロキネチシン　1]	-	+
置換 (SEQ ID NO:17)	AAAAAA[プロキネチシン　1]	-	ND
挿入 (SEQ ID NO:18)	MAVITGA[プロキネチシン　1]	-	+
キメラ	AVITGA[コリバーゼ]	-	-
キメラ	AVITGA[dickkopf4]	-	ND
ペプチド (SEQ ID NO:19)	AVITGACERDVQCG	-	-
Cys変異体	AVITGA[プロキネチシン　1]18S	-	-
Cys変異体	AVITGA[プロキネチシン　1]60R	-	-

プロキネチシン１由来のＮ末端配列とコリバーゼまたはＤｉｃｋｋｏｐｆ　４のいずれか由来のＣ末端の１０個のシステイン領域を含むキメラ組換えタンパク質がまた、構築された。これらの２つのキメラ組換えタンパク質は、回腸の平滑筋調製物を用いて２５０ｎＭまでの濃度で試験した場合、機能しなかった。また、Ｎ末端ペプチド（配列番号１９）も試験されたが、これもまた機能しなかった。

【０１４７】

これらの結果は、Ｎ末端保存配列およびＣ末端のシステインリッチなドメインの両方が、プロキネチシンの収縮活性に不可欠であることを示す。

【０１４８】

（キメラプロキネチシン）

照会としてプロキネチシンｃＤＮＡを用いたドラフトヒトゲノムデータベースのサーチは、プロキネチシン１およびプロキネチシン２をコードする遺伝子が、３つのエキソンから構成されることを示した。そのシグナル伝達ペプチドとＮ末端領域に保存されたＡＶＩＴＧ配列が第１エキソンにコードされており、一方システインリッチドメインは、エキソン２およびエキソン３によってコードされる。プロキネチシン２の２１個のアミノ酸挿入は、選択的にスプライスされたミニエキソンによってコードされる。プロキネチシン１およびプロキネチシン２の機能的差異を調査するために、スワッピングされたそれらのエキソン３を用いて、キメラポリペプチドが作製された（図６参照のこと）。このキメラポリペプチドは、図６に示すように構築されたキメラ１２（配列番号１３）およびキメラ２１（配列番号１４）であり、スワッピングされたエキソンを示している。

【０１４９】

再折り畳みされたキメラプロキネチシン１２およびキメラプロキネチシン２１の機能アッセイは、これらのキメラポリペプチドの両方が、ＧＩ平滑筋の収縮において活性であることを示した（図７Ａ）。しかし、このキメラプロキネチシン２１（配列番号１４）のEC_{50}は、プロキネチシン１またはプロキネチシン２より約８倍高かった。さらに、ピーク

収縮は影響されなかったが、キメラプロキネチシンポリペプチドは、回腸片の収縮を結果的に延長した（図７Ｂ）。野生型プロキネチシンに関して、中途の収縮に対する時定数（ピーク収縮から持続的な停滞期の中間）は、約１５分であった。対照的に、キメラポリペプチドに関して、これらの時定数は、約４０分まで延長された。

【０１５０】

これらの結果は、キメラプロキネチシンが、野生型のプロキネチシンとは僅かに異なってレセプターと相互作用し、そしてより少なく明白なタキフィラキシーを引き起こすことを示唆する。従って、このキメラプロキネチシン（配列番号１３および１４）は、インビボで、野生型のプロキネチシンよりも強力な薬理学的活性を有し得る。

【０１５１】

（インビボでのモルモットの回腸平滑筋におけるプロキネチシンの影響）

インビボでの回腸平滑筋収縮におけるプロキネチシンの影響をモニターするために、外部管腔（ｅｘｔｒａｌｕｍｉｎａｌ）力トランスデューサは、モルモットの回腸の漿膜表面上に移植された。次いで、組換えプロキネチシン１は、１０秒間にわたり、頸静脈内へのボーラスとして投与された。図８に示すように、プロキネチシン１の静脈内ボーラスは、用量依存様式でモルモットの回腸平滑筋を収縮させた。プロキネチシン１の閾値は、約０．０３μｇ／ｋｇであり、３０μｇ／ｋｇの投与量は、最大の効果を生じる。

【０１５２】

従って、プロキネチシンはまた、エキソビボでの調製物中の回腸平滑筋を収縮させ得ると以上に示され、インビボでも効果的である。

【０１５３】

（プロキネチシンシグナル伝達）

プロキネチシンの潜在的なシグナル伝達機構を調査するために、内因的にプロキネチシンレセプターを発現する細胞株を同定した。ヨウ素標識したプロキネチシン１への結合について、２０を越える細胞株をスクリーニングした。１つの細胞株、Ｍ２Ａ７黒色腫細胞（ＡＴＣＣ　ＣＲＬ－２５００；Ｃｕｎｎｉｎｇｈａｍら、Ｓｃｉｅｎｃｅ　２５５：３２５－３２７（１９９２））は、約１５０ｆｍｏｌｅ／ｍｇタンパク質のレセプターレベルに対し、特異的結合を明瞭に示した。プロキネチシンに特異的に結合する他の細胞株としては、Ｍ２黒色腫細胞（Ｃｕｎｎｉｎｇｈａｍら、Ｓｃｉｅｎｃｅ　２５５；３２５－３２７（１９９２））およびＲＣ－４Ｂ／Ｃ下垂体腫瘍細胞（ＡＴＣＣ　ＣＲＬ－１９０３）が挙げられる。プロキネチシンに結合しない細胞株としては、ＨＥＫ２９３細胞、ＣＯＳ－７細胞、ＣＯＳ－１細胞、Ｌｔｋ－細胞、ＮＩＨ３Ｔ３細胞、Ｃ６細胞、ＮＳ１０Ｙ細胞およびＨＴ－２９細胞が挙げられる。

【０１５４】

Ｍ２Ａ７細胞におけるシグナル伝達を調査するために、ＦＬＩＰＲシステム（Ｆｌｕｏｒｏｍｅｔｒｉｃ　Ｉｍａｇｉｎｇ　Ｐｌａｔｅ　Ｒｅａｄｅｒ；Ｍｏｌｅｃｕｌａｒ　Ｄｅｖｉｃｅｓ）を用いる、ｆｕｒａ－３蛍光によって、細胞基質のカルシウムを測定した。細胞をＨＥＰＥＳ媒体中に懸濁し、そして２μＭのｆｕｒａ－３　ＡＭと一緒に３１℃で２０分間インキュベートした。次いでこの細胞を遠心し、洗浄し、ｆｕｒａ－３を含まない媒体中に再懸濁し、そして９６ウェル中に１ウェルにつき4×10^{4}細胞を播種した。この細胞を、０．１％　ウシ胎仔血清を含む標準緩衝溶液（１３０ｍＭ　ＮａＣｌ、２ｍＭ　$CaCl_2$、５ｍＭ　ＫＣ１、１０ｍＭ　グルコース、０．４５ｍＭ　KH_2PO_4、０．４ｍＭ　Na_2HPO_4、８ｍＭ　$MgSO_4$、４．２ｍＭ　$NaHCO_3$、２０ｍＭ　ＨＥＰＥＳおよび１０μＭ　プロベネシド）中のＦｌｕｏ－３　ＡＭ（Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ）と一緒に３７℃で１時間ロードし、次いで標準緩衝溶液で洗浄した。プロキネチシン（０．０１ｎＭ、０．１ｎＭ、０．３ｎＭ、１ｎＭ、３ｎＭ、１０ｎＭ、１００ｎＭ）によって惹起された$[Ca_{2+}]_i$での過渡変化を、９６－ウェルプレートにおいて４８８ｎｍで２１０秒間ＦＬＩＰＲシステムを使用して観測した。

【０１５５】

図９に示されるように、プロキネチシンは、Ｍ２Ａ７黒色腫細胞においてカルシウムを動

員（ｍｏｂｉｌｉｚｅ）し得、組換えプロキネチシン１および組換えプロキネチシン２のＥＣ$_{50}$は、それぞれ、約１２ｎＭと約２１ｎＭであった。このシグナル伝達は、ＨＥＫ２９３細胞では応答しないので、特異的なものである。プロキネチシンによって動員されるカルシウムシグナル伝達は、コントロールＭＣＨ（メラニン凝集ホルモン）レセプターＳＬＣ１によって活性化されるカルシウムシグナルと同程度である（Ｓａｉｔｏら、Ｎａｔｕｒｅ　４００：２６５－２６９（１９９９））。プロキネチシンによって誘発されるカルシウムシグナルは、代表的なレセプターチロシンキナーゼの活性化によって誘導されるわずかな（ｍｏｄｅｓｔ）カルシウムシグナルよりも非常に強力である。この結果は、上記、チロシンタンパク質キナーゼインヒビターであるゲニステイン（ｇｅｎｉｓｔｅｉｎ）（５ｍＭ）が、回腸平滑筋でのプロキネチシンの収縮活性を引き起こさないという知見と、一致する。

【０１５６】

これらの結果は、このプロキネチシンレセプターが、ＧＰＣＲであり且つＧαｑを介するシグナルである可能性が高いことを、示す。

【０１５７】

（考察）

上記の結果は、カエルＢＶ８およびヘビＭＩＴ１の哺乳動物のホモログの存在を明らかにする。ＧＩ平滑筋におけるそれらの強力且つ特異的な効果を示すために、これらのタンパク質は、プロキネチシンと名付けられている。ハムスターの回腸平滑筋の収縮を特異的に刺激するが、他の平滑筋（大動脈、大腿動脈、気管および胆嚢を含む）を刺激しない、それらの高い効力は、プロキネチシンがＧＩ運動性の重要な内因性調節因子であり得ることを示す。プロキネチシンは、神経分泌性シグナル伝達分子または神経分泌性循環ホルモン、あるいはパラクリン液性因子としてＧＩ平滑筋を調節し得る。プロキネチシンはまた、ＧＩ系以外でも広く発現されているため、プロキネチシンが遠隔器官から放出され、そしてＧＩ活性を調節し得る得る可能性がある。この点において、プロキネチシンは、プロテアーゼ処理に対して耐性であることが決定され、このことは、プロキネチシンの潜在的な長距離効果（ｌｏｎｇ－ｒａｎｇｅ）および長期的（ｌｏｎｇ－ｔｅｒｍ）効果を支持する

プロキネチシンの分子サイズおよびプロセシングは、それらを代表的な神経ペプチドと識別可能にし、そしてそれらがサイトカインに非常に類似していることを示す。病原菌を排除するための１つの機構として、胃腸管の運動性を増強し、そして攻撃した生物を胃腸管の外に押し出すことであり、プロキネチシンがまた、防御免疫応答の一部であり得る、すなわち、ＧＩ運動性を増強させる炎症性サイトカインとして機能し得る。

【０１５８】

ＧＩ収縮性における組換えプロキネチシンの高い効力は、おそらく、プロキネチシンが細胞表面レセプターと相互作用することを示唆する。この考察は、上記のレセプター結合実験によって促進され（ｒｅｉｎｆｏｒｃｅ）、ヨウ素標識された組換えプロキネチシンに対し飽和可能に（ｓａｔｕｒａｂｌｙ）高親和性を示す。さらに、１０μＭのＧＴＰγＳが特異的結合のほとんど全てを置換し得るという知見は、プロキネチシンレセプターのシグナル伝達におけるＧタンパク質の関与を示す。さらに、プロキネチシンの収縮効果におけるカルシウムチャネル遮断薬（ベラパミル）の阻害効果は、プロキネチシンに対するレセプターの介在機構と一致し、そしてまた、ＧＩ平滑筋の収縮における、Ｍ３ムスカリン性レセプターおよびモチリンレセプターのシグナル伝達機構と同様のプロキネチシンのシグナル伝達機構を示唆し：電位型カルシウムチャネルを解するカルシウムの流入は、不可欠な要素である。従って、プロキネチシンレセプターは、おそらく、Ｇタンパク質共役レセプターである。

【０１５９】

しかし、代替的解釈は可能である。例えば、プロキネチシンは直接的に非選択的カチオン性イオンチャネルを活性化することによってか、またはＧＩ平滑筋細胞での阻害性カリウムチャネルを遮断することにより、平滑筋収縮を引き起こし得る。

【０１６０】
配列解析は、プロキネチシンが、２つの機能ドメイン（短いＮ末端領域およびシステインリッチなＣ末端領域）を含み得ることを示す。第１システインの前にあるＮ末端配列は、プロキネチシン内に完全に保存されており（図１）、この領域は、機能的重要性を有する可能性が高い。他の種由来のプロキネチシンとそれらのアイソフォームに加えて、同様の１０個のシステインモチーフはまた、コリパーゼ（腸性脂質消化酵素リパーゼのコファクター）（ｖａｎ　Ｔｉｌｂｅｕｒｇｈら、Ｎａｔｕｒｅ　３５９：１５９－１６２（１９９２））およびｄｉｋｋｏｐｆ（初期胚発達において重要な役割を有する、タンパク質のファミリー）（Ｇｌｉｎｋａら、Ｎａｔｕｒｅ　３９１：３５７－３６２（１９９８）およびＡｒａｖｉｎｄら、Ｃｕｒｒ．Ｂｉｏｌ．８：Ｒ４７７－４７８　（１９９８））を含む、多数の他の分泌タンパク質において見出される。興味深いことに、ｄｉｃｋｋｏｐｆは、実際的に、鏡面対称を有する１０個のシステインドメイン２つを有する。Ｘ線結晶学および溶液構造解析は、ＭＩＴ１が５対のジスルフィド結合により形成され、そしてコリパーゼと類似の構造に折り込まれることを示している（Ｂｏｉｓｂｏｕｖｉｅｒら、Ｊ．Ｍｏｌ．Ｂｉｏｌ．２８３：２０５－２１９（１９９８））。

【０１６１】
５対のジスルフィド結合を有するタンパク質の成功した再折り畳みは、これまでは、インビトロでは達成されていなかった。３対より多くのジスルフィド結合を有するタンパク質の再折り畳みは、まだなお興味深く且つ困難であると考えられる（Ｇｅｏｒｇｉｏｕら、Ｃｕｒｒ．Ｏｐｉｎ．Ｂｉｏｔｅｃｈｎｏｌ．７：１９０－１９７（１９９６）およびＬｉｈｅら、Ｃｕｒｒ．Ｏｐｉｎ．Ｂｉｏｔｅｃｈｎｏｌ．９：４９７－５０１（１９９８））。Ｅ．ｃｏｌｉでのこのようなジスルフィド結合リッチなタンパク質の発現は、結果として、しばしばジスルフィド結合を形成し得ないか、あるいはよりおおまかには、分子内または分子間の誤ったジスルフィド結合を形成し得る。これらの事象は、通常、細菌封入体内で不活性な組換えタンパク質の産生およびそれらの凝集を誘導する。

【０１６２】
本研究において、緩徐な置換方法は、５対のジスルフィド結合を有するプロキネチシンの再折り畳みに利用される。多数の因子は、最終的に、プロキネチシンの首尾良い再折り畳みに貢献した：１）低速な変性剤の除去；２）ジスルフィド結合の緩徐な形成を可能にする、レドックスフォールディング混合物中での還元剤のみの使用；３）低い温度；４）タンパク質凝集を防止するための、透析緩衝液中の高濃度の尿素およびグリセロール；５）分子間のジスルフィド結合ではなく、分子内のジスルフィド結合の形成を有利にする、低濃度の組換えタンパク質。これらの再折り畳み条件を用いて、複数のジスルフィド結合を有する他の組換えタンパク質を再折り畳みするためのプロトコルを設計し得る。

【０１６３】
要約すると、２つのプロキネチシンをコードするｃＤＮＡが記載されている。再折り畳みした組換えプロキネチシンは、ＧＩ平滑筋の収縮を強力且つ特異的に刺激する。低下したＧＩ運動性は、過敏性結腸、糖尿性胃不全麻痺、術後の腸閉塞、慢性収縮性心膜炎、および胃食道逆流疾患（Ｌｏｎｇｏら、Ｄｉｓ　Ｃｏｌｏｎ　Ｒｅｃｔｕｍ　３６：６９６－７０８（１９９３）；Ｔｏｎｉｎｉ，Ｐｈａｒｍａｃｏｌ．Ｒｅｓ．３３：２１７－２２６（１９９６）；ＳａｍｓｏｍおよびＳｍｏｕｔ、Ｄｉｇ　Ｄｉｓ．１５：２６３－２７４（１９９８）；ＡｃｈｅｍおよびＲｏｂｉｎｓｏｎ、Ｄｉｇ　Ｄｉｓ．１６：３８－４６（１９９８）ならびにＢｒｉｅｊｅｒら、Ｔｒｅｎｄｓ　Ｐｈａｒｍａｃｏｌ　Ｓｃｉ．２０：１－３（１９９９））を含む多数の一般的な疾患において非常に多発する臨床症状であるため、ＧＩ平滑筋の内因性調節因子の発見は、変化したＧＩ運動性によって恩恵を受ける、このような疾患の新規治療の開発を容易にする。

【０１６４】
括弧内、またはその他の上記に記載された全ての学術論文、引用文献および特許引用例は、以前に記載されようとされまいと、本明細書中でその全体が参考として援用される。

【０１６５】

本発明は、上記の例を参照して記載されているが、本発明の精神から逸脱することなく、種々の改変が行われ得ることが理解される。

【図面の簡単な説明】

【図１】

図１は、Ａ）プロキネチシン１前駆体（配列番号２）のアミノ酸配列；Ｂ）プロキネチシン２前駆体（配列番号４）のアミノ酸配列；Ｃ）カエルＢＶ８（配列番号１１）のアミノ酸配列およびＤ）ＭＩＴ１の部分的配列（配列番号１２）を、示す。１０個の保存性システイン残基に（＊）を付ける。シグナルペプチドに下線を引く。矢印は、イントロンスプライス部位を示す。

【図２】

図２は、プロキネチシンの発現パターンを示す。ヒトＲＮＡマスターブロットを、Ａ）プロキネチシン１、およびＢ）プロキネチシン２　ｃＤＮＡを用いて、プローブした。図２Ｃは、各ドットについてのＲＮＡ供給源を示すブロット図を示す。図２Ｄは、プロキネチシン１を用いたＮｏｒｔｈｅｒｎ　ｂｌｏｔ分析を示す。各レーンは、以下に示すような異なる脳組織由来のＲＮＡを含む：１．大脳；２．大脳皮質；３．髄質；４．脊髄；５．後頭極；６．前頭葉；７．側頭葉；８．被殻；９．小脳扁桃；１０．尾状核；１１．脳梁；１２．海馬；１３．脳全体；１４．黒質（Ｓｕｂｓｔａｎｔｉ　ａｎｉｇｒａ）：１５．視床下核；１６．視床。

【図３】

図３は、ヒトプロキネチシンの生成および精製を示す：Ａ）Ｃｏｏｍａｓｓｉｅ　ｂｌｕｅ　Ｇ－２５０を用いて染色したプロキネチシンサンプルのＳＤＳ－ＰＡＧＥ（１８％）。レーン１、分子量標準；レーン２、インキュベーション後の全細菌ライセート；レーン３、Ｎｉ－ＮＴＡアフィニティークロマトグラフィーで精製したプロキネチシン；レーン４、Ｆａｃｔｏｒ　Ｘａで切断したプロキネチシン；レーン５、ＨＰＬＣ精製後に再折り畳みさせたプロキネチシン。各レーンに、１０μｇ～１５μｇの全タンパク質をロードした。Ｂ）再折り畳みさせたタンパク質混合物の逆相ＨＰＬＣ分離。ピーク２が再折り畳みさせたプロキネチシンを含む。Ｃ）再折り畳みさせたプロキネチシン１のエレクトロスプレー質量分析。

【図４】

図４は、モルモット回腸縦平滑筋の収縮性に対するプロキネチシンの効果を示す。プロキネチシン１（２ｎＭ）に対する収縮応答は、テトロドトキシン（０．１μＭ）およびベラパミル（１μＭ）の非存在下（Ａ）、およびテトロドトキシン（０．１μＭ；Ｂ）存在下およびベラパミル（１μＭ；Ｃ）存在下で、回腸内で測定される。図４Ｄは、プロキネチシンの収縮効果に関する濃度－応答の関係を示す。結果は、最大収縮性のパーセント（％）として与えられる。データは３回の独立した実験からのものである。ベラパミル（１μＭ）の非存在下（Ｅ）およびベラパミル（１μＭ；Ｆ）の存在下での回腸内のオキソトレモリン－Ｍの収縮効果もまた、示される。矢印は、薬物が投与された時を示す。

【図５】

図５Ａは、モルモット回腸膜への^{125}Ｉ－プロキネチシンの特異的結合のスキャッチャード分析を示す。図５Ｂは、異なる濃度の非標識プロキネチシン１（黒四角）および非標識プロキネチシン２（白四角）による、^{125}Ｉ－プロキネチシン（２０ｐＭ）の結合の阻害を示す。白丸は、異なる濃度のＧＴＰγＳを用いた^{125}Ｉ－プロキネチシン１（２０ｐＭ）の置換を示す。

【図６】

図６は、プロキネチシン１とプロキネチシン２との間で構築されたキメラ（キメラ１２（配列番号１３）およびキメラ２１（配列番号１４）と命名された）の説明図を示す。

【図７】

図７は、キメラプロキネチシンの機能的特徴付けを示す。図７Ａは、モルモット回腸を収縮する能力についてアッセイされた、キメラプロキネチシンおよび野生型プロキネチシンの用量応答曲線を示す。図７Ｂは、キメラプロキネチシンおよび野生型プロキネチシンの

時定数を示す。この時定数は、ピークの収縮から中間の収縮までの経過時間（ピークから持続するプラトー収縮までの半分の時間）を示す。オキソトレモリンＭ誘導収縮に対して規格化した後、プロキネチシンによって導き出されるピーク収縮および持続するプラトー収縮は、各々約８０％および４０％だった。従って、中間の収縮は、最大収縮の約６０％である。

【図８】

図８Ａは、インビボでのモルモット回腸における収縮に対するＩＶボーラスとしての、種々の用量のプロキネチシン１の投与の効果を示す。図８Ｂは、１０００ｎｇ／ｋｇのプロキネチシン１への収縮応答を示す。

【図９】

図９は、ＦＬＩＰＲアッセイにおいて決定されたように、望ましい濃度のプロキネチシン１またはプロキネチシン２によって、ＨＥＫ２９３細胞またはＭ２Ａ７細胞中で誘導されたカルシウム動員を示す。

【図２】

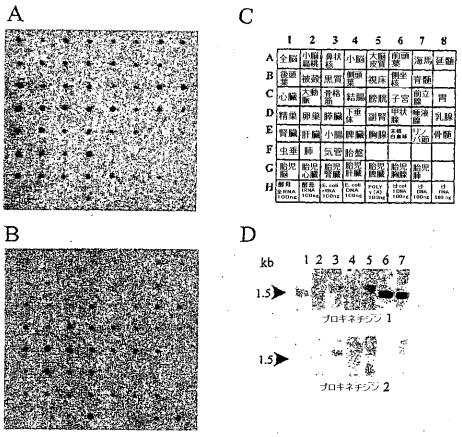

FIGURE 2

【図３】

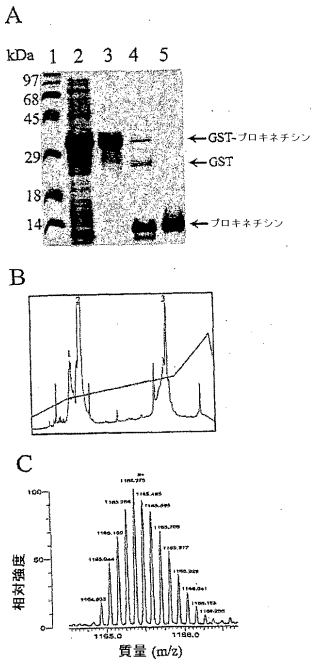

FIGURE 3

【図４】

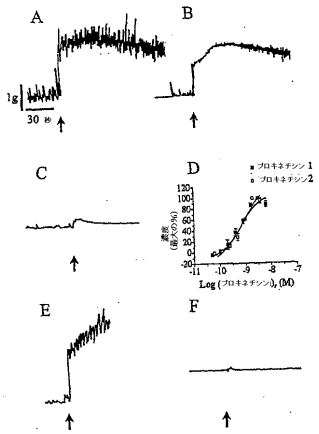

FIGURE 4

【図５】

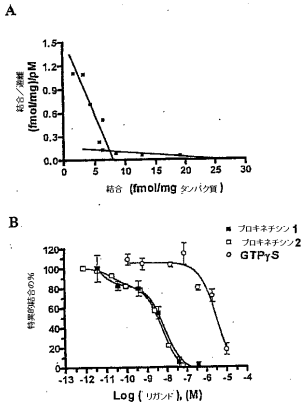

FIGURE 5

【図６】

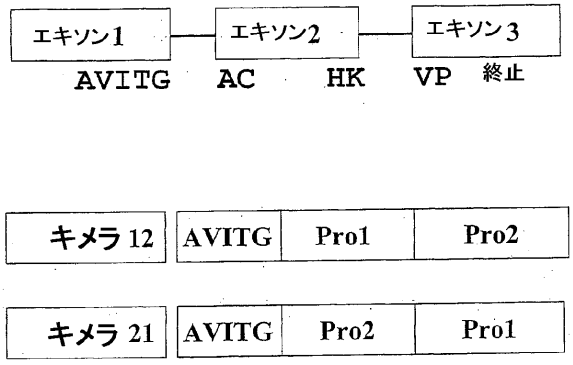

FIGURE 6

【図７】

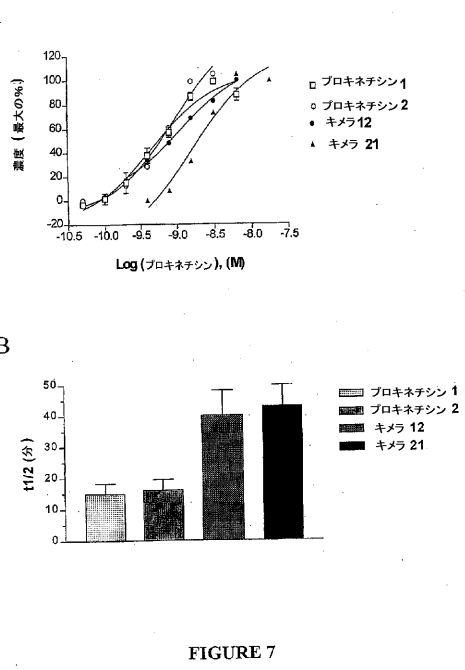

FIGURE 7

【図８】

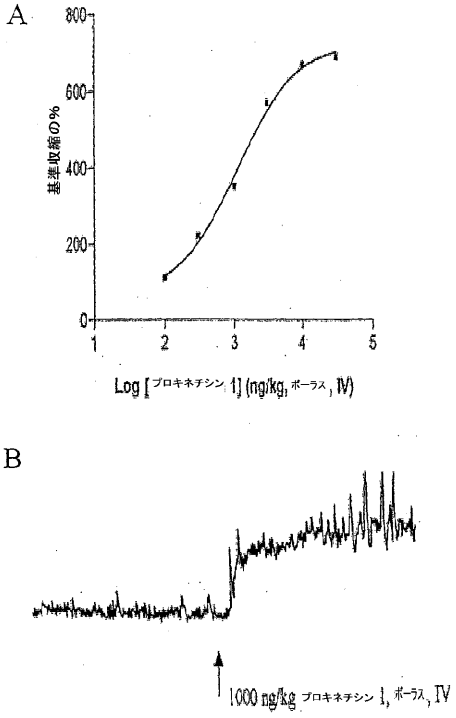

FIGURE 8

【図９】

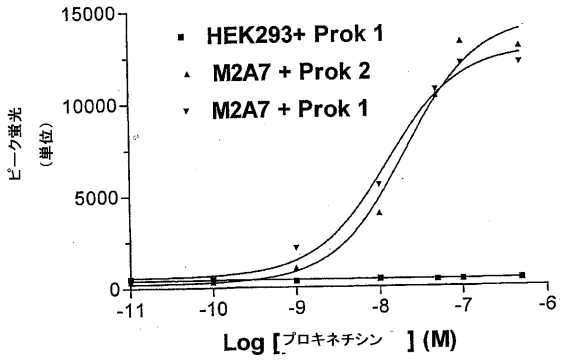

FIGURE 9

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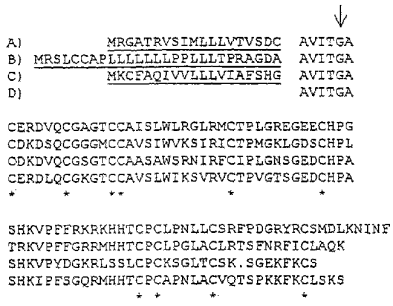
(54) **Title:** PROKINETICIN POLYPEPTIDES, RELATED COMPOSITIONS AND METHODS



(57) **Abstract:** The invention provides isolated polypeptides that stimulate gastroeintestinal smooth muscle contraction, including human prokineticin 1 and human prokineticin 2 polypeptides, and functional fragments and modifications thereof. Also provided are methods of stimulating gastrointestinal smooth muscle contraction in a mammal, by administering to the mammal an effective amount of a prokineticin polypeptide. The invention also provides nucleic acid molecules encoding a prokineticin polypeptide, and antibodies that selectively bind a prokineticin polypeptide. Further provided are methods of identifying a prokineticin receptor ligand, agonist or antagonist.

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PROKINETICIN POLYPEPTIDES, RELATED COMPOSITIONS AND METHODS

BACKGROUND OF THE INVENTION

The main function of gastrointestinal (GI) smooth muscle is to mix and propel intralumenal contents, which enables efficient digestion of food, progressive absorption of nutrients, and eventual evacuation of residual components. The activity of GI smooth muscle is regulated by intrinsic and extrinsic neural signals, including classical neurotransmitters, co-existing neuropeptides, and circulating peptide hormones. In addition, a number of humoral agents including histamine, serotonin, and adenosine that are produced by nonneural GI cells also influence the activity of smooth muscle cells.

A number of clinical conditions are associated with altered GI motility, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, gastrointestinal reflux disease, chronic diarrhea, infectious diseases, malabsorptive disorders, inflammatory bowel disorders, and intestinal cancers. The identification of regulators of gastrointestinal motility should facilitate the development of novel therapeutics for disorders that involve impaired or enhanced gastrointestinal motility.

Two potential regulators of gastrointestinal motility have recently been identified. Mamba intestinal toxin (MITl), a small protein that potently stimulates the contraction of guinea-pig ileum, has been purified from mamba snake venom (Schweitz et al., Toxicon 28:847-856 (1990) and Schweitz et al., FEBS Letters 461:183-188 (1999)). Recently, a protein of similar size and having

greater than 40% identity with MIT1, including all 10 conserved cysteines, has been purified from frog skin secretions (Mollay et al., Eur. J. Pharmacol. 374:189-196 (1999)). The frog protein, named Bv8, was also found to potently stimulate the contraction of GI smooth muscle.

Methods of recombinantly preparing these snake and frog polypeptides, or of recombinantly preparing other polypeptides containing 10 cysteines, have not previously been described, limiting the utility of these regulators for therapeutic use. Additionally, snake and frog polypeptides could elicit antibodies if administered to mammals that would likely reduce their efficacy as therapeutics.

Accordingly, there exists a need to identify endogenous human polypeptides that stimulate or inhibit gastrointestinal motility, and to develop methods of preparing these compounds recombinantly as therapeutics. There also exists a need to identify small molecule agonists and antagonists of endogenous gastrointestinal regulators that can be used therapeutically. The present invention satisfies this need, and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides isolated polypeptides that stimulate gastrointestinal smooth muscle contraction. In one embodiment, the polypeptide contains an amino acid sequence at least 80% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), wherein the sequence contains the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3. In another embodiment, the polypeptide

containis an amino acid sequence at least 80% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), wherein the sequence contains the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6.

Also provided are methods of stimulating gastrointestinal smooth muscle contraction in a mammal, by administering to the mammal an effective amount of a prokineticin polypeptide.

The invention also provides nucleic acid molecules encoding a prokineticin polypeptide.

Further provided are antibodies that selectively bind a prokineticin polypeptide.

The invention also provides methods of identifying a prokineticin receptor ligand, by contacting a preparation containing prokineticin receptor with one or more candidate compounds, and identifying a compound that specifically binds to the receptor. Such a compound is characterized as a prokineticin receptor ligand.

Also provided are methods of identifying a prokineticin receptor agonist, by contacting a preparation containing a prokineticin receptor with one or more candidate compounds, and identifying a compound that selectively promotes production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor agonist.

Further provided are methods of identifying a prokineticin receptor antagonist, by contacting a preparation containing a prokineticin receptor with one

or more candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor antagonist.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences of A) prokineticin 1 precursor (SEQ ID NO:2); B) prokineticin 2 precursor (SEQ ID NO:4); C) frog BV8 (SEQ ID NO:11) and D) partial sequence of MIT 1 (SEQ ID NO:12). Ten conservative cysteine residues are marked (*). Signal peptides are underlined. The arrow indicates an intron splice site.

Figure 2 shows the expression pattern of prokineticins. A human RNA master blot was probed with A) prokineticin 1 and B) prokineticin 2 cDNA. Figure 2C show the blot diagram indicating the RNA sources for each dot. Figure 2D shows a Northern blot analysis with prokineticin 1. Each lane contains RNA from different brain tissues as indicated: 1. Cerebellum; 2. Cerebral cortex; 3. Medulla; 4. Spinal cord; 5. Occipital pole; 6. Frontal lobe; 7. Temporal lobe; 8. Putamen; 9, Amygdala; 10. Caudate nucleus; 11. Corpus callosum; 12. Hippocampus; 13. Whole brain; 14. Substanti anigra.; 15. Subthalamic nucleus; 16. Thalamus.

Figure 3 shows the production and purification of human prokineticins: A) SDS-PAGE (18%) of prokineticin samples stained with Coomassie blue G-250. Lane 1, molecular weight standards; lane 2, whole bacterial lysate after induction; lane 3, Ni-NTA affinity chromatography-purified prokineticin; lane 4, Factor Xa digested prokineticin; lane 5, refolded prokineticin

after HPLC purification. Each lane was loaded with 10-15 µg total protein. B) Reverse phase HPLC separation of refolded protein mixture. Peak 2 contains refolded prokineticin. C) Electrospray mass spectrum of refolded prokineticin 1.

Figure 4 shows the effects of prokineticins on the contractility of guinea-pig ileal longitudinal smooth muscle. The contractile responses to prokineticin 1 (2 nM) were measured in ileum in the absence (A) and in the presence of tetrodotoxin (0.1µM; B) and verapamil (1 µM; C). Figure 4D shows the concentration-response relationship for the contractile effects of prokineticins. Results are given as percentage of maximum contractility. Data are from three independent experiments. Contractile effects of oxotremorine-M in ileum in the absence (E) and in the presence of verapamil(1µM; F) are also shown. Arrows indicate when drugs were added.

Figure 5A shows Scatchard analysis of the specific binding of ^{125}I-prokineticin 1 to guinea pig ileal membrane. Figure 5B shows the inhibition of binding of ^{125}I-prokineticin 1 (20 pM) by different concentrations of unlabeled prokineticin 1 (filled squares) and unlabeled prokineticin 2 (open squares). Open circles show displacement of ^{125}I-prokineticin 1 (20 pM) with different concentrations of GTPγS.

Figure 6 shows a schematic diagram of chimeras constructed between prokineticin 1 and prokineticin 2, designated chimera 12 (SEQ ID NO:13) and chimera 21 (SEQ ID NO:14).

Figure 7 shows functional characterization of chimeric prokineticins. Figure 7A shows a dose-response curve of chimeric and wild type prokineticins assayed for their ability to contract guinea-pig ileum. Figure 7B shows time constants of chimeric and wild type prokineticins. The time constant indicates the time elapsed from peak contraction to midway contraction (half way from peak to sustained plateau contraction). After normalizing against the oxotremorine M-induced contraction, the peak and sustained plateau contraction elicited by prokineticins are about 80% and 40%, respectively. The midway contraction is thus about 60% of maximum contraction.

Figure 8A shows the effect of administration of various doses of prokineticin 1 as an IV bolus on contractions in guinea pig ileum *in vivo*. Figure 8B shows the contractile response to 1000 ng/kg of prokineticin 1.

Figure 9 shows calcium mobilization, as determined in a FLIPR assay, elicited in HEK293 or M2A7 cells by the indicated concentrations of prokineticin 1 or prokineticin 2.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides an isolated prokineticin polypeptide that is able to stimulate gastrointestinal (GI) smooth muscle contraction. The prokineticin polypeptides of the invention can be used, for example, in therapeutic methods to treat disorders involving impaired GI motility. Such polypeptides can also be used, for example, in screening methods to identify prokineticin receptor ligands, including receptor

agonists and antagonists, which can be used therapeutically to treat disorders involving impaired or enhanced GI motility.

As used herein, the term "prokineticin polypeptide" refers to a polypeptide comprising the amino acid sequence of human prokineticin 1 shown as the non-underlined sequence in Figure 1A (SEQ ID NO:3), or comprising the amino acid sequence of human prokineticin 2 shown as the non-underlined sequence in Figure 1B (SEQ ID NO:6); and to a polypeptide containing minor modifications to SEQ ID NOS:3 or 6 that has GI smooth muscle contractile activity; and to a fragment of the reference polypeptide that has GI smooth muscle contractile activity.

As used herein, the terms "comprising," "having," "encoding," and "containing," and derivatives of these terms, are intended to be open-ended. The term "consisting" is intended to be closed-ended.

As used herein, the term "minor modification" to the sequences designated SEQ ID NOS:3 or 6 refers to one or more additions, deletions or substitutions compared with the recited amino acid sequence; one or more chemical or enzymatic modifications to the polypeptide; or substitution of one or more L-configuration amino acids with corresponding D-configuration amino acids. Such modifications can be advantageous, for example, in enhancing the stability, expression, bioactivity, or receptor affinity of the polypeptide, or for facilitating its identification or purification.

The GI smooth muscle contractile activity of a modified polypeptide can be determined by *ex vivo* or *in vivo* methods known in the art, such as the *ex vivo* and *in vivo* guinea pig ileal bioassays described in the Example, to confirm that it has GI smooth muscle contractile activity. Suitable assays for determining GI smooth muscle contractile activity can alternatively be performed using other GI smooth muscle tissue that responds to prokineticin 1 or 2, such as fundic muscle strip or proximal colon (see Example). Likewise, suitable assays can be performed using other mammals, including, for example, mice, rats, cats, dogs, sheep, goats, pigs, cows and primates.

A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can elicit at least 10%, 25%, 50%, 75%, 100% or more of the maximal GI smooth muscle contraction of human prokineticin 1 or 2, under the same conditions. A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can be less potent, similarly potent, or more potent than human prokineticin 1 or 2, under the same conditions. For example, a modified polypeptide can have an EC_{50} that is 5-fold, 10-fold, 50-fold or 100-fold higher or lower than the EC_{50} for human prokineticin 1 or 2. A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can also elicit contractions for the same duration or for a longer or shorter duration than human prokineticin 1 or 2, under the same conditions.

A chimeric polypeptide encoded by exons 1 and 2 of prokineticin 1 and exon 3 of prokineticin 2, designated chimera 12 (SEQ ID NO:13) (see Figure 6) is an example of a modified prokineticin that elicits ileal contractions with a similar potency as prokineticins 1 or

2 (see Figure 7A), but which causes prolonged contractions in comparison with prokineticins 1 or 2 (see Figure 7B).

A chimeric polypeptide encoded by exons 1 and 2 of prokineticin 2, and exon 3 of prokineticin 1, designated chimera 21 (SEQ ID NO:13) (see Figure 6) is an example of a modified prokineticin that elicits ileal contractions with an 8-fold lower potency than prokineticins 1 or 2 (see Figure 7A), and which causes prolonged contractions in comparison with prokineticins 1 or 2 (see Figure 7B).

Modifications to the amino acid sequence designated SEQ ID NOS:3 or 6 can be randomly generated, such as by random insertions, deletions or substitutions of nucleotides in a nucleic acid molecule encoding SEQ ID NOS:3 or 6. Alternatively, modifications can be directed, such as by site-directed mutagenesis of a nucleic acid molecule encoding SEQ ID NOS:3 or 6.

Computer programs known in the art can provide guidance in predicting which amino acid residues can be modified without abolishing the function of the polypeptide (see, for example, Eroshkin et al., Comput. Appl. Biosci. 9:491-497 (1993)).

Furthermore, guidance in modifying amino acid residues of SEQ ID NOS:3 or 6 while retaining activity can be provided by comparison of SEQ ID NOS:3 or 6 with the sequence of their mammalian homologs, such as homologs in non-human primates, mouse, rat, rabbit, bovine, porcine, ovine, canine or feline species, as well as sequences of their homologs in non-mammalian vertebrates, including frog Bv8 polypeptide (SEQ ID NO:11) and snake MIT1 polypeptide (SEQ ID NO:12) (see

Figure 1). It is well known in the art that evolutionarily conserved amino acid residues and domains are more likely to be important for maintaining biological activity than less well-conserved residues and domains. Thus, it would be expected that substituting a residue that is highly conserved among mammalian prokineticins, frog Bv8 polypeptide and snake MIT1 polypeptide, such as the N-terminal sequence, or any of the 10 cysteines, would likely be detrimental to activity, whereas substitution of less highly conserved residues, such as the C-terminal residues, is likely to be tolerated.

As described in the Example, retention of the six N-terminal residues (AVITGA) of prokineticin, without addition, deletion or substitution, is apparently required to retain smooth muscle contractile activity (see Table 1). However, modifications of the AVITGA sequence can result in polypeptides that do not exhibit GI smooth muscle contractile activity, but that antagonize the smooth muscle contractile activity of wild-type prokineticins. The N-terminal mutants designated SEQ ID NOS:16 and 18 are examples of modified prokineticin polypeptides with antagonistic activity.

The N-terminal domain of prokineticins, while apparently required for GI smooth muscle contractile activity, is not sufficient for GI smooth muscle contractile activity. Specifically, a prokineticin N-terminal peptide (SEQ ID NO:19), or a polypeptide with the cysteine-rich domain of prokineticin substituted with the cysteine-rich domain of either co-lipase or dickkopf4, did not exhibit smooth muscle contractile activity.

The cysteine-rich domain of prokineticins was shown to also be required for GI smooth muscle contractile activity (see Table 1). Specifically, correct cysteine pairing was shown to be required in order to retain activity, as substitutions at either of two cysteines abolished activity.

The sequence C-terminal to the final cysteine is presumably not required, as evidenced by GI smooth muscle contractile activity of prokineticin polyepeptides with a 6XHis tag inserted at this position. Additionally, as evidenced by similar activities of prokineticins 1 and 2, despite only being 44% identical, amino acid sequence substitutions at positions other than the N-terminus and conserved cysteine residues are well tolerated.

Substitutions to the amino acid sequences designated SEQ ID NOS:3 or 6 can either be conservative or non-conservative. Conservative amino acid substitutions include, but are not limited to, substitution of an apolar amino acid with another apolar amino acid (such as replacement of leucine with an isoleucine, valine, alanine, proline, tryptophan, phenylalanine or methionine); substitution of a charged amino acid with a similarly charged amino acid (such as replacement of a glutamic acid with an aspartic acid, or replacement of an arginine with a lysine or histidine); substitution of an uncharged polar amino acid with another uncharged polar amino acid (such as replacement of a serine with a glycine, threonine, tyrosine, cysteine, asparagine or glutamine); or substitution of a residue with a different functional group with a residue of similar size and shape (such as replacement of a serine with an alanine; an arginine with a methionine; or a tyrosine with a phenylalanine).

Specifically contemplated substitutions to the amino acid sequences designated SEQ ID NOS:3 and 6 include replacement of residues from wild-type prokineticin 1 with residues from prokineticin 2, and vice versa. The replacements can be of single residues, multiple residues throughout the polypeptide, or multiple contiguous residues. Because chimeras between SEQ ID NOS:3 and 6, namely SEQ ID NOS:13 and 14, were demonstrated to exhibit prolonged contractile activity in comparison with wild-type prokineticins, it is contemplated that substituted prokineticins can be potent therapeutics *in vivo*.

Additions to the amino acid sequence designated SEQ ID NOS:3 or 6 include, but are not limited to, the addition of "tag" sequences, which are preferably added at the C terminus. Such tag sequence include, for example, epitope tags, histidine tags, glutathione-S-transferase (GST), and the like, or sorting sequences. Such additional sequences can be used, for example, to facilitate recombinant expression, purification or characterization of a prokineticin. Exemplary polypeptides containing additions to the sequences designated SEQ ID NOS:3 or 6 are the active prokineticins prepared as described in the Example by the insertion of a 6XHis-tag after the C-terminal cysteine.

Deletions to the amino acid sequences designated SEQ ID NOS:3 or 6 include, but are not limited to, deletion of one or more residues at the C-termini that are not highly conserved among the active polypeptides shown in Figure 1. Deleted sequences can optionally be replaced by tag sequences, as described previously.

Chemical and enzymatic modifications to the polypeptide containing the amino acid sequence designated SEQ ID NOS:3 or 6 include, but are not limited to the following: replacement of hydrogen by an alkyl, acyl, or amino group; esterification of a carboxyl group with a suitable alkyl or aryl moiety; alkylation of a hydroxyl group to form an ether derivative; phosphorylation or dephosphorylation of a serine, threonine or tyrosine residue; or N- or O-linked glycosylation.

As used herein, the term "isolated" indicates that the molecule is altered by the hand of man from how it is found in its natural environment. Preferably, an "isolated" prokineticin polypeptide can be a "substantially purified" molecule, that is at least 60%, 70%, 80%, 90 or 95% free from cellular components with which it is naturally associated. An isolated polypeptide can be in any form, such as in a buffered solution, a suspension, a lyophilized powder, recombinantly expressed in a heterologous cell, bound to a receptor or attached to a solid support.

The invention provides isolated polypeptides that stimulate gastrointestinal smooth muscle contraction. In one embodiment, the polypeptide contains an amino acid sequence at least 50% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), and including the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3. The encoded polypeptide can thus have at least 60%, 65%, 70%, 75% identity, including at least 80%, 85%, 90%, 95%, 96%, 98%, 99% or greater identity to SEQ ID NO:3. An exemplary polypeptide contains the amino acid sequence designated SEQ ID NO:3, or amino acids 1-77 thereof.

In one embodiment, the isolated polypeptide does not contain the amino acid sequence NNFGNGRQERRKRKRSKRKKE (SEQ ID NO:7). In another embodiment, the isolated polypeptide does not contain the amino acid sequence SHVANGRQERRRAKRRKRKKE (SEQ ID NO:8).

In another embodiment, the polypeptide contains an amino acid sequence at least 50% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), and including the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6. The encoded polypeptide can thus have at least 60%, 65%, 70%, 75% identity, including at least 80%, 85%, 90%, 95%, 96%, 98%, 99% or greater identity to SEQ ID NO:6. An exemplary polypeptide contains the amino acid sequence designated SEQ ID NO:6, or amino acids 1-77 thereof.

As used herein, the term "percent identity" with respect to two molecules is intended to refer to the number of identical nucleotide or amino acid residues between the aligned portions of two sequences, expressed as a percentage of the total number of aligned residues, as determined by comparing the entire sequences using an optimized manual alignment or computer alignment, such as a BLAST 2.0 alignment (Tatusova et al., FEMS Microbiol Lett. 174:247-250 (1999)).

For certain applications, such as in the screening methods disclosed herein, a prokineticin polypeptide can be labeled with a detectable moiety, such as a radiolabel, a fluorochrome, a ferromagnetic substance, a luminescent tag or a detectable binding agent such as biotin. Other suitable labeled moieties are well known in the art.

The invention also provides methods for preparing an isolated prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, by culturing host cells (described below) so as to express a recombinant prokineticin polypeptide, and refolding the polypeptide under conditions that minimize protein aggregation.

Recombinant expression of polypeptides containing multiple cysteine residues often results in the incorrect formation of inter- and intra-molecular disulfide bonds, which leads to the production of inactive, aggregated bacterial proteins. As disclosed herein, these problems can be overcome using conditions that minimize protein aggregation during refolding of the expressed polypeptide. Exemplary conditions that minimize protein aggregation are described in the Example, and differ from conventional conditions for preparing recombinant protein by including one or more of the following refolding conditions: 1) keeping protein concentration low (e.g. about 100 µg/ml); 2) dialysing, rather than diluting, the peptides to remove denaturing agent; 3) omitting oxidants from buffers; 4) maintaining high concentrations of urea in all buffers; 5) maintaining high concentrations of glycerol (e.g. at least about 10%) in buffers; and 6) keeping peptides and buffers at low temperature (e.g. about 4°C). Of these conditions, it is contemplated that low protein concentration (ie. less than about 250 µg/ml, preferably less than 200 µg/ml, 150 µg/ml, 100 µg/ml, or 50 µg/ml) and high urea concentration (e.g. at least about 1.5M, such as about 2M, 4M, 6M, 8M or higher) are the most important factors in successful refolding of active prokineticins.

It is expected that the same or similar conditions as those described herein can be used to recombinantly express and refold other polypeptides containing multiple cysteines, including dickkopf, co-lipase, MIT-1 and Bv8, so as to isolate a biologically active polypeptide.

In a preferred method for preparing an isolated prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, a prokineticin polypeptide is recombinantly expressed in bacteria as a fusion protein (e.g. as a GST fusion) containing a tag (e.g. a 6XHis tag), and partially purified by affinity isolation (e.g. on a nickel column). The fused polypeptide is then cleaved so as to remove the heterologous protein (e.g. using protease factor Xa cleavage between GST and prokineticin), and the prokineticin polypeptide refolded under conditions described above to minimize protein aggregation. To obtain more highly purified polypeptide, the polypeptide can further be purified by column chromatography (e.g. reverse-phase HPLC). Those skilled in the art recognize that modification to these preferred methods for recombinantly expressing, refolding and purifying active prokineticin polypeptides can readily be determined, such as employing alternative heterologous sequences, cleavable sequences, tags, host cells and buffer conditions.

Alternatively, an isolated prokineticin polypeptide can be prepared by biochemical procedures. As disclosed herein, prokineticins 1 and 2 are expressed in a variety of human tissues (see Example, and particularly Figure 2). Therefore, an isolated prokineticin polypeptide can be isolated from tissues or cells that normally express these polypeptides, by biochemical procedures routinely used in the art,

including membrane fractionation, chromatography, electrophoresis and ligand affinity methods, or using immunoaffinity methods with the prokineticin antibodies described herein. Following biochemical isolation, an inactive prokineticin can be refolded by the methods described above to restore activity.

Likewise, an isolated prokineticin polypeptide can be prepared by chemical synthesis procedures known in the art. Following chemical synthesis, an inactive prokineticin can be refolded by the methods described herein to restore activity.

If desired, such as to optimize their functional activity, selectivity, stability or bioavailability, chemically synthesized polypeptides can be modified to include D-stereoisomers, non-naturally occurring amino acids, and amino acid analogs and mimetics. Examples of modified amino acids and their uses are presented in Sawyer, Peptide Based Drug Design, ACS, Washington (1995) and Gross and Meienhofer, The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983). For certain applications, it can also be useful to incorporate one or more detectably labeled amino acids into a chemically synthesized polypeptide or peptide, such as radiolabeled or fluorescently labeled amino acids.

The invention also provides isolated peptides containing, or consisting of, at least 10 contiguous amino acids of the amino acid sequences designated SEQ ID NOS:3 or 6 which can, but need not, be able to stimulate gastrointestinal (GI) smooth muscle contraction. Such isolated peptides are useful, for example, in preparing and purifying prokineticin antibodies of the invention. Such peptides can also act as antagonists to block

signaling through a prokineticin receptor, and thus can be used in therapeutic and screening methods. An isolated prokineticin peptide can thus contain, or consist of, at least 12, 15, 20, 25 or more contiguous amino acids of SEQ ID NOS:3 or 6, including at least, or not more than, 30, 40, 50, 60, 70, 80, 81 or 86 contiguous amino acids.

In one embodiment, an isolated prokineticin peptide contains, or consists of, at least 10 contiguous residues from within amino acid residues 6 and 48 of SEQ ID NO:3. In another embodiment, an isolated prokineticin peptide contains, or consists of, at least 10 contiguous residues from within amino acid residues 6 and 48 of SEQ ID NO:6.

An isolated peptide containing at least 10 contiguous amino acids of SEQ ID NOS:3 or 6 can be immunogenic. As used herein, the term "immunogenic" refers to a peptide that either is capable of inducing prokineticin-specific antibodies, or is capable of competing with prokineticin-specific antibodies for binding to a prokineticin. Peptides that are likely to be immunogenic can be predicted using methods and algorithms known in the art and described, for example, by Irnaten et al., Protein Eng. 11:949-955 (1998), and Savoie et al., Pac. Symp. Biocomput. 1999:182-189 (1999). The immunogenicity of the peptides of the invention can be confirmed by methods known in the art.

The isolated prokineticin polypeptide and peptides of the invention can optionally be conjugated to a carrier, such as KLH, serum albumin, tetanus toxoid and the like, using standard linking techniques, to enhance their immunogenicity. Additionally or alternatively, the isolated polypeptides and peptides can be formulated with

an adjuvant known in the art, such as Freund's complete or incomplete adjuvant.

An isolated prokineticin peptide of at least 10 contiguous residues can conveniently be prepared by chemical synthesis, or by chemical or enzymatic digestion of longer peptides, prepared as described above. An isolated prokineticin peptide of at least 10 contiguous residues can also be prepared recombinantly, such as fused to a protein tag. Those skilled in the art can determine an appropriate method of preparing an isolated prokineticin peptide, depending on its size, sequence, and intended application.

The invention also provides an isolated nucleic acid molecule encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction. The invention nucleic acid molecules are suitable for a variety of screening, therapeutic and diagnostic applications. For example, an invention nucleic acid molecule can be expressed *in vitro* and the encoded prokineticin polypeptide isolated. An invention nucleic acid molecule can also be expressed *in vivo*, to restore normal prokineticin activity in patients, or expressed in an antisense orientation to block prokineticin expression in patients in need thereof. Additionally, the invention nucleic acid molecules can be used as probes or primers to identify and isolate prokineticin-encoding nucleic acid molecules from other species, or to identify structurally related molecules. Such probes and primers are also useful diagnostically to determine normal and abnormal expression of prokineticin in human tissues, and thus to predict susceptibility to conditions associated with altered prokineticin expression.

As used herein, the term "isolated nucleic acid molecule" is intended to mean that the nucleic acid molecule is altered, by the hand of man, from how it is found in its natural environment. For example, an isolated nucleic acid molecule can be a molecule operatively linked to an exogenous nucleic acid sequence. An isolated nucleic acid molecule can also be a molecule removed from some or all of its normal flanking nucleic acid sequences.

An isolated molecule can alternatively, or additionally, be a "substantially pure" molecule, in that the molecule is at least 60%, 70%, 80%, 90 or 95% free from cellular components with which it is naturally associated. An isolated nucleic acid molecule can be in any form, such as in a buffered solution, a suspension, a lyophilized powder, attached to a solid support (e.g. as a component of a DNA array), or in a cell.

As used herein, the term "nucleic acid molecule" refers to a polynucleotide of natural or synthetic origin, which can be single- or double-stranded, can correspond to genomic DNA, cDNA or RNA, and can represent either the sense or antisense strand or both.

The term "nucleic acid molecule" is intended to include nucleic acid molecules that contain one or more non-natural nucleotides, such as nucleotides having modifications to the base, the sugar, or the phosphate portion, or having one or more non-natural linkages, such as phosphothioate linkages. Such modifications can be advantageous in increasing the stability of the nucleic acid molecule, particularly when used in hybridization applications.

Furthermore, the term "nucleic acid molecule" is intended to include nucleic acid molecules modified to contain a detectable moiety, such as a radiolabel, a fluorochrome, a ferromagnetic substance, a luminescent tag or a detectable binding agent such as biotin. Nucleic acid molecules containing such moieties are useful as probes for detecting the presence or expression of prokineticin nucleic acid molecule.

Prokineticin polypeptides that are able to stimulate GI smooth muscle contraction have been described above. Accordingly, it is routine for those skilled in the art to prepare isolated nucleic acid molecules encoding such polypeptides. Exemplary isolated nucleic acid molecules encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction contains, or consists of, a) the nucleotide sequences designated SEQ ID NOS:1 or 4; b) the portion of the nucleotide sequences designated SEQ ID NOS:1 or 4 that encodes SEQ ID NOS:3 or 6 (i.e. nucleotides 55-370 of the nucleotide sequence designated SEQ ID NO:1 and nucleotides 10-334 of the nucleotide sequence designated SEQ ID NO:4); c) a nucleotide sequence that encodes an active modification or active fragment of SEQ ID NOS:3 or 6; and d) a sequence that is degenerate with respect to either a), b) or c).

In one embodiment, the isolated nucleic acid molecule does not encode the amino acid sequence NNFGNGRQERRKRKRSKRKKE (SEQ ID NO:7). In another embodiment, the isolated nucleic acid molecule does not encode the amino acid sequence SHVANGRQERRRAKRRKRKKE(SEQ ID NO:8). In yet another embodiment, an isolated nucleic acid molecule encoding a prokineticin polypeptide excludes naturally occuring signal polypeptides, such as nucleic acid molecules encoding the underlined portions

of the amino acid sequences shown in Figure 1A and 1B (MRGATRVSIMLLLVTVSDC (SEQ ID NO:9) and MRSLCCAPLLLLLLLPLLLTPPAGDA (SEQ ID NO:10)).

In certain embodiments, an isolated nucleic acid molecule encoding a prokineticin polypeptide specifically excludes nucleic acid molecules having the exact sequence of genomic fragments ESTs and cDNAs whose sequences are compiled in publically available databases, such as GenBank Accession Nos. AI277349, AA883760, AQ426386, AC068519, AC026973, AL358215 and AL390797 or sequences which encode amino acid sequences having GenBank Accession Nos. AF182066, AF182064, AF182069 and AF182065.

In one embodiment, an isolated nucleic acid molecule encoding a prokineticin polypeptide excludes mammalian sequences present in the GenBank database that contain sequences which do not encode SEQ ID NOS:3 and 6 (e.g. nucleic acid molecules that encode 5' and 3' untranslated regions, introns or other exons present on chromosomes 1 or 3).

The invention further provides an isolated nucleic acid molecule encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, wherein the nucleic acid molecule is operatively linked to a promoter of gene expression. As used herein, the term "operatively linked" is intended to mean that the nucleic acid molecule is positioned with respect to either the endogenous promoter, or a heterologous promoter, in such a manner that the promoter will direct the transcription of RNA using the nucleic acid molecule as a template.

Methods for operatively linking a nucleic acid to a heterologous promoter are well known in the art and include, for example, cloning the nucleic acid into a vector containing the desired promoter, or appending the promoter to a nucleic acid sequence using PCR. A nucleic acid molecule operatively linked to a promoter of RNA transcription can be used to express prokineticin transcripts and polypeptides in a desired host cell or *in vitro* transcription-translation system.

The choice of promoter to operatively link to an invention nucleic acid molecule will depend on the intended application, and can be determined by those skilled in the art. For example, if a particular gene product may be detrimental to a particular host cell, it may be desirable to link the invention nucleic acid molecule to a regulated promoter, such that gene expression can be turned on or off. Alternatively, it may be preferred to have expression driven by either a weak or strong constitutive promoter. Exemplary promoters suitable for mammalian cell systems include, for example, the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, and the Moloney murine leukemia virus (MMLV) promoter. Exemplary promoters suitable for bacterial cell systems include, for example, T7, T3, SP6, lac and trp promoters.

The invention further provides a vector containing an isolated nucleic acid molecule encoding a prokineticin polypeptide. Exemplary vectors include vectors derived from a virus, such as a bacteriophage, a baculovirus or a retrovirus, and vectors derived from bacteria or a combination of bacterial sequences and sequences from other organisms, such as a cosmid or a plasmid. The vectors of the invention will generally

contain elements such as an origin of replication compatible with the intended host cells; transcription termination and RNA processing signals; one or more selectable markers compatible with the intended host cells; and one or more multiple cloning sites. Optionally, the vector will further contain sequences encoding tag sequences, such as GST tags, and/or a protease cleavage site, such as a Factor Xa site, which facilitate expression and purification of the encoded polypeptide.

The choice of particular elements to include in a vector will depend on factors such as the intended host cells; the insert size; whether expression of the inserted sequence is desired; the desired copy number of the vector; the desired selection system, and the like. The factors involved in ensuring compatibility between a host cell and a vector for different applications are well known in the art.

In applications in which the vectors are to be used for recombinant expression of the encoded polypeptide, the isolated nucleic acid molecules will generally be operatively linked to a promoter of gene expression, as described above, which may be present in the vector or in the inserted nucleic acid molecule. An exemplary vector suitable for fusion protein expression in bacterial cells is the pGEX-3X vector (Amersham Pharmacia Biotech, Piscataway, NJ).

Also provided are cells containing an isolated nucleic acid molecule encoding a prokineticin polypeptide. The isolated nucleic acid molecule will generally be contained within a vector. The isolated nucleic acid molecule can be maintained episomally, or incorporated into the host cell genome.

The cells of the invention can be used, for example, for molecular biology applications such as expansion, subcloning or modification of the isolated nucleic acid molecule. For such applications, bacterial cells, such as laboratory strains of *E. coli*, are useful, and expression of the encoded polypeptide is not required.

The cells of the invention can also advantageously be used to recombinantly express and isolate the encoded polypeptide. For such applications bacterial cells (e.g. *E. coli*), insect cells (e.g. *Drosophila*), yeast cells (e.g. *S. cerevisiae*, *S. pombe*, or *Pichia pastoris*), and vertebrate cells (e.g. mammalian primary cells and established cell lines; and amphibian cells, such as *Xenopus* embryos and oocytes). An exemplary cell suitable for recombinantly expressing prokineticin polypeptides is an *E. coli* BL21 cell.

The invention further provides isolated polynucleotides that contain at least 20 contiguous nucleotides from SEQ ID NOS:1 or 4, such as portions of SEQ ID NOS:1 or 4 that encode SEQ ID NOS:2, 3, 5 or 6, or from the complement thereof. The polynucleotides of the invention are thus of sufficient length to be useful as sequencing primers, PCR primers and hybridization probes to detect or isolate nucleic acid molecules encoding prokineticin polypeptides, and are also useful as therapeutic antisense reagents to inhibit prokineticin expression. The polynucleotides of the invention can, but need not, encode prokineticin polypeptides that are able to stimulate GI smooth muscle contraction. Those skilled in the art can determine the appropriate length and sequence of a polynucleotide of the invention for a particular application.

As used herein, the term "polynucleotide" refers to a nucleic acid molecule that contains at least 20 contiguous nucleotides from the reference sequence and which may, but need not, encode a functional polypeptide. Thus, a polynucleotide of the invention can contain at least 20, 22 or 25 contiguous nucleotides, such as at least, or not more than, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, or 300 contiguous nucleotides from SEQ ID NOS:1 or 4, or from their complement. A polynucleotide of the invention does not consist of the exact sequence of an EST present in publically available databases, including the sequences designated by GenBank Accession Nos. AI277349, AA883760, AQ426386, AC068519, AC026973, AL358215 and AL390797 or sequences which encode amino acid sequences having GenBank Accession Nos. AF182066, AF182064, AF182069 and AF182065.

For certain applications, such as for detecting prokineticin expression in a sample, it is desirable to use isolated polynucleotide molecules of the invention that specifically hybridize to a nucleic acid molecule encoding a prokineticin. The term "specifically hybridize" refers to the ability of a nucleic acid molecule to hybridize, under stringent hybridization conditions as described below, to a nucleic acid molecule that encodes a prokineticin, without hybridizing to a substantial extent under the same conditions with nucleic acid molecules that do not encode a prokineticin, such as unrelated molecules that fortuitously contain short regions of identity with a prokineticin. Thus, a nucleic acid molecule that "specifically hybridizes" is of a sufficient length and contains sufficient distinguishing sequence from a prokineticin for use in expression analysis, such as tissue blots and Northern blots (see Figure 2).

As used herein, the term "stringent conditions" refers to conditions equivalent to hybridization of a filter-bound nucleic acid molecule to a nucleic acid in a solution containing 50% formamide, 5X Denhart's solution, 5X SSC, 0.2% SDS at 42°C, followed by washing the filter in 0.1X SSC and 0.1% SDS at 65°C twice for 30 minutes. Equivalent conditions to the stringent conditions set forth above are well known in the art, and are described, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992).

The invention further provides a kit containing a pair of polynucleotides of the invention packaged together, either in a single container or separate containers. The pair of polynucleotides are preferably suitable for use in polymerase chain reaction (PCR) applications. Thus, the pair of polynucleotides can be used to detect or quantitate normal or abnormal expression of a nucleic acid molecule encoding a prokineticin. The pair of polynucleotides can also be used to amplify a nucleic acid molecule encoding a prokineticin, or any portion thereof, for sequencing, subcloning or for preparing sequence modifications. The kit can further contain written instructions for use of the pair of polynucleotides in PCR applications, or solutions and buffers suitable for such applications.

The isolated prokineticin nucleic acid molecules of the invention can be prepared by methods known in the art. An exemplary method for preparing an isolated prokineticin nucleic acid molecule involves amplification of the nucleic acid molecule using prokineticin-specific primers and the polymerase chain reaction (PCR). Using PCR, a prokineticin nucleic acid molecule having any desired boundaries can be amplified

exponentially starting from only a few DNA or RNA molecules, such as from a single cell. PCR methods, including methods of isolating homologs of a given nucleic acid molecule in other species using degenerate primers, are well known in the art.

Alternatively, an isolated prokineticin nucleic acid molecule can be prepared by screening a library, such as a genomic library, cDNA library or expression library, with a detectable prokineticin nucleic acid molecule or with an antibody. Human libraries, and libraries from a large variety of mammalian species, are commercially available or can be produced from species or cells of interest. The library clones identified as containing a prokineticin nucleic acid molecule can be isolated, subcloned or sequenced by routine methods.

Furthermore, an isolated prokineticin nucleic acid molecule can be prepared by direct synthetic methods. For example, a single stranded nucleic acid molecule can be chemically synthesized in one piece, or in several pieces, by automated synthesis methods known in the art. The complementary strand can likewise be synthesized in one or more pieces, and a double-stranded molecule made by annealing the complementary strands. Direct synthesis is particularly advantageous for producing relatively short molecules, such as probes and primers, and also for producing nucleic acid molecules containing modified nucleotides or linkages.

The invention also provides an antibody specific for a prokineticin polypeptide or peptide, such as an antibody specific for a polypeptide having the amino acid sequence of SEQ ID NOS:3 or 6. Also provided

is an antibody specific for an isolated immunogenic peptide that contains at least 10 contiguous amino acids of SEQ ID NOS:3 or 6.

The antibodies of the invention can be used, for example, to detect prokineticin expression in research and diagnostic applications. Such antibodies are also useful for identifying nucleic acid molecules that encode prokineticin polypeptides present in mammalian expression libraries, and for purifying prokineticin polypeptides by immunoaffinity methods. Furthermore, such antibodies can be administered therapeutically to bind to and block the activity of prokineticin, such as in applications in which it is desirable to inhibit GI smooth muscle contractions.

The term "antibody," as used herein, is intended to include molecules having specific binding activity for a prokineticin peptide or polypeptide of at least about 1×10^5 M^{-1}, preferably at least 1×10^7 M^{-1}, more preferably at least 1×10^9 M^{-1}. The term "antibody" includes both polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies (e.g. Fab, $F(ab')_2$, Fd and Fv fragments and the like). In addition, the term "antibody" is intended to encompass non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric antibodies, bifunctional antibodies, CDR-grafted antibodies and humanized antibodies, as well as antigen-binding fragments thereof.

Methods of preparing and isolating antibodies, including polyclonal and monoclonal antibodies, using peptide and polypeptide immunogens, are well known in the art and are described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor

Laboratory Press (1988). Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains. Such methods are described, for example, in Huse et al. Science 246:1275-1281 (1989); Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

The invention provides a method of identifying a prokineticin receptor ligand. The method is practiced by contacting a preparation containing prokineticin receptor with one or more candidate compounds, and identifying a candidate compound that specifically binds the receptor. Such a compound is characterized as a prokineticin receptor ligand.

The term "ligand," as used herein, includes compounds that bind to the prokineticin receptor at the same or different site as prokineticin.

As used herein, the term "candidate compound" refers to any biological or chemical compound. For example, a candidate compound can be a naturally occurring macromolecule, such as a polypeptide, nucleic acid, carbohydrate, lipid, or any combination thereof. A candidate compound also can be a partially or completely synthetic derivative, analog or mimetic of such a macromolecule, or a small organic molecule prepared by combinatorial chemistry methods. If desired in a particular assay format, a candidate compound can be detectably labeled or attached to a solid support.

Methods for preparing large libraries of compounds, including simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like, are well known in the art and are described, for example, in Huse, U.S. Patent No. 5,264,563; Francis et al., Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze et al., Curr. Biol., 2:363-371 (1998); Sofia, Mol. Divers. 3:75-94 (1998); Eichler et al., Med. Res. Rev. 15:481-496 (1995); and the like. Libraries containing large numbers of natural and synthetic compounds also can be obtained from commercial sources.

The number of different candidate compounds to test in the methods of the invention will depend on the application of the method. For example, one or a small number of candidate compounds can be advantageous in manual screening procedures, or when it is desired to compare efficacy among several predicted ligands, agonists or antagonists. However, it will be appreciated that the larger the number of candidate compounds, the greater the likelihood of identifying a compound having the desired activity in a screening assay. Additionally, large numbers of compounds can be processed in high-throughput automated screening assays. Therefore, "one or more candidate compounds" can be, for example, 2 or more, such as 5, 10, 15, 20, 50 or 100 or more different compounds, such as greater than about 10^3, 10^5 or 10^7 different compounds.

A suitable preparation for a identifying a prokineticin receptor ligand can employ a tissue, cell, cell membrane, or purified prokineticin receptor, so long as the preparation contains a prokineticin receptor in a suitable conformation for binding prokineticin with a

similar affinity and specificity as a prokineticin receptor expressed on GI smooth muscle tissues.

In one embodiment, the preparation is an intestinal smooth muscle preparation, such as a mammalian ileal, fundic muscle or proximal colon preparation, or membrane preparation thereof. A suitable intestinal smooth muscle preparation is a guinea pig ileal preparation prepared by the methods described in the Example.

In another embodiment, the preparation is a cell line that expresses prokineticin receptor, or membrane preparation thereof. A cell line that expresses prokineticin receptor can be identified by methods known in the art, such as the competitive binding assays described in the Example. An exemplary cell line that expresses prokineticin receptor is the melanoma cell line M2A7 (available from American Type Culture Collection as ATCC CRL-2500). Other cell lines that express prokineticin receptor include M2 melanoma cells (Cunningham et al., Science 255;325-327 (1992)) and RC-4B/C pituitary tumor cells (ATCC CRL-1903).

A suitable control cell line that does not express prokineticin receptor is HEK293 (available from American Type Culture Collection as CRL-1573). Other control cell include COS-7, COS-1, Ltk-, NIH3T3, C6, NS10Y and HT-29 cells.

Appropriate assays to identify receptor ligands are known in the art. Such assays can involve directly determining binding of the candidate compound to the receptor preparation. Direct assays are suitable when an appropriate control preparation is available that does not contain the prokineticin receptor. Such assays can

also involve determining the ability of the candidate compound to compete with a prokineticin polypeptide for binding to the receptor preparation. Competition assays can be performed by detectably labeling a candidate compound and competing the compound with an unlabeled prokineticin polypeptide, or competing an unlabeled candidate compound with a detectably labeled prokineticin polypeptide.

As used herein, the term "detectably labeled" refers to derivation with, or conjugation to, a moiety that is detectable by any analytical means. An exemplary detectable moiety is a radioisotope (e.g. ^{14}C, ^{131}I, ^{32}P or ^{3}H), fluorochrome (e.g. fluoroscein, green fluorescent protein), ferromagnetic substance, or luminescent substance. Methods of detectably labeling organic and inorganic compounds with such moieties are well known in the art.

An exemplary competitive binding assay suitable for detecting a prokineticin receptor ligand is described in the Example, below. Other suitable receptor binding assays, including high-throughput assays, are described, for example, in Mellentin-Micelotti et al., Anal. Biochem. 272:P182-190 (1999); Zuck et al., Proc. Natl. Acad. Sci. USA 96:11122-11127 (1999); and Zhang et al., Anal. Biochem. 268;134-142 (1999).

Other suitable assays for detecting binding include, for example, scintillation proximity assays (SPA) (Alouani, Methods Mol. Biol. 138:135-41 (2000)), UV or chemical cross-linking (Fancy, Curr. Opin. Chem. Biol. 4:28-33 (2000)), competition binding assays (Yamamura et al., Methods in Neurotransmitter Receptor Analysis, Raven Press, New York, 1990), biomolecular interaction analysis (BIA) such as surface plasmon resonance (SPR) (Weinberger

et al., Pharmacogenomics 1:395-416 (2000)), mass spectrometry (MS) (McLafferty et al., Science 284:1289-1290 (1999) and Degterev, et al., Nature Cell Biology 3:173-182 (2001)), nuclear magnetic resonance (NMR) (Shuker etal., Science 274:1531-1534 (1996), Hajduk et al., J. Med. Chem. 42:2315-2317 (1999), and Chen and Shapiro, Anal. Chem. 71:669A-675A (1999)), and fluorescence polarization assays (FPA) (Degterev et al., *supra*, 2001). An appropriate binding assay can be chosen depending on the nature and purity of the receptor preparation and the number and nature of the candidate compounds.

A compound that is determined to be a prokineticin receptor ligand can further be tested to determine whether it is an agonist or antagonist of prokineticin receptor. Likewise, a compound that is determined to be a prokineticin receptor ligand can further be tested to determine whether it modulates, either positively or negatively, GI smooth muscle contractility, using an *in vitro* or *in vivo* assay known in the art, such as the assays described herein.

The invention further provides a method of identifying a prokineticin receptor agonist. The method is practiced by contacting a preparation containing a prokineticin receptor with one or more candidate compounds, and identifying a compound that selectively promotes production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor agonist.

The invention also provides a method of identifying a prokineticin receptor antagonist. The method is practiced by contacting a preparation containing a prokineticin receptor with one or more

candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor antagonist. Using the invention method, prokineticin mutants designated SEQ ID NOS:16 and 18 were identified as prokineticin receptor antagonists.

The invention methods can be performed in the presence of a suitable concentration of a prokineticin, such as within 10-fold of its EC_{50}. Thus, an agonist that competes with prokineticin for signaling through the prokineticin receptor, or indirectly potentiates the signaling activity of prokineticin, can be readily identified. Likewise, an antagonist that prevents prokineticin from binding the prokineticin receptor, or indirectly decreases the signaling activity of prokineticin, can also be identified.

As used herein, the term "prokineticin receptor agonist" refers to a molecule that selectively activates or increases normal signal transduction through the prokineticin receptor. As used herein, the term "prokineticin receptor antagonist" refers to a compound that selectively inhibits or decreases normal signal transduction through the prokineticin receptor.

For therapeutic applications, a prokineticin receptor agonist preferably has an EC_{50}, and a prokineticin receptor antagonist preferably has an IC_{50}, of less than about 10^{-7} M, such as less than 10^{-8} M, and more preferably less than 10^{-9} or 10^{-10} M. However, depending on the stability, selectivity and toxicity of the compound, a prokineticin receptor agonist with a higher EC_{50}, or a prokineticin receptor antagonist with a higher IC_{50}, can also be useful therapeutically.

As described herein, the endogenous prokineticin receptor appears to be a G-protein coupled receptor. Signaling through the prokineticin receptor promotes intracellular calcium ion mobilization, suggesting that the prokineticin receptor normally couples to Gαq-containing G proteins. Therefore, signaling through the prokineticin receptor can be detected by any assay known in the art that detects intracellular calcium ion mobilization. Such an assay can be performed in the presence or absence of a prokineticin.

A suitable preparation for detecting calcium ion mobilization can be a tissue or cell line expressing the prokineticin receptor, such as an intestinal smooth muscle preparation, or the M2A7 cell line.

Calcium ion mobilization can conveniently be measured using detectably labeled Ca^{2+} ion indicators, such as fluorescently labeled or radiolabeled indicators, and suitable detection systems. Exemplary Ca^{2+} ion indicators include FLUO-3 AM, FLUO-4 AM, FURA-2, INDO-1, FURA RED, CALCIUM GREEN, CALCIUM ORANGE, CALCIUM CRIMSON, BTC, and OREGON GREEN BAPTA (see, for example, Grynkiewitz et al., J. Biol. Chem. 260:3440-3450 (1985); Sullivan et al., in Calcium Signal Protocol, Methods in Molecular Biology 114: 125-133, Edited by David G. Lambert, Human Press, Totowa, New Jersey (1999); Miyawaki et al., Proc. Natl. Acad. Sci. USA 96:2135-2140 (1999); and Coward et al., Analyt. Biochem. 270:242-248 (1999)). A suitable detection system for monitoring calcium ion mobilization is the FLIPR (Fluorometric Imaging Plate Reader) system available from Molecular Devices.

The specificity of Gα subunits for cell-surface receptors is determined by the C-terminal five amino acids of the Gα. Thus, a variety of signal transduction pathways can be assayed to determine transduction of a G-protein coupled signal by a prokineticin receptor, by recombinantly expressing a chimeric Gα containing the five C-terminal residues of a Gα known or predicted to couple to ADP-glucose receptor (such as Gαq or a promiscuous Gα such as Gα16), with the remainder of the protein corresponding to a Gα that couples to the signal transduction pathway to be assayed (e.g. Gαs, to assay increased cAMP production, or Gαq to assay intracellular Ca^{2+} mobilization). Based on the known sequences of Gα subunits, nucleic acid molecules encoding chimeric Gα can be constructed and expressed by methods known in the art and described, for example, in Conklin et al., Nature 363:274-276 (1993), and Komatsuzaki et al., FEBS Letters 406:165-170 (1995).

Thus, depending on the Gα subunit endogenously or recombinantly expressed in the assay system, prokineticin receptor signals that can be determined include, but are not limited to, calcium ion mobilization; increased or decreased production or liberation of arachidonic acid, acetylcholine, diacylglycerol, cGMP, cAMP, inositol phosphate and ions; altered cell membrane potential; GTP hydrolysis; influx or efflux of amino acids; increased or decreased phosphorylation of intracellular proteins; and activation of transcription of an endogenous gene or promoter-reporter construct downstream of any of the above-described second messenger pathways.

Suitable assays for detecting agonistic and antagonistic activity of G protein coupled receptors, including high-throughput signaling assays, are well

known in the art and reviewed, for example, in reviewed, for example, in Tate et al., Trends in Biotech. 14:426-430 (1996).

Assay methods for identifying compounds that selectively bind to or modulate signaling through a prokineticin receptor (e.g. ligands, agonists and antagonists) generally involve comparison to a control. One type of a "control" is a preparation that is treated identically to the test preparation, except the control is not exposed to the candidate compound. Another type of "control" is a preparation that is similar to the test preparation, except that the control preparation does not express the receptor, or has been modified so as not to respond selectively to prokineticin. In this situation, the response of the test preparation to a candidate compound is compared to the response (or lack of response) of the control preparation to the same compound under substantially the same reaction conditions.

A compound that is determined to be a prokineticin receptor agonist or antagonist can further be tested to determine whether it modulates, either positively or negatively, GI smooth muscle contractility, using an *in vitro* or *in vivo* assay known in the art, such as the assays described herein.

The invention also provides compositions suitable for use in assays to identify prokineticin ligands, agonists and antagonists. Suitable compositions contain a cell or tissue containing a prokineticin receptor and a prokineticin polypeptide, which optionally can be detectably labeled. An exemplary composition comprises a GI smooth muscle preparation, such as an ileal smooth muscle preparation. A further exemplary composition comprises a cell line, such as M2A7.

The prokineticin polypeptides described herein, as well as prokineticin ligands, agonists and antagonists identified by the described screening methods, are potential therapeutic compounds that can be administered to individuals with conditions associated with abnormal gastrointestinal motility, or other conditions associated with altered expression, or activity of a prokineticin or its receptor. The invention compounds can be formulated and administered in a manner and in an amount appropriate for the condition to be treated; the weight, gender, age and health of the individual; the biochemical nature, bioactivity, bioavailability and side effects of the particular compound; and in a manner compatible with concurrent treatment regimens. An appropriate amount and formulation for a particular therapeutic application in humans can be extrapolated based on the activity of the compound in the *in vitro* binding and signaling assays described herein, or from recognized animal models of the particular disorder.

The total amount of therapeutic compound can be administered as a single dose or by infusion over a relatively short period of time, or can be administered in multiple doses administered over a more prolonged period of time. Additionally, the compound can be administered in a slow-release matrice, which can be implanted for systemic delivery at or near the site of the target tissue. Contemplated matrices useful for controlled release of therapeutic compounds are well known in the art, and include materials such as DepoFoam™, biopolymers, micropumps, and the like.

The therapeutic compounds can be administered to a mammal by routes known in the art including, for example, intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally,

intracisternally, intra-articularly, intracerebrally, orally, intravaginally, rectally, topically, intranasally, or transdermally. Preferred routes for human administration are oral and intravenous administration, with oral routes particulary preferred.

Preferably, the therapeutic compounds are administered to a mammal as a pharmaceutical composition comprising the compound and a pharmaceutically acceptable carrier. The choice of pharmaceutically acceptable carrier depends on the route of administration of the compound and on its particular physical and chemical characteristics. Pharmaceutically acceptable carriers are well known in the art and include sterile aqueous solvents such as physiologically buffered saline, and other solvents or vehicles such as glycols, glycerol, oils such as olive oil and injectable organic esters. A pharmaceutically acceptable carrier can further contain physiologically acceptable compounds that stabilize the compound, increase its solubility, or increase its absorption. Such physiologically acceptable compounds include carbohydrates such as glucose, sucrose or dextrans; antioxidants, such as ascorbic acid or glutathione; chelating agents; and low molecular weight proteins.

For applications that require the compounds and compositions to cross the blood-brain barrier, or to cross cell membranes, formulations that increase the lipophilicity of the compound are particularly desirable. For example, the compounds of the invention can be incorporated into liposomes (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed. (CRC Press, Boca Raton FL (1993)). Liposomes, which consist of phospholipids or other lipids, are nontoxic,

physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

In one embodiment, a pharmaceutical composition containing a prokineticin polypeptide or a prokineticin agonist is administered to a mammal in an effective amount to stimulate gastrointestinal motility. Impaired GI motility is a common clinical manifestation of a variety of disorders, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastrointestinal reflux disease, and the compositions of the invention can thus be used to ameliorate the symptoms of such disorders.

In another embodiment, a pharmaceutical composition containing a prokineticin antagonist is administered to a mammal in an effective amount to inhibit gastrointestinal motility. Enhanced GI motility is associated with diarrhea, which is a common symptom of infectious diseases, malabsorptive disorders, inflammatory bowel disorders, and intestinal cancers, and antagonistic compositions of the invention can thus be used to ameliorate the symptoms of such disorders.

Injection of Bv8 or MIT1 into the brain ventricles of rats leads to hyperalgesia (Mollay et al., Eur J Pharmacol. 374:189-196 (1999)). Therefore, prokineticin antagonists (e.g. prokineticin antibodies, mutant polypeptides comprising SEQ ID NOS:16 or 18, and other compounds determined by the methods described herein) can be administered to a mammal in an effective amount to act as an analgesic (pain killer).

Those skilled in the art can determine other conditions for which it is appropriate to administer a pharmaceutical composition of the invention, and can monitor the safety and efficacy of the therapy.

Preferably, the mammal administered a pharmaceutical composition of the invention is a human, but for certain applications the mammal can alternatively be a veterinary animal or a research animal. For example, in preclinical studies, the methods of the invention can be practiced with animals that serve as credible models of human disease, such as non-human primates, pigs, dogs, cats, and rodents (e.g. rats, mice and guinea pigs). Those skilled in the art understand which animals serve as appropriate models for a human disease of interest.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Identification, Preparation and Characterization of Prokineticins 1 and 2

This example shows the cloning, recombinant expression, purification and biological activities of human prokineticins 1 and 2, as well as modifications thereof.

Materials and methods

RNA blot

Human multiple tissue RNA blots containing normalized samples of polyA RNA were used as described by the manufacture's instructions (Clontech). The blots were probed with random primer-labeled probes (nucleotides

1-550 and 1-1178 for prokineticin 1 and prokineticin 2 cDNAs), and signals were visualized by exposing to Kodak XAR film.

Production, refolding and purification of recombinant prokineticins

The coding sequences for mature prokineticins were cloned into prokacyotic expression vector pGEX-3X (Pharmacia). The extra nucleotidcs between the factor Xa protease digestion site of GST (Glutathione-s-transferase) tag and mature prokineticins were removed by site-directed mutagenesis and confirmed by sequencing. To facilitate protein purification, a 6xHis-tag was added to the C-terminus so that the fusion proteins could be purified with Ni-NTA affinity chromatography (Qiagen).

The method for production of fusion proteins is as follows. The *E. coli* cells (BL21) were grown to OD 0.8 and induced with 600 nM IPTG for 2 hours at 37°C. The cells were then pelleted, washed, and lysed with buffer A (6 M guanidine hydrochloride, 100 mM NaH_2PO_4 and 10mM Tris, pH 8.0). Fusion proteins were allowed to bind to Ni-NTA beads and then washed extensively with buffer C (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 6.3) and buffer D (8M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 5.9). Fusion protein-bound beads were equilibrated with factor Xa digestion buffer (50 mM Tris, 150 nM NaCl, and 1 mM CaCl, pH 7.5). Factor Xa digestion was performed overnight at room temperature with 10 ng/μ fusion protein. Cleaved GST tag was then washed away with buffer D. Mature prokineticins were then eluted with buffer E (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 4.5). Fractions were analyzed by SDS-PAGE. The pooled recombinant prokineticins were then refolded as follows. Proteins were diluted to 100 μg/ml with buffer E, and

dialyzed against renaturing buffer (4 M urea, 5 mM cysteine, 0.02% Tween-20, 10% glycerol, 10 mM Tris, 150 mM NaCI, 100 mM NaH_2PO_4, pH 8.3). New renaturing buffer (same component except 2 M urea) was then added, and dialysis was continued for four more days with at least one more change of renaturing buffer. The refolded protein was then desalted with a spin column (Qiagen) and analyzed by receptor binding or bioassay. The final purification was performed with reverse phase-HPLC (LKB). Functional proteins were eluted with 0.08% trifluoroacetic acid and 10-50% acetonitrile gradient. The elution of protein was monitored at 206 nm. Trifluoroacetic acid and acetonitrile were then evaporated by lyophilization.

Mass spectrometry

The electrospray ionization mass spectrometry was pwformed with a 6.5 T HiResESI Fourier Transform mass spectrometer (IonSpec, Irvine, CA) as previously described (Li et al., Anal. Chem. 66:2077-2083 (1994)). Protein eluted from RP-HPLC was lyophilized and dissolved in nanopure water and then diluted to a concentration of 1nM with methanol-water- acetic acid (49.5%:49.5%:1%, v/v/v). 100 µl of sample was infused.

Measurement of smooth muscle contraction in isolated organ preparations

Guinea pigs were euthanized with CO_2, and a section of ileum (2-3 cm) approximately 10 cm rostral to the cecum was removed. The ileum was washed clean with Krebs-Ringer bicarbonate (KRB) buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 nM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM CaCl, and 10 mM glucose) and mounted longitudinally in an organ bath containing KRB buffer. Isometric contractions were measured with a force-displacement transducer and polygraph as described previously (Thomas et al.,

Biochem. Pharmacol. 51:779-788 (1993)). The ileum was allowed to incubate for 1 hr, and then three test doses of the muscarinic agonist, oxotremorine-M, were added to ensure that the contractions were reproducible and of sufficient magnitude. The ileum was washed and allowed to rest for 5 min between each test dose. The longitudinal fundic strip and the zig-zag tracheal preparation were prepared as described previously (15). Isolated colon (proximal and distal) was prepared as described (Sawyer et al., J. Pharmacol. Exp. Ther. 284:269-277 (1998)). Aorta and femoral artery were taken from adult rats. A 10 ml bath was used for aorta and femoral artery experiments. Tension was recorded on a Grass polygraph with initial preloads of 0.5 g for intestinal tissues and tracheal preparations and 2 g for aorta and femoral artery.

Iodination

Prokineticin 1 was iodinated by the iodogen method as described (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849-857 (1978)). Briefly, refolded prokineticin 1 (7.5 µg) was incubated with 50 µg of iodogen in 50 µL of 0.5 M PBS buffer, pH 7.2 for 15 minutes at room temperature. The reaction was stopped by removal of the mixture from the iodogen tube and placing it in a microfuge tube with 100 µL of PBS containing 1 mM NaI. Following the addition of 100 µL of PBS with 1mM NaI and 0.1% BSA, the free iodine was removed by gel filtration on Bio-Gel P2 and the radioactivity was counted. Assuming all the radioactivity was incorporated into 6.0 µg prokineticin 1 recovered (80% recovery rate), specific radioactivity was calculated as 819 cpm/fmol or 372 Ci/mole.

Receptor binding

Membranes were prepared from guinea pig ileum as described (Li et al., Mol. Pharmacol. 57:446-452 (2000)), except additional steps of differential centrifugation (800g, 10,000g, 100,000g, 4°C, 20 min each) were applied to reduce the background binding. Incubation was performed in 4 ml in 20 mM Tris-HCl pH 7.4 buffer containing 0.1% BSA at room temperature. For saturation binding, 1.5-200 pM of labeled prokineticin 1 was used. Non-specific binding was defined in the presence of 20nM unlabeled prokineticin 1. For displacement experiments, unlabeled protein was pre-incubated with membrane in 3ml total reaction volume for 1hr, then ^{125}I-prokineticin 1 (20pM) was added. The membrane was incubated for an additional 3 hrs at room temperature. The binding mixture was filtered through GF-C glass filters and washed with 10 ml of 20 mM Tris-HCI, pH 7.4. Radioactivity retained on filters was counted in gamma counter. The data were analyzed with the LIGAND program.

Results

Identification and analysis of two mammalian homologues for frog Bv8 and snake MIT1

In an effort to identify mammalian homologues of frog Bv8 and snake MIT1, multiple databases (EST and HGTS) were searched using the BLAST 2.1 algorithm (Altschul et al. Nucleic Acids Res. 25:3389-3400 (1997)), with their protein sequences as queries. A search of the EST database revealed the presence of two human EST sequences (ai277349 and aa883760). Sequence analysis of these two EST clones revealed that aa 883760 encodes a predicted protein (Heijne Nucleic Acids Res. 14:4683-4690 (1986)) with a signal peptide of 19 amino acids and a mature protein of 86 amino acids. Clone ai277349 was

found to be a partial cDNA. Full-length sequence for EST clone ai277349, cloned by 5' RACE with human brain cDNA as template, was found to contain a signal peptide of 27 amino acids and a mature protein of 81 amino acids (Figure 1). These proteins were respectively named as prokineticin 1 and prokineticin 2 (see below).

Sequence analysis reveals that prokineticin 1 and 2 have about 44% amino acid identity, including ten conserved cysteines. Both prokineticins possess about 43% identity with frog Bv8 and snake MIT1. Interestingly, the N-terminal sequences before the first cysteine (AVITGA) is completely conserved among all species (Figure 1), suggesting the functional significance of this region. Preliminary analysis of the mouse prokineticin 1 gene indicates that the N-terminal sequence AVITG is derived from the first exon that also contains the signal peptide sequence, whereas the cysteine-rich sequences are from other exon(s).

Prokineticins are expressed in various adult and embryonic tissues

As an initial survey of prokineticin expression, a human masterr blot was probed using fragments of human prokineticin cDNAS. Both prokineticins were widely expressed in various adult tissues, with a generally higher expression level of prokineticin 1 compared to prokineticin 2 (Figure 2A, 2B). The exception was found in GI tract, liver and spleen, whereas prokineticin 2 expression seemed comparable to that of prokineticin 1. The highest level of prokineticin 1 expression is found in testis and placenta. Among human fetal tissues, all showed a similar level of expression, again with an expression level of prokineticin 1 higher than that of prokineticin 2.

The expression of prokineticins in human brain was further examined by Northern blot analysis. Figure 2D showed that prokineticin 1 mRNA size is about 1.5kb with the highest expression in the putamen, thalamus, temporal lobe, and corpus callosum. Prokineticin 2 expression in human brain was undetectable (data not shown).

Production, refolding and purification of human prokineticins

As the N-terminal sequences were completely conserved (Figure 1), recombinant proteins with authentic N-terminal residue were produced first as GST-fusion proteins, followed by digestion with protease factor Xa to remove the GST tag. Figure 3 shows that a protein with correct molecular weight was produced by factor Xa digestion.

Bioassay with guinea-pig ileum preparations revealed the unfolded recombinant proteins were inactive. As NMR examination indicated that 10 cysteines of MIT1 are formed into 5 disulfide bonds (Boisbouvier et al., J. Mol. Biol. 283:205-219 (1998)) and these 10 cysteines are all conserved in human prokineticins, it was considered that these disulfide bonds were probably essential for protein bioactivities. Thus considerable effort was devoted to ensure proper disulfide bond formation (out of 945 possible combinations).

Initial refolding in a single dilution into refolding buffer was unsuccessful, as almost all recombinant proteins were precipitated, probably due to the formation of inter-molecular disulfide bonds. A series of modifications to control protein aggregation and to slow disulfide bond formation were then adopted. These modifications included: 1) reduction of protein concentration to 100 µg/ml or less to favor forming

intra- but not inter-molecular disulfide bonds; 2) refolding proteins by dialysis method instead of direct dilution; 3) using higher levels of urea (4 M and then 2 M) in all dialysis buffers; 4) omitting oxidants cystine or oxidized glutathione from redox pairs, leaving only 5 mM cysteine or 3 mM reduced glutathione; 5) adding glycerol to further reduce protein aggregation; 6) cooling proteins and buffers to 4°C before initiating the refolding process. These carefully controlled steps allowed the successful refolding of recombinant prokineticins with minimal protein aggregarion.

The refolded proteins were finally purified by RP-HPLC (Figure 3A, lane 5). Mass spectrometry confirmed the formation of five disulfide bonds in refolded recombinant prokineticin 1. The molecular weight of 6xHis-tagged prokineticin 1, determined with a Fourier transform mass spectrometer, was found to be 10480.30 Da (Figure 3C). As the calculated molecular weight with all ten cysteines present in reduced form was 10490.20, five pairs of disulfide bonds were clearly formed.

Refolded recombinant prokineticins potently contract gastrointestinal smooth muscle

The refolded recombinant prokineticins were then tested on isolated smooth muscle preparations. Figure 4 shows that both recombinant prokineticin 1 and prokineticin 2 potently stimulated the contraction of guinea-pig ileum longitudinal muscle with ED50 values of about 0.46 and 0.90 nM, respectively. Prokineticin 1 (5 nM) also stimulated the contraction of fundic muscle strip and proximal colon, but had no effect on distal colon (25 nM, data not shown). Recombinant prokineticin 1 (25 nM) also had no effect on other smooth muscle tissues, including aorta and femoral artery, trachea and gallbladder. Thus, the contractile effect of

prokineticins appears to be specific for GI smooth muscle.

To probe the possible signaling mechanisms of prokineticins, a number of kinase and ion channel inhibitors were tested. Tetrodotoxin (TTX), which is known to block nerve action potential propagation, had no effect on prokineticin 1-stimulated ileum longitudinal muscle contraction (Figure 4B), indicating that prokineticin 1 acts directly on the smooth muscle. The contractile mechanism of prokineticin was further investigated with a number of compounds, including the protein kinase C inhibitor calphostin C (1 μM), the phospholipase A2 inhibitor 7, 7-dimethyl-(5Z,8Z)-eicosa-dienoic acid (10 μM), the tyrosine kinase inhibitor genistein (5 μM), the MEK inhibitor PD 098059 (10 μM) and L-type calcium channel blocker verapamil. Only verapamil was effective, with 1 μM completely inhibiting the contractile effect of 2 nM prokineticin I (Figure 4C). The same concentration of verapamil also completely blocked the contractile action of 100 nM oxotremorine-M (Figure 4F). This result indicates that, like muscarinic M3 receptor mediated contraction of the ileum (Eglen et al., Pharmacol. Rev. 48:531-565 (1996) and Ehlert et al., Muscurinic Receptors and Gastrointestinal Smooth Muscle, ed. Eglen, CRC Press, pgs 92-147 (1997)), calcium entry via the voltage-gated calcium channel is an essential component of prokineticin signaling.

Bioactivities of prokineticins are mediated by membrane receptors

The potent contractile action of recombinant prokineticins on guinea-pig GI smooth muscle and the inhibitory effect of the calcium channel blocker verapamil suggest a receptor-mediated mechanism for

prokineticins. To provide direct evidence that prokineticins are interacting with selective membrane receptors, recombinant prokineticin was labeled with ^{125}I and receptor binding experiments were carried out.

Scatchard analysis indicated that the specific binding of prokineticin 1 was best fitted with two-site model (F=38.78, P<0.001 versus one site model; Figure 5A). The high- and low-affinity constants (K_d) were 5.0 ± 0.8 pM and 227 ± 63 pM (n = 3), respectively. The B_{max} for high- and low-affinity sites were 7.8 ± 1.2 and 26.4 ± 8.4 fmol/mg of protein, respectively (n = 3). Competition experiments revealed that the specific binding was displaced by recombinant prokineticin 1. The displacement curves were also best fitted with two-site model (with K_i of 8.0 ± 3.9 pM, and 1.50 ± 0.9 nM, n = 3 for high- and low-affinity sites, respectively) (Figure 5B). Figure 5B also shows that prokineticin 2 displaced labeled prokineticin 1 with similar affinity (K_i of 4.2 pM for high affinity and 1.22 nM for low affinity site, average of two experiments).

Because agonist binding to many G protein-coupled receptors is inhibited by GTP, it was investigated whether GTPγS had any effect on specific ^{125}I-labeled prokineticin 1 binding. As shown in Figure 5B, GTPγS caused a concentration-dependent inhibition of ^{125}I-prokineticin 1 binding. At the highest concentration tested (10 µM), GTPγS displaced 85% of the specific prokineticin binding to ileal membranes. These results suggest that prokineticin receptor(s) belong to the G protein-coupled receptor family.

Stability of prokineticins

Experiments were also performed to determine the half-life of prokineticins. The half-life of intravenously injected iodinated human prokineticin 1 was approximately 3 hours, compared to 10 min for motilin, a small peptide that also increases GI motility. A reasonably long half-life in the blood circulation is crucial for achieving therapeutic effect. Therefore, prokineticins are likely to be effective as therapeutics.

Structure/activity relationship studies of prokineticins

Sequence analysis indicated that prokineticins may contain two functional domains, namely the short N-terminus and the cysteine-rich C-terminus. As the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Figure 1), it was predicted that this region has functional importance.

In addition to prokineticins, the ten-cysteine motif is also found in a number of secreted proteins, including colipase, a cofactor for intestinal lipid digestive enzyme lipase, and dickkopfs, a family of proteins that have an important role in early embryonic development.

A number of N-terminal substitution, deletion, and insertion mutants were constructed, and recombinant, refolded proteins produced. Bioassays with ileal smooth muscle preparations revealed that these mutant proteins at concentrations up to 250 nM are not able to elicit contractions (Table 1). However, an N-terminal deletion mutant (SEQ ID NO:16) and an N-terminal insertion mutant (SEQ ID NO:18) were able to weakly antagonize the contractile effect of prokineticin 1. Therefore,

N-terminal variants of prokineticins, such as SEQ ID NOS:16 and 18, are potential therapeutics for inhibiting GI contractility.

Table 1

	Polypeptide	Contractile Activity	Antagonistic Activity
Wild Type (SEQ ID NO:3)	AVITGA[Prokineticin 1]	+	-
Insertion (SEQ ID NO:15)	GILAVITGA[Prokineticin 1]	-	ND
Deletion (SEQ ID NO:16)	VITGA[Prokineticin 1]	-	+
Substitution (SEQ ID NO:17)	AAAAAA[Prokineticin 1]	-	ND
Insertion (SEQ ID NO:18)	MAVITGA[Prokineticin 1]	-	+
Chimera	AVITGA[Co-lipase]	-	-
Chimera	AVITGA[dickkopf4]	-	ND
peptide (SEQ ID NO:19)	AVITGACERDVQCG	-	-
Cys mutation	AVITGA[Prokineticin 1]18S	-	-
Cys mutation	AVITGA[Prokineticin 1]60R	-	-

Chimeric recombinant proteins containing N-terminal sequences from prokineticin 1 and the C-terminal ten-cysteine domain from either colipase or Dickkopf 4 were also constructed. These two chimeric recombinant proteins were non-functional when tested with ileal smooth muscle preparation at concentrations up to 250 nM. Also tested was an N-terminal peptide (SEQ ID NO:19), which also was non-functional.

These results indicate that both N-terminal conserved sequence and C-terminal cysteine-rich domain are essential for the contractile activity of prokineticins.

Chimeric prokineticins

A search of the draft human genome database with prokineticin cDNAs as queries revealed that genes encoding prokineticin 1 and 2 are composed of three exons. The signaling peptide and N-terminal conserved AVITG sequence are encoded in the first exon, while the cysteine-rich domain is encoded by exons 2 and 3. The 21 amino acid insertion of prokineticin 2 is encoded by an alternatively spliced mini-exon. To explore the functional difference of prokineticin 1 and 2, chimeric polypeptides were made with their exons 3 swapped (see Figure 6). The chimeric polypeptides were designated chimera 12 (SEQ ID NO:13) and chimera 21 (SEQ ID NO:14), designating the swapped exons, as shown in Figure 6.

Functional assays of refolded chimeric prokineticins 12 and 21 indicated that both of these chimeric polypeptides are active in contracting GI smooth muscle (Figure 7A) . However, the EC_{50} for the chimeric prokineticin 21 (SEQ ID NO:14) was about 8-fold higher than prokineticin 1 or prokineticin 2. Additionally, although the peak contractions were not affected, chimeric prokineticin polypeptides resulted in prolonged contraction of ileal strips (Figure 7B). For wild type prokineticins, the time constants to midway contraction (half way from peak contraction to sustained plateau) were about 15 mins. In contrast, for the chimeric polypeptides, these time constants were prolonged to about 40 mins.

These results suggest that the chimeric prokineticins interact slightly differently with the receptor than wild type prokineticins, and cause less pronounced tachyphylaxis. Thus, the chimeric prokineticins (SEQ ID NOS:13 and 14) may have more potent pharmacological activity *in vivo* than wild-type prokineticins.

Effects of prokineticin on guinea pig ileum smooth muscle in vivo.

To monitor the effects of prokineticin on the contraction of ileal smooth muscle *in vivo*, extraluminal force transducers were implanted on the serosal surface of the guinea pig ileum. Recombinant prokineticin 1 was then administered as a bolus into the jugular vein over a 10-second period. As shown in Figure 8, an intravenous bolus of prokineticin 1 contracts guinea pig ileal smooth muscle in a dose-dependent manner. The threshold dose of prokineticin 1 is about 0.03 µg/kg, and a dose of 30 µg/kg produces the maximum effect.

Therefore, prokineticins, demonstrated above to be able to contract ileal smooth muscle in *ex vivo* preparations, are also effective *in vivo*.

Prokineticin signal transduction

To probe the potential signaling mechanisms of prokineticins, cell lines were identified that express prokineticin receptor endogenously. Over twenty cell lines were screened for binding to iodinated prokineticin 1. One cell line, M2A7 melanoma cells (ATCC CRL-2500; Cunningham et al., Science 255;325-327 (1992)), clearly displayed specific binding, with a receptor level of about 150 fmole/mg protein. Other cell lines that specifically bound prokineticin included M2 melanoma

cells (Cunningham et al., Science 255;325-327 (1992)) and RC-4B/C pituitary tumor cells (ATCC CRL-1903). Cell lines that did not bind prokineticin included HEK293, COS-7, COS-1, Ltk-, NIH3T3, C6, NS10Y and HT-29 cells.

To assess signaling in M2A7 cells, cytosolic calcium was measured by fura-3 fluorescence using a FLIPR system (Fluorometric Imaging Plate Reader; Molecular Devices). Cells were suspended in HEPES medium and incubated with 2 µM of fura-3 AM for 20 min at 31°C. The cells were then centrifuged, washed, resuspended in fura-3-free medium and seeded into 96 wells at 4 x 10^4 cells per well. The cells were loaded with Fluo-3 AM (Molecular Probes) in standard buffer solution (130 mM NaCl,2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 0.45 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 8 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 20 mM HEPES and 10 µM probenecid) with 0.1% fetal bovine serum for 1 h at 37°C, then washed with standard buffer solution. Transient changes in $[Ca_{2+}]_i$ evoked by prokineticin (0.01, 0.1, 0.3, 1, 3, 10, 100 nM) were monitored using the FLIPR system in 96-well plates at 488 nm for 210 s.

As shown in Figure 9, prokineticins can mobilize calcium in M2A7 melanoma cells, with EC_{50} of about 12 and 21 nM for recombinant prokineticin 1 and prokineticin 2, respectively. The signaling is specific, as there was no response in HEK 293 cells. The calcium signaling mobilized by prokineticins is comparable to calcium signal activated by control MCH (melanin-concentrating hormone) receptor SLC1 (Saito et al., Nature 400:265-269 (1999)). The calcium signals elicited by prokineticins are more much robust than the modest calcium signal induced by activation of a typical receptor tyrosine kinase. This result is consistent with

the observation, described above, that the tyrosine protein kinase inhibitor genistein (5mM) had no effect on the contractile activity of prokineticin on ileal smooth muscle.

These results indicate that the prokineticin receptor(s) is/are likely to be GPCR(s), and to signal through Gαq.

Discussion

The results described above establish the existence of mammalian homologues of frog BV8 and snake MITl. To reflect their potent and specific effects on GI smooth muscle, these proteins have been named prokineticins. Their high potency in specifically stimulating the contraction of guinea-pig ileum smooth muscle but not other smooth muscles including aorta, femoral artery, trachea, and gallbladder indicate that prokineticins may be important endogenous regulators of GI motility. Prokineticins may regulate GI smooth muscle as neurocrine signaling molecules, or circulating hormones, or paracrine humoral agents. Since prokineticins are also widely expressed outside the GI system, it is possible that prokineticins may be released from remote organs and regulate GI activity. In this respect, it has also been determined that prokineticins are resistant to protease treatment, which supports their potential long-range and long-term effects.

The molecular size and the processing of prokineticins distinguish them from typical neuropeptides, and indicate they are more similar to cytokines. As one mechanism for eliminating pathogenic organisms is to enhance motility and push the offending

organisms out of the GI tract, prokineticins may also be part of defending immune response, i.e. functioning as inflammatory cytokines that increase the GI motility.

The high potency of recombinant prokineticins on GI contractility suggests that prokineticins probably interact with cell surface receptor(s). This conclusion is reinforced by the receptor binding experiments described above, which demonstrate a saturably high affinity for the iodinated recombinant prokineticin. Moreover, the observation that 10 μM GTPγS can displace almost all of the specific binding indicates the involvement of G protein in prokineticin receptor signaling. Furthermore, the inhibitory effect of the calcium channel blocker verapamil on the contractile effect of prokineticin is consistent with a receptor-mediated mechanism for prokineticins, and also suggests a similar signaling mechanism of prokineticins as those of the M3 muscarinic and motilin receptor in contracting GI smooth muscle: calcium entry via voltage-gated calcium channel is an essential component. Thus, prokineticin receptor most likely is a G protein coupled receptor.

However, alternative interpretations are possible. For instance, prokineticins may cause smooth muscle contraction by directly activating non-selective cation ion channels, or blocking inhibitory potassium channels on GI smooth muscle cells.

Sequence analysis indicates that prokineticins may contain two functional domains: the short N-terminus and the cysteine-rich C-terminus. Since the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Figure 1), this region is likely to have functional importance. In addition to

prokineticins and their isoforms from other species, a similar ten-cysteine motif is also found in a number of other secreted proteins, including colipase, a cofactor for intestinal lipid digestive enzyme lipase (van Tilbeurgh et al., Nature 359:159-162 (1992)) and dikkopfs, a family of proteins that have important roles in early embryonic development (Glinka et al., Nature 391:357-362 (1998) and Aravind et al., Curr. Biol. 8:R477-478 (1998)). Interestingly, dickkopfs actually possess two ten-cysteine domains that have mirror symmetry. X-ray crystallography and solution structural analysis have demonstrated that MIT1 is formed of five pairs of disulfide bonds and folded into a structure similar to colipase (Boisbouvier et al., J. Mol. Biol. 283:205-219 (1998)).

Successful refolding of proteins with five pairs of disulfide bonds has not hitherto been accomplished *in vitro*. Refolding of proteins with more than three pairs of disulfide bonds is still regarded as challenging and difficult (Georgiou et al., Curr. Opin. Biotechnol. 7:190-197 (1996) and Lihe et al., Curr. Opin. Biotechnol. 9:497-501 (1998)). The expression of such disulfide bond-rich proteins in *E. coli* often results in no formation of disulfide bonds, or more probably the formation of incorrect intramolecular or intermolecular disulfide bonds. These events routinely lead to production of inactive recombinant proteins and their aggregation in bacterial inclusion bodies.

In this study, a slow exchange method was utilized to refold prokineticins that have five pairs of disulfide bonds. A number of factors eventually contributed to the successful refolding of prokineticins: 1) a slow rate of removal of denaturing agent; 2) using only reducing agents in the redox refolding mixture,

allowing slow formation of disulfide bonds; 3) low temperature; 4) high concentration of urea and glycerol in dialyzing buffer to prevent protein aggregation; 5) low concentration of recombinant protein to favor forming intra- but not inter-molecular disulfide bonds. These refolding conditions can be used to design protocols for refolding other recombinant proteins that possess multiple disulfide bonds.

In summary, cDNAs encoding two prokineticins have been described. Refolded recombinant prokineticins potently and specifically stimulate the contraction of GI smooth muscle. As impaired GI motility is a very common clinical manifestation in many common disorders including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastroesophageal reflux disease (Longo et al., Dis Colon Rectum 36:696-708 (1993); Tonini, Pharmacol. Res. 33:217-226 (1996); Samsom and Smout, Dig Dis. 15:263-274 (1998); Achem and Robinson, Dig Dis. 16:38-46 (1998) and Briejer et al., Trends Pharmacol Sci. 20:1-3 (1999)), the discovery of endogenous regulators of GI smooth muscle should facilitate the development of novel therapeutics for such disorders that will benefit from altered GI motility.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. An isolated polypeptide that stimulates gastrointestinal smooth muscle contraction, comprising an amino acid sequence at least 80% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), said sequence comprising the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3.

2. The isolated polypeptide of claim 1, wherein amino acid residues that differ from residues in SEQ ID NO:3 are conservative substitutions thereof.

3. The isolated polypeptide of claim 1, wherein amino acid residues that differ from residues in SEQ ID NO:3 consist of the corresponding residues from SEQ ID NO:6.

4. The isolated polypeptide of claim 3, comprising SEQ ID NO:13.

5. The isolated polypeptide of claim 1, comprising amino acids 1-77 of SEQ ID NO:3.

6. The isolated polypeptide of claim 1, comprising SEQ ID NO:3.

7. The isolated polypeptide of claim 1, comprising a 6XHis tag.

8. The isolated polypeptide of claim 1, which is detectably labeled.

9. An isolated peptide comprising at least 10 contiguous amino acids of SEQ ID NO:3, wherein said peptide is immunogenic.

10. A pharmaceutical composition, comprising the isolated polypeptide of claim 1 and a pharmaceutically acceptable carrier.

11. A method of stimulating gastrointestinal smooth muscle contraction in a mammal, comprising administering to said mammal an effective amount of the polypeptide of claim 1.

12. A nucleic acid molecule encoding the polypeptide of claim 1.

13. An expression vector containing the nucleic acid molecule of claim 12 operatively linked to a promoter of gene expression.

14. A host cell comprising the expression vector of claim 13.

15. A method of preparing the isolated polypeptide of claim 1, comprising culturing the host cell of claim 14 so as to express said polypeptide, substantially purifying said polypeptide, and refolding said polypeptide.

16. An antibody that selectively binds the polypeptide of claim 1.

17. An isolated polypeptide that stimulates gastrointestinal smooth muscle contraction, comprising an amino acid sequence at least 80% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), said sequence comprising the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6.

18. The isolated polypeptide of claim 17, wherein amino acid residues that differ from residues in SEQ ID NO:6 are conservative substitutions thereof.

19. The isolated polypeptide of claim 17, wherein amino acid residues that differ from residues in SEQ ID NO:6 consist of the corresponding residues from SEQ ID NO:3.

20. The isolated polypeptide of claim 19, comprising SEQ ID NO:14.

21. The isolated polypeptide of claim 17, comprising amino acids 1-77 of SEQ ID NO:6.

22. The isolated polypeptide of claim 17, comprising SEQ ID NO:6.

23. The isolated polypeptide of claim 17, comprising a 6XHis tag.

24. The isolated polypeptide of claim 17, which is detectably labeled.

25. An isolated peptide comprising at least 10 contiguous amino acids of SEQ ID NO:6, wherein said peptide is immunogenic.

26. A pharmaceutical composition, comprising the isolated polypeptide of claim 17 and a pharmaceutically acceptable carrier.

27. A method of stimulating gastrointestinal smooth muscle contraction in a mammal, comprising administering to said mammal an effective amount of the polypeptide of claim 17.

28. A nucleic acid molecule encoding the polypeptide of claim 17.

29. An expression vector containing the nucleic acid molecule of claim 17 operatively linked to a promoter of gene expression.

30. A host cell comprising the expression vector of claim 29.

31. A method of preparing the isolated polypeptide of claim 17, comprising culturing the host cell of claim 30 so as to express said polypeptide, substantially purifying said polypeptide, and refolding said polypeptide.

32. An antibody that selectively binds the polypeptide of claim 17.

33. A method of identifying a prokineticin receptor ligand, comprising contacting a preparation comprising prokineticin receptor with one or more candidate compounds, and identifying a compound that specifically binds to said receptor, said compound being characterized as a prokineticin receptor ligand.

34. The method of claim 33, wherein said preparation is an intestinal smooth muscle preparation or membrane preparation thereof.

35. The method of claim 33, wherein said preparation is a cell line or membrane preparation thereof.

36. The method of claim 35, wherein said cell line is M2A7 (ATCC CRL-2500).

37. The method of claim 33, wherein the ability of said ligand to selectively agonize or antagonize prokineticin receptor signaling is further determined.

38. The method of claim 37, wherein said signaling is determined in a cell line.

39. The method of claim 38, wherein said cell line is M2A7 (ATCC CRL-2500).

40. The method of claim 37, wherein said signaling is determined by monitoring calcium mobilization.

41. The method of claim 33, wherein the ability of said ligand to modulate smooth muscle contractility is further determined.

42. A method of identifying a prokineticin receptor agonist, comprising contacting a preparation comprising a prokineticin receptor with one or more candidate compounds, and identifying a compound that

selectively promotes production of a prokineticin receptor signal, said compound being characterized as a prokineticin receptor agonist.

43. The method of claim 42, wherein said preparation is a cell line.

44. The method of claim 43, wherein said cell line is M2A7 (ATCC CRL-2500).

45. The method of claim 42, wherein said signaling is determined by monitoring calcium mobilization.

46. The method of claim 42, wherein the ability of said agonist to modulate smooth muscle contractility is further determined.

47. A method of identifying a prokineticin receptor antagonist, comprising contacting a preparation comprising a prokineticin receptor with one or more candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal, said compound being characterized as a prokineticin receptor antagonist.

48. The method of claim 47, wherein said prokineticin comprises an amino acid sequence selected from the group consisting of amino acids 1-77 of SEQ ID NOS:3 and amino acids 1-77 of SEQ ID NO:6.

49. The method of claim 47, wherein said preparation is a cell line.

50. The method of claim 49, wherein said cell line is M2A7 (ATCC CRL-2500).

51. The method of claim 47, wherein said signaling is determined by monitoring calcium mobilization.

52. The method of claim 47, wherein the ability of said antagonist to modulate smooth muscle contractility is further determined.

```
                                                  ⇓
A)          MRGATRVSIMLLLVTVSDC  AVITGA
B) MRSLCCAPLLLLLLLPPLLLTPRAGDA  AVITGA
C)          MKCFAQIVVLLLVIAFSHG  AVITGA
D)                               AVITGA

CERDVQCGAGTCCAISLWLRGLRMCTPLGREGEECHPG
CDKDSQCGGGMCCAVSIWVKSIRICTPMGKLGDSCHPL
CDKDVQCGSGTCCAASAWSRNIRFCIPLGNSGEDCHPA
CERDLQCGKGTCCAVSLWIKSVRVCTPVGTSGEDCHPA
*     *    **           *         *

SHKVPFFRKRKHHTCPCLPNLLCSRFPDGRYRCSMDLKNINF
TRKVPFFGRRMHHTCPCLPGLACLRTSFNRFICLAQK
SHKVPYDGKRLSSLCPCKSGLTCSK.SGEKFKCS
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FIGURE 1

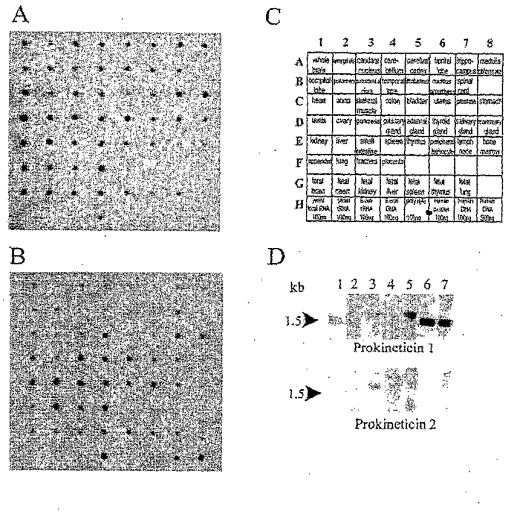

A
B
C
1 2 3 4 5 6 7 8
A
B
C
D
E
F
G
H
D
kb
1 2 3 4 5 6 7
1.5
Prokineticin 1
1.5
Prokineticin 2

FIGURE 2

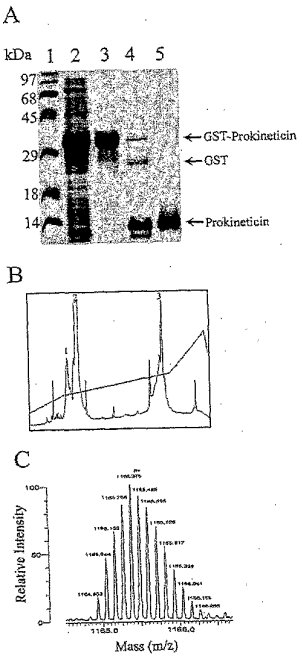

A
kDa 1 2 3 4 5
97
68
45
29
18
14
GST-Prokineticin
GST
Prokineticin
B
C
Relative Intensity
100
50
0
1163.0
1166.0
Mass (m/z)

FIGURE 3

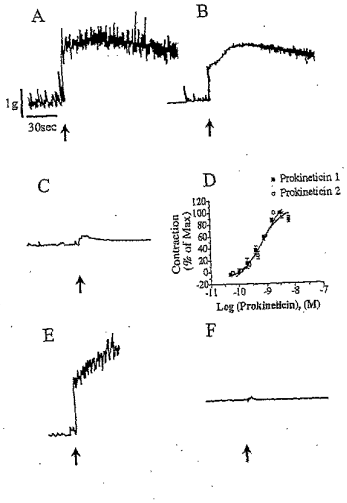

A
B
1g
30sec
C
D
Prokineticin 1
Prokineticin 2
Contraction (% of Max)
120
100
80
60
40
20
0
-20
-11
-10
-9
-8
-7
Log (Prokineticin), (M)
E
F

FIGURE 4

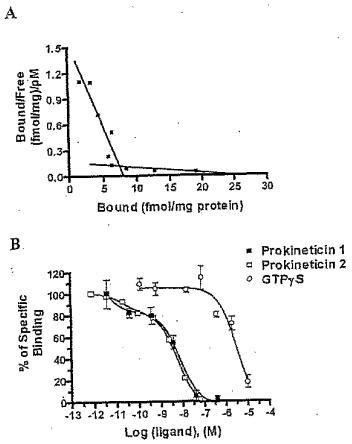

A
Bound/Free (fmol/mg)/pM
Bound (fmol/mg protein)
B
Prokineticin 1
Prokineticin 2
GTPγS
% of Specific Binding
Log (ligand), (M)

FIGURE 5

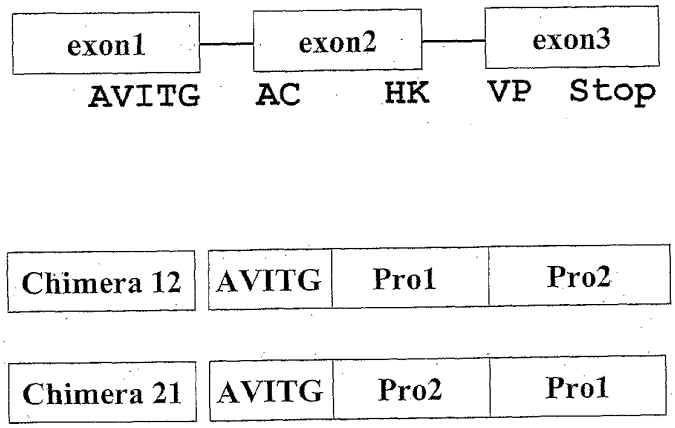
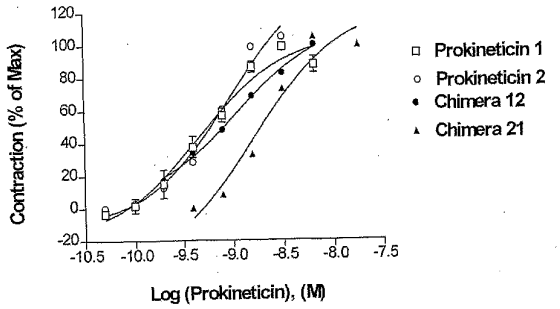

exon1
exon2
exon3
AVITG
AC
HK
VP
Stop
Chimera 12
AVITG
Pro1
Pro2
Chimera 21
AVITG
Pro2
Pro1

FIGURE 6

A


Contraction (% of Max)
120
100
80
60
40
20
0
-20
-10.5
-10.0
-9.5
-9.0
-8.5
-8.0
-7.5
Log (Prokineticin), (M)
Prokineticin 1
Prokineticin 2
Chimera 12
Chimera 21

B

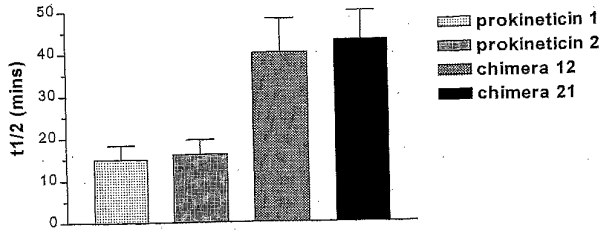

t1/2 (mins)
50
40
30
20
10
0
prokineticin 1
prokineticin 2
chimera 12
chimera 21

FIGURE 7

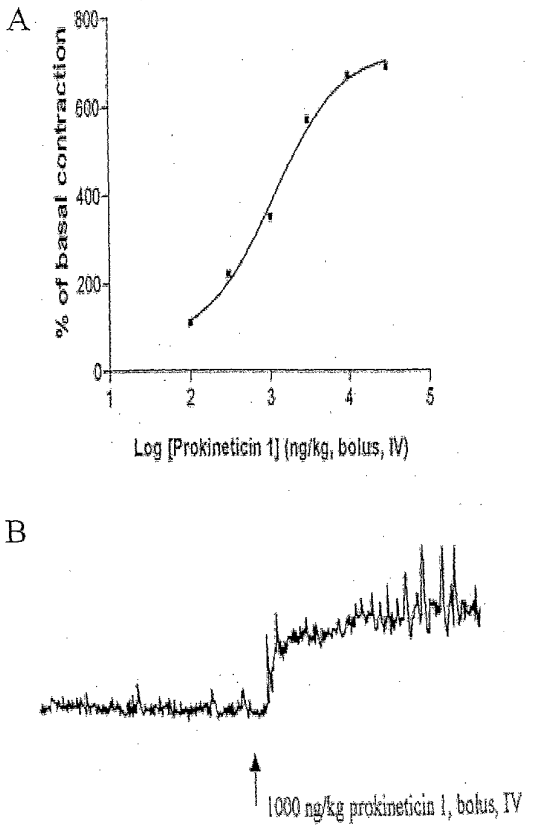

A
% of basal contraction
800
600
400
200
0
1
2
3
4
5
Log [Prokineticin 1] (ng/kg, bolus, IV)
B
1000 ng/kg prokineticin 1, bolus, IV

FIGURE 8

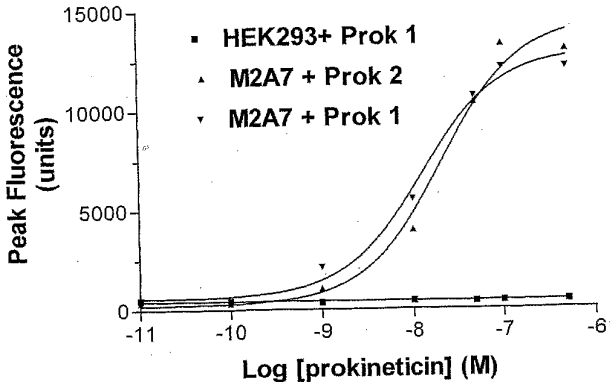

15000
10000
5000
0
Peak Fluorescence (units)
HEK293+ Prok 1
M2A7 + Prok 2
M2A7 + Prok 1
-11
-10
-9
-8
-7
-6
Log [prokineticin] (M)

FIGURE 9

SEQUENCE LISTING

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<120> Prokineticin Polypeptides, Related
Compositions and Methods

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<150> 60/245,882
<151> 2000-11-03

<160> 19

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                                                           Met
                                                            1

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Arg Gly Ala Thr Arg Val Ser Ile Met Leu Leu Leu Val Thr Val Ser
            5                  10                  15

gac tgt gct gtg atc aca ggg gcc tgt gag cgg gat gtc cag tgt ggg   153
Asp Cys Ala Val Ile Thr Gly Ala Cys Glu Arg Asp Val Gln Cys Gly
        20                  25                  30

gca ggc acc tgc tgt gcc atc agc ctg tgg ctt cga ggg ctg cgg atg   201
Ala Gly Thr Cys Cys Ala Ile Ser Leu Trp Leu Arg Gly Leu Arg Met
    35                  40                  45

tgc acc ccg ctg ggg cgg gaa ggc gag gag tgc cac ccc ggc agc cac   249
Cys Thr Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His
 50                  55                  60                  65

aag gtc ccc ttc ttc agg aaa cgc aag cac cac acc tgt cct tgc ttg   297
Lys Val Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu
                70                  75                  80

ccc aac ctg ctg tgc tcc agg ttc ccg gac ggc agg tac cgc tgc tcc   345
Pro Asn Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser
            85                  90                  95
```

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Met Asp Leu Lys Asn Ile Asn Phe
        100                 105

catccttttc tgagcacagc ctggattttt atttctgcca tgaaacccag ctcccatgac 459
tctcccagtc cctacactga ctaccctgat ctctcttgtc tagtacgcac atatgcacac 519
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<212> PRT
<213> Homo sapiens

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            20                  25                  30
Gly Ala Gly Thr Cys Cys Ala Ile Ser Leu Trp Leu Arg Gly Leu Arg
        35                  40                  45
Met Cys Thr Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser
    50                  55                  60
His Lys Val Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys
65                  70                  75                  80
Leu Pro Asn Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys
                85                  90                  95
Ser Met Asp Leu Lys Asn Ile Asn Phe
            100                 105


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<213> Homo sapiens

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            20                  25                  30
Pro Leu Gly Arg Glu Gly Glu Glu Cys His Pro Gly Ser His Lys Val
```

```
       35                  40                  45
Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
    50                  55                  60
Leu Leu Cys Ser Arg Phe Pro Asp Gly Arg Tyr Arg Cys Ser Met Asp
65                  70                  75                  80
Leu Lys Asn Ile Asn Phe
                85
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<220>
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<222> (10)...(333)

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          Met Arg Ser Leu Cys Cys Ala Pro Leu Leu Leu Leu Leu Leu
           1               5                  10

ctg ccg ccg ctg ctg ctc acg ccc cgc gct ggg gac gcc gcc gtg atc   99
Leu Pro Pro Leu Leu Leu Thr Pro Arg Ala Gly Asp Ala Ala Val Ile
 15                  20                  25                  30

acc ggg gct tgt gac aag gac tcc caa tgt ggt gga ggc atg tgc tgt   147
Thr Gly Ala Cys Asp Lys Asp Ser Gln Cys Gly Gly Gly Met Cys Cys
                35                  40                  45

gct gtc agt atc tgg gtc aag agc ata agg att tgc aca cct atg ggc   195
Ala Val Ser Ile Trp Val Lys Ser Ile Arg Ile Cys Thr Pro Met Gly
            50                  55                  60

aaa ctg gga gac agc tgc cat cca ctg act cgt aaa gtt cca ttt ttt   243
Lys Leu Gly Asp Ser Cys His Pro Leu Thr Arg Lys Val Pro Phe Phe
        65                  70                  75

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(54) Title: PROKINETICIN POLYPEPTIDES, RELATED COMPOSITIONS AND METHODS

(57) Abstract: The invention provides isolated polypeptides that stimulate gastrointestinal smooth muscle contraction, including human prokineticin 1 and human prokineticin 2 polypeptides, and functional fragments and modifications thereof. Also provided are methods of stimulating gastrointestinal smooth muscle contraction in a mammal, by administering to the mammal an effective amount of a prokineticin polypeptide. The invention also provides nucleic acid molecules encoding a prokineticin polypeptide, and antibodies that selectively bind a prokineticin polypeptide. Further provided are methods of identifying a prokineticin receptor ligand, agonist or antagonist.

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ZHOU, Qun-Yong** [CN/US]; 42 Harvey Court, Irvine, CA 92612 (US). **EHLERT, Frederick, J.** [US/US]; 29 Whitman Court, Irvine, CA 92612 (US).

(74) Agents: **WEBSTER, Melanie, K.** et al.; Campbell & Flores LLP, 7th Floor, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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PROKINETICIN POLYPEPTIDES, RELATED COMPOSITIONS AND METHODS

BACKGROUND OF THE INVENTION

The main function of gastrointestinal (GI) smooth muscle is to mix and propel intralumenal contents, which enables efficient digestion of food, progressive absorption of nutrients, and eventual evacuation of residual components. The activity of GI smooth muscle is regulated by intrinsic and extrinsic neural signals, including classical neurotransmitters, co-existing neuropeptides, and circulating peptide hormones. In addition, a number of humoral agents including histamine, serotonin, and adenosine that are produced by nonneural GI cells also influence the activity of smooth muscle cells.

A number of clinical conditions are associated with altered GI motility, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, gastrointestinal reflux disease, chronic diarrhea, infectious diseases, malabsorptive disorders, inflammatory bowel disorders, and intestinal cancers. The identification of regulators of gastrointestinal motility should facilitate the development of novel therapeutics for disorders that involve impaired or enhanced gastrointestinal motility.

Two potential regulators of gastrointestinal motility have recently been identified. Mamba intestinal toxin (MITl), a small protein that potently stimulates the contraction of guinea-pig ileum, has been purified from mamba snake venom (Schweitz et al., Toxicon 28:847-856 (1990) and Schweitz et al., FEBS Letters 461:183-188 (1999)). Recently, a protein of similar size and having

greater than 40% identity with MIT1, including all 10 conserved cysteines, has been purified from frog skin secretions (Mollay et al., Eur. J. Pharmacol. 374:189-196 (1999)). The frog protein, named Bv8, was also found to potently stimulate the contraction of GI smooth muscle.

Methods of recombinantly preparing these snake and frog polypeptides, or of recombinantly preparing other polypeptides containing 10 cysteines, have not previously been described, limiting the utility of these regulators for therapeutic use. Additionally, snake and frog polypeptides could elicit antibodies if administered to mammals that would likely reduce their efficacy as therapeutics.

Accordingly, there exists a need to identify endogenous human polypeptides that stimulate or inhibit gastrointestinal motility, and to develop methods of preparing these compounds recombinantly as therapeutics. There also exists a need to identify small molecule agonists and antagonists of endogenous gastrointestinal regulators that can be used therapeutically. The present invention satisfies this need, and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides isolated polypeptides that stimulate gastrointestinal smooth muscle contraction. In one embodiment, the polypeptide contains an amino acid sequence at least 80% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), wherein the sequence contains the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3. In another embodiment, the polypeptide

containis an amino acid sequence at least 80% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), wherein the sequence contains the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6.

Also provided are methods of stimulating gastrointestinal smooth muscle contraction in a mammal, by administering to the mammal an effective amount of a prokineticin polypeptide.

The invention also provides nucleic acid molecules encoding a prokineticin polypeptide.

Further provided are antibodies that selectively bind a prokineticin polypeptide.

The invention also provides methods of identifying a prokineticin receptor ligand, by contacting a preparation containing prokineticin receptor with one or more candidate compounds, and identifying a compound that specifically binds to the receptor. Such a compound is characterized as a prokineticin receptor ligand.

Also provided are methods of identifying a prokineticin receptor agonist, by contacting a preparation containing a prokineticin receptor with one or more candidate compounds, and identifying a compound that selectively promotes production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor agonist.

Further provided are methods of identifying a prokineticin receptor antagonist, by contacting a preparation containing a prokineticin receptor with one

or more candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor antagonist.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences of A) prokineticin 1 precursor (SEQ ID NO:2); B) prokineticin 2 precursor (SEQ ID NO:4); C) frog BV8 (SEQ ID NO:11) and D) partial sequence of MIT 1 (SEQ ID NO:12). Ten conservative cysteine residues are marked (*). Signal peptides are underlined. The arrow indicates an intron splice site.

Figure 2 shows the expression pattern of prokineticins. A human RNA master blot was probed with A) prokineticin 1 and B) prokineticin 2 cDNA. Figure 2C show the blot diagram indicating the RNA sources for each dot. Figure 2D shows a Northern blot analysis with prokineticin 1. Each lane contains RNA from different brain tissues as indicated: 1. Cerebellum; 2. Cerebral cortex; 3. Medulla; 4. Spinal cord; 5. Occipital pole; 6. Frontal lobe; 7. Temporal lobe; 8. Putamen; 9, Amygdala; 10. Caudate nucleus; 11. Corpus callosum; 12. Hippocampus; 13. Whole brain; 14. Substanti anigra.; 15. Subthalamic nucleus; 16. Thalamus.

Figure 3 shows the production and purification of human prokineticins: A) SDS-PAGE (18%) of prokineticin samples stained with Coomassie blue G-250. Lane 1, molecular weight standards; lane 2, whole bacterial lysate after induction; lane 3, Ni-NTA affinity chromatography-purified prokineticin; lane 4, Factor Xa digested prokineticin; lane 5, refolded prokineticin

after HPLC purification. Each lane was loaded with 10-15 µg total protein. B) Reverse phase HPLC separation of refolded protein mixture. Peak 2 contains refolded prokineticin. C) Electrospray mass spectrum of refolded prokineticin 1.

Figure 4 shows the effects of prokineticins on the contractility of guinea-pig ileal longitudinal smooth muscle. The contractile responses to prokineticin 1 (2 nM) were measured in ileum in the absence (A) and in the presence of tetrodotoxin (0.1µM; B) and verapamil (1 µM; C). Figure 4D shows the concentration-response relationship for the contractile effects of prokineticins. Results are given as percentage of maximum contractility. Data are from three independent experiments. Contractile effects of oxotremorine-M in ileum in the absence (E) and in the presence of verapamil(1µM; F) are also shown. Arrows indicate when drugs were added.

Figure 5A shows Scatchard analysis of the specific binding of ^{125}I-prokineticin 1 to guinea pig ileal membrane. Figure 5B shows the inhibition of binding of ^{125}I-prokineticin 1 (20 pM) by different concentrations of unlabeled prokineticin 1 (filled squares) and unlabeled prokineticin 2 (open squares). Open circles show displacement of ^{125}I-prokineticin 1 (20 pM) with different concentrations of GTPγS.

Figure 6 shows a schematic diagram of chimeras constructed between prokineticin 1 and prokineticin 2, designated chimera 12 (SEQ ID NO:13) and chimera 21 (SEQ ID NO:14).

Figure 7 shows functional characterization of chimeric prokineticins. Figure 7A shows a dose-response curve of chimeric and wild type prokineticins assayed for their ability to contract guinea-pig ileum. Figure 7B shows time constants of chimeric and wild type prokineticins. The time constant indicates the time elapsed from peak contraction to midway contraction (half way from peak to sustained plateau contraction). After normalizing against the oxotremorine M-induced contraction, the peak and sustained plateau contraction elicited by prokineticins are about 80% and 40%, respectively. The midway contraction is thus about 60% of maximum contraction.

Figure 8A shows the effect of administration of various doses of prokineticin 1 as an IV bolus on contractions in guinea pig ileum *in vivo*. Figure 8B shows the contractile response to 1000 ng/kg of prokineticin 1.

Figure 9 shows calcium mobilization, as determined in a FLIPR assay, elicited in HEK293 or M2A7 cells by the indicated concentrations of prokineticin 1 or prokineticin 2.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides an isolated prokineticin polypeptide that is able to stimulate gastrointestinal (GI) smooth muscle contraction. The prokineticin polypeptides of the invention can be used, for example, in therapeutic methods to treat disorders involving impaired GI motility. Such polypeptides can also be used, for example, in screening methods to identify prokineticin receptor ligands, including receptor

agonists and antagonists, which can be used therapeutically to treat disorders involving impaired or enhanced GI motility.

As used herein, the term "prokineticin polypeptide" refers to a polypeptide comprising the amino acid sequence of human prokineticin 1 shown as the non-underlined sequence in Figure 1A (SEQ ID NO:3), or comprising the amino acid sequence of human prokineticin 2 shown as the non-underlined sequence in Figure 1B (SEQ ID NO:6); and to a polypeptide containing minor modifications to SEQ ID NOS:3 or 6 that has GI smooth muscle contractile activity; and to a fragment of the reference polypeptide that has GI smooth muscle contractile activity.

As used herein, the terms "comprising," "having," "encoding," and "containing," and derivatives of these terms, are intended to be open-ended. The term "consisting" is intended to be closed-ended.

As used herein, the term "minor modification" to the sequences designated SEQ ID NOS:3 or 6 refers to one or more additions, deletions or substitutions compared with the recited amino acid sequence; one or more chemical or enzymatic modifications to the polypeptide; or substitution of one or more L-configuration amino acids with corresponding D-configuration amino acids. Such modifications can be advantageous, for example, in enhancing the stability, expression, bioactivity, or receptor affinity of the polypeptide, or for facilitating its identification or purification.

The GI smooth muscle contractile activity of a modified polypeptide can be determined by *ex vivo* or *in vivo* methods known in the art, such as the *ex vivo* and *in vivo* guinea pig ileal bioassays described in the Example, to confirm that it has GI smooth muscle contractile activity. Suitable assays for determining GI smooth muscle contractile activity can alternatively be performed using other GI smooth muscle tissue that responds to prokineticin 1 or 2, such as fundic muscle strip or proximal colon (see Example). Likewise, suitable assays can be performed using other mammals, including, for example, mice, rats, cats, dogs, sheep, goats, pigs, cows and primates.

A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can elicit at least 10%, 25%, 50%, 75%, 100% or more of the maximal GI smooth muscle contraction of human prokineticin 1 or 2, under the same conditions. A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can be less potent, similarly potent, or more potent than human prokineticin 1 or 2, under the same conditions. For example, a modified polypeptide can have an EC_{50} that is 5-fold, 10-fold, 50-fold or 100-fold higher or lower than the EC_{50} for human prokineticin 1 or 2. A modified prokineticin polypeptide that elicits GI smooth muscle contractile activity can also elicit contractions for the same duration or for a longer or shorter duration than human prokineticin 1 or 2, under the same conditions.

A chimeric polypeptide encoded by exons 1 and 2 of prokineticin 1 and exon 3 of prokineticin 2, designated chimera 12 (SEQ ID NO:13) (see Figure 6) is an example of a modified prokineticin that elicits ileal contractions with a similar potency as prokineticins 1 or

2 (see Figure 7A), but which causes prolonged contractions in comparison with prokineticins 1 or 2 (see Figure 7B).

A chimeric polypeptide encoded by exons 1 and 2 of prokineticin 2, and exon 3 of prokineticin 1, designated chimera 21 (SEQ ID NO:13) (see Figure 6) is an example of a modified prokineticin that elicits ileal contractions with an 8-fold lower potency than prokineticins 1 or 2 (see Figure 7A), and which causes prolonged contractions in comparison with prokineticins 1 or 2 (see Figure 7B).

Modifications to the amino acid sequence designated SEQ ID NOS:3 or 6 can be randomly generated, such as by random insertions, deletions or substitutions of nucleotides in a nucleic acid molecule encoding SEQ ID NOS:3 or 6. Alternatively, modifications can be directed, such as by site-directed mutagenesis of a nucleic acid molecule encoding SEQ ID NOS:3 or 6.

Computer programs known in the art can provide guidance in predicting which amino acid residues can be modified without abolishing the function of the polypeptide (see, for example, Eroshkin et al., Comput. Appl. Biosci. 9:491-497 (1993)).

Furthermore, guidance in modifying amino acid residues of SEQ ID NOS:3 or 6 while retaining activity can be provided by comparison of SEQ ID NOS:3 or 6 with the sequence of their mammalian homologs, such as homologs in non-human primates, mouse, rat, rabbit, bovine, porcine, ovine, canine or feline species, as well as sequences of their homologs in non-mammalian vertebrates, including frog Bv8 polypeptide (SEQ ID NO:11) and snake MIT1 polypeptide (SEQ ID NO:12) (see

Figure 1). It is well known in the art that evolutionarily conserved amino acid residues and domains are more likely to be important for maintaining biological activity than less well-conserved residues and domains. Thus, it would be expected that substituting a residue that is highly conserved among mammalian prokineticins, frog Bv8 polypeptide and snake MIT1 polypeptide, such as the N-terminal sequence, or any of the 10 cysteines, would likely be detrimental to activity, whereas substitution of less highly conserved residues, such as the C-terminal residues, is likely to be tolerated.

As described in the Example, retention of the six N-terminal residues (AVITGA) of prokineticin, without addition, deletion or substitution, is apparently required to retain smooth muscle contractile activity (see Table 1). However, modifications of the AVITGA sequence can result in polypeptides that do not exhibit GI smooth muscle contractile activity, but that antagonize the smooth muscle contractile activity of wild-type prokineticins. The N-terminal mutants designated SEQ ID NOS:16 and 18 are examples of modified prokineticin polypeptides with antagonistic activity.

The N-terminal domain of prokineticins, while apparently required for GI smooth muscle contractile activity, is not sufficient for GI smooth muscle contractile activity. Specifically, a prokineticin N-terminal peptide (SEQ ID NO:19), or a polypeptide with the cysteine-rich domain of prokineticin substituted with the cysteine-rich domain of either co-lipase or dickkopf4, did not exhibit smooth muscle contractile activity.

The cysteine-rich domain of prokineticins was shown to also be required for GI smooth muscle contractile activity (see Table 1). Specifically, correct cysteine pairing was shown to be required in order to retain activity, as substitutions at either of two cysteines abolished activity.

The sequence C-terminal to the final cysteine is presumably not required, as evidenced by GI smooth muscle contractile activity of prokineticin polyepeptides with a 6XHis tag inserted at this position. Additionally, as evidenced by similar activities of prokineticins 1 and 2, despite only being 44% identical, amino acid sequence substitutions at positions other than the N-terminus and conserved cysteine residues are well tolerated.

Substitutions to the amino acid sequences designated SEQ ID NOS:3 or 6 can either be conservative or non-conservative. Conservative amino acid substitutions include, but are not limited to, substitution of an apolar amino acid with another apolar amino acid (such as replacement of leucine with an isoleucine, valine, alanine, proline, tryptophan, phenylalanine or methionine); substitution of a charged amino acid with a similarly charged amino acid (such as replacement of a glutamic acid with an aspartic acid, or replacement of an arginine with a lysine or histidine); substitution of an uncharged polar amino acid with another uncharged polar amino acid (such as replacement of a serine with a glycine, threonine, tyrosine, cysteine, asparagine or glutamine); or substitution of a residue with a different functional group with a residue of similar size and shape (such as replacement of a serine with an alanine; an arginine with a methionine; or a tyrosine with a phenylalanine).

Specifically contemplated substitutions to the amino acid sequences designated SEQ ID NOS:3 and 6 include replacement of residues from wild-type prokineticin 1 with residues from prokineticin 2, and vice versa. The replacements can be of single residues, multiple residues throughout the polypeptide, or multiple contiguous residues. Because chimeras between SEQ ID NOS:3 and 6, namely SEQ ID NOS:13 and 14, were demonstrated to exhibit prolonged contractile activity in comparison with wild-type prokineticins, it is contemplated that substituted prokineticins can be potent therapeutics *in vivo*.

Additions to the amino acid sequence designated SEQ ID NOS:3 or 6 include, but are not limited to, the addition of "tag" sequences, which are preferably added at the C terminus. Such tag sequence include, for example, epitope tags, histidine tags, glutathione-S-transferase (GST), and the like, or sorting sequences. Such additional sequences can be used, for example, to facilitate recombinant expression, purification or characterization of a prokineticin. Exemplary polypeptides containing additions to the sequences designated SEQ ID NOS:3 or 6 are the active prokineticins prepared as described in the Example by the insertion of a 6XHis-tag after the C-terminal cysteine.

Deletions to the amino acid sequences designated SEQ ID NOS:3 or 6 include, but are not limited to, deletion of one or more residues at the C-termini that are not highly conserved among the active polypeptides shown in Figure 1. Deleted sequences can optionally be replaced by tag sequences, as described previously.

Chemical and enzymatic modifications to the polypeptide containing the amino acid sequence designated SEQ ID NOS:3 or 6 include, but are not limited to the following: replacement of hydrogen by an alkyl, acyl, or amino group; esterification of a carboxyl group with a suitable alkyl or aryl moiety; alkylation of a hydroxyl group to form an ether derivative; phosphorylation or dephosphorylation of a serine, threonine or tyrosine residue; or N- or O-linked glycosylation.

As used herein, the term "isolated" indicates that the molecule is altered by the hand of man from how it is found in its natural environment. Preferably, an "isolated" prokineticin polypeptide can be a "substantially purified" molecule, that is at least 60%, 70%, 80%, 90 or 95% free from cellular components with which it is naturally associated. An isolated polypeptide can be in any form, such as in a buffered solution, a suspension, a lyophilized powder, recombinantly expressed in a heterologous cell, bound to a receptor or attached to a solid support.

The invention provides isolated polypeptides that stimulate gastrointestinal smooth muscle contraction. In one embodiment, the polypeptide contains an amino acid sequence at least 50% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), and including the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3. The encoded polypeptide can thus have at least 60%, 65%, 70%, 75% identity, including at least 80%, 85%, 90%, 95%, 96%, 98%, 99% or greater identity to SEQ ID NO:3. An exemplary polypeptide contains the amino acid sequence designated SEQ ID NO:3, or amino acids 1-77 thereof.

In one embodiment, the isolated polypeptide does not contain the amino acid sequence NNFGNGRQERRKRKRSKRKKE (SEQ ID NO:7). In another embodiment, the isolated polypeptide does not contain the amino acid sequence SHVANGRQERRRAKRRKRKKE (SEQ ID NO:8).

In another embodiment, the polypeptide contains an amino acid sequence at least 50% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), and including the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6. The encoded polypeptide can thus have at least 60%, 65%, 70%, 75% identity, including at least 80%, 85%, 90%, 95%, 96%, 98%, 99% or greater identity to SEQ ID NO:6. An exemplary polypeptide contains the amino acid sequence designated SEQ ID NO:6, or amino acids 1-77 thereof.

As used herein, the term "percent identity" with respect to two molecules is intended to refer to the number of identical nucleotide or amino acid residues between the aligned portions of two sequences, expressed as a percentage of the total number of aligned residues, as determined by comparing the entire sequences using an optimized manual alignment or computer alignment, such as a BLAST 2.0 alignment (Tatusova et al., FEMS Microbiol Lett. 174:247-250 (1999)).

For certain applications, such as in the screening methods disclosed herein, a prokineticin polypeptide can be labeled with a detectable moiety, such as a radiolabel, a fluorochrome, a ferromagnetic substance, a luminescent tag or a detectable binding agent such as biotin. Other suitable labeled moieties are well known in the art.

The invention also provides methods for preparing an isolated prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, by culturing host cells (described below) so as to express a recombinant prokineticin polypeptide, and refolding the polypeptide under conditions that minimize protein aggregation.

Recombinant expression of polypeptides containing multiple cysteine residues often results in the incorrect formation of inter- and intra-molecular disulfide bonds, which leads to the production of inactive, aggregated bacterial proteins. As disclosed herein, these problems can be overcome using conditions that minimize protein aggregation during refolding of the expressed polypeptide. Exemplary conditions that minimize protein aggregation are described in the Example, and differ from conventional conditions for preparing recombinant protein by including one or more of the following refolding conditions: 1) keeping protein concentration low (e.g. about 100 µg/ml); 2) dialysing, rather than diluting, the peptides to remove denaturing agent; 3) omitting oxidants from buffers; 4) maintaining high concentrations of urea in all buffers; 5) maintaining high concentrations of glycerol (e.g. at least about 10%) in buffers; and 6) keeping peptides and buffers at low temperature (e.g. about 4°C). Of these conditions, it is contemplated that low protein concentration (ie. less than about 250 µg/ml, preferably less than 200 µg/ml, 150 µg/ml, 100 µg/ml, or 50 µg/ml) and high urea concentration (e.g. at least about 1.5M, such as about 2M, 4M, 6M, 8M or higher) are the most important factors in successful refolding of active prokineticins.

It is expected that the same or similar conditions as those described herein can be used to recombinantly express and refold other polypeptides containing multiple cysteines, including dickkopf, co-lipase, MIT-1 and Bv8, so as to isolate a biologically active polypeptide.

In a preferred method for preparing an isolated prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, a prokineticin polypeptide is recombinantly expressed in bacteria as a fusion protein (e.g. as a GST fusion) containing a tag (e.g. a 6XHis tag), and partially purified by affinity isolation (e.g. on a nickel column). The fused polypeptide is then cleaved so as to remove the heterologous protein (e.g. using protease factor Xa cleavage between GST and prokineticin), and the prokineticin polypeptide refolded under conditions described above to minimize protein aggregation. To obtain more highly purified polypeptide, the polypeptide can further be purified by column chromatography (e.g. reverse-phase HPLC). Those skilled in the art recognize that modification to these preferred methods for recombinantly expressing, refolding and purifying active prokineticin polypeptides can readily be determined, such as employing alternative heterologous sequences, cleavable sequences, tags, host cells and buffer conditions.

Alternatively, an isolated prokineticin polypeptide can be prepared by biochemical procedures. As disclosed herein, prokineticins 1 and 2 are expressed in a variety of human tissues (see Example, and particularly Figure 2). Therefore, an isolated prokineticin polypeptide can be isolated from tissues or cells that normally express these polypeptides, by biochemical procedures routinely used in the art,

including membrane fractionation, chromatography, electrophoresis and ligand affinity methods, or using immunoaffinity methods with the prokineticin antibodies described herein. Following biochemical isolation, an inactive prokineticin can be refolded by the methods described above to restore activity.

Likewise, an isolated prokineticin polypeptide can be prepared by chemical synthesis procedures known in the art. Following chemical synthesis, an inactive prokineticin can be refolded by the methods described herein to restore activity.

If desired, such as to optimize their functional activity, selectivity, stability or bioavailability, chemically synthesized polypeptides can be modified to include D-stereoisomers, non-naturally occurring amino acids, and amino acid analogs and mimetics. Examples of modified amino acids and their uses are presented in Sawyer, Peptide Based Drug Design, ACS, Washington (1995) and Gross and Meienhofer, The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983). For certain applications, it can also be useful to incorporate one or more detectably labeled amino acids into a chemically synthesized polypeptide or peptide, such as radiolabeled or fluorescently labeled amino acids.

The invention also provides isolated peptides containing, or consisting of, at least 10 contiguous amino acids of the amino acid sequences designated SEQ ID NOS:3 or 6 which can, but need not, be able to stimulate gastrointestinal (GI) smooth muscle contraction. Such isolated peptides are useful, for example, in preparing and purifying prokineticin antibodies of the invention. Such peptides can also act as antagonists to block

signaling through a prokineticin receptor, and thus can be used in therapeutic and screening methods. An isolated prokineticin peptide can thus contain, or consist of, at least 12, 15, 20, 25 or more contiguous amino acids of SEQ ID NOS:3 or 6, including at least, or not more than, 30, 40, 50, 60, 70, 80, 81 or 86 contiguous amino acids.

In one embodiment, an isolated prokineticin peptide contains, or consists of, at least 10 contiguous residues from within amino acid residues 6 and 48 of SEQ ID NO:3. In another embodiment, an isolated prokineticin peptide contains, or consists of, at least 10 contiguous residues from within amino acid residues 6 and 48 of SEQ ID NO:6.

An isolated peptide containing at least 10 contiguous amino acids of SEQ ID NOS:3 or 6 can be immunogenic. As used herein, the term "immunogenic" refers to a peptide that either is capable of inducing prokineticin-specific antibodies, or is capable of competing with prokineticin-specific antibodies for binding to a prokineticin. Peptides that are likely to be immunogenic can be predicted using methods and algorithms known in the art and described, for example, by Irnaten et al., Protein Eng. 11:949-955 (1998), and Savoie et al., Pac. Symp. Biocomput. 1999:182-189 (1999). The immunogenicity of the peptides of the invention can be confirmed by methods known in the art.

The isolated prokineticin polypeptide and peptides of the invention can optionally be conjugated to a carrier, such as KLH, serum albumin, tetanus toxoid and the like, using standard linking techniques, to enhance their immunogenicity. Additionally or alternatively, the isolated polypeptides and peptides can be formulated with

an adjuvant known in the art, such as Freund's complete or incomplete adjuvant.

An isolated prokineticin peptide of at least 10 contiguous residues can conveniently be prepared by chemical synthesis, or by chemical or enzymatic digestion of longer peptides, prepared as described above. An isolated prokineticin peptide of at least 10 contiguous residues can also be prepared recombinantly, such as fused to a protein tag. Those skilled in the art can determine an appropriate method of preparing an isolated prokineticin peptide, depending on its size, sequence, and intended application.

The invention also provides an isolated nucleic acid molecule encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction. The invention nucleic acid molecules are suitable for a variety of screening, therapeutic and diagnostic applications. For example, an invention nucleic acid molecule can be expressed *in vitro* and the encoded prokineticin polypeptide isolated. An invention nucleic acid molecule can also be expressed *in vivo*, to restore normal prokineticin activity in patients, or expressed in an antisense orientation to block prokineticin expression in patients in need thereof. Additionally, the invention nucleic acid molecules can be used as probes or primers to identify and isolate prokineticin-encoding nucleic acid molecules from other species, or to identify structurally related molecules. Such probes and primers are also useful diagnostically to determine normal and abnormal expression of prokineticin in human tissues, and thus to predict susceptibility to conditions associated with altered prokineticin expression.

As used herein, the term "isolated nucleic acid molecule" is intended to mean that the nucleic acid molecule is altered, by the hand of man, from how it is found in its natural environment. For example, an isolated nucleic acid molecule can be a molecule operatively linked to an exogenous nucleic acid sequence. An isolated nucleic acid molecule can also be a molecule removed from some or all of its normal flanking nucleic acid sequences.

An isolated molecule can alternatively, or additionally, be a "substantially pure" molecule, in that the molecule is at least 60%, 70%, 80%, 90 or 95% free from cellular components with which it is naturally associated. An isolated nucleic acid molecule can be in any form, such as in a buffered solution, a suspension, a lyophilized powder, attached to a solid support (e.g. as a component of a DNA array), or in a cell.

As used herein, the term "nucleic acid molecule" refers to a polynucleotide of natural or synthetic origin, which can be single- or double-stranded, can correspond to genomic DNA, cDNA or RNA, and can represent either the sense or antisense strand or both.

The term "nucleic acid molecule" is intended to include nucleic acid molecules that contain one or more non-natural nucleotides, such as nucleotides having modifications to the base, the sugar, or the phosphate portion, or having one or more non-natural linkages, such as phosphothioate linkages. Such modifications can be advantageous in increasing the stability of the nucleic acid molecule, particularly when used in hybridization applications.

Furthermore, the term "nucleic acid molecule" is intended to include nucleic acid molecules modified to contain a detectable moiety, such as a radiolabel, a fluorochrome, a ferromagnetic substance, a luminescent tag or a detectable binding agent such as biotin. Nucleic acid molecules containing such moieties are useful as probes for detecting the presence or expression of prokineticin nucleic acid molecule.

Prokineticin polypeptides that are able to stimulate GI smooth muscle contraction have been described above. Accordingly, it is routine for those skilled in the art to prepare isolated nucleic acid molecules encoding such polypeptides. Exemplary isolated nucleic acid molecules encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction contains, or consists of, a) the nucleotide sequences designated SEQ ID NOS:1 or 4; b) the portion of the nucleotide sequences designated SEQ ID NOS:1 or 4 that encodes SEQ ID NOS:3 or 6 (i.e. nucleotides 55-370 of the nucleotide sequence designated SEQ ID NO:1 and nucleotides 10-334 of the nucleotide sequence designated SEQ ID NO:4); c) a nucleotide sequence that encodes an active modification or active fragment of SEQ ID NOS:3 or 6; and d) a sequence that is degenerate with respect to either a), b) or c).

In one embodiment, the isolated nucleic acid molecule does not encode the amino acid sequence NNFGNGRQERRKRKRSKRKKE (SEQ ID NO:7). In another embodiment, the isolated nucleic acid molecule does not encode the amino acid sequence SHVANGRQERRRAKRRKRKKE(SEQ ID NO:8). In yet another embodiment, an isolated nucleic acid molecule encoding a prokineticin polypeptide excludes naturally occuring signal polypeptides, such as nucleic acid molecules encoding the underlined portions

of the amino acid sequences shown in Figure 1A and 1B (MRGATRVSIMLLLVTVSDC (SEQ ID NO:9) and MRSLCCAPLLLLLLLPLLLTPPAGDA (SEQ ID NO:10)).

In certain embodiments, an isolated nucleic acid molecule encoding a prokineticin polypeptide specifically excludes nucleic acid molecules having the exact sequence of genomic fragments ESTs and cDNAs whose sequences are compiled in publically available databases, such as GenBank Accession Nos. AI277349, AA883760, AQ426386, AC068519, AC026973, AL358215 and AL390797 or sequences which encode amino acid sequences having GenBank Accession Nos. AF182066, AF182064, AF182069 and AF182065.

In one embodiment, an isolated nucleic acid molecule encoding a prokineticin polypeptide excludes mammalian sequences present in the GenBank database that contain sequences which do not encode SEQ ID NOS:3 and 6 (e.g. nucleic acid molecules that encode 5' and 3' untranslated regions, introns or other exons present on chromosomes 1 or 3).

The invention further provides an isolated nucleic acid molecule encoding a prokineticin polypeptide that is able to stimulate GI smooth muscle contraction, wherein the nucleic acid molecule is operatively linked to a promoter of gene expression. As used herein, the term "operatively linked" is intended to mean that the nucleic acid molecule is positioned with respect to either the endogenous promoter, or a heterologous promoter, in such a manner that the promoter will direct the transcription of RNA using the nucleic acid molecule as a template.

Methods for operatively linking a nucleic acid to a heterologous promoter are well known in the art and include, for example, cloning the nucleic acid into a vector containing the desired promoter, or appending the promoter to a nucleic acid sequence using PCR. A nucleic acid molecule operatively linked to a promoter of RNA transcription can be used to express prokineticin transcripts and polypeptides in a desired host cell or *in vitro* transcription-translation system.

The choice of promoter to operatively link to an invention nucleic acid molecule will depend on the intended application, and can be determined by those skilled in the art. For example, if a particular gene product may be detrimental to a particular host cell, it may be desirable to link the invention nucleic acid molecule to a regulated promoter, such that gene expression can be turned on or off. Alternatively, it may be preferred to have expression driven by either a weak or strong constitutive promoter. Exemplary promoters suitable for mammalian cell systems include, for example, the SV40 early promoter, the cytomegalovirus (CMV) promoter, the mouse mammary tumor virus (MMTV) steroid-inducible promoter, and the Moloney murine leukemia virus (MMLV) promoter. Exemplary promoters suitable for bacterial cell systems include, for example, T7, T3, SP6, lac and trp promoters.

The invention further provides a vector containing an isolated nucleic acid molecule encoding a prokineticin polypeptide. Exemplary vectors include vectors derived from a virus, such as a bacteriophage, a baculovirus or a retrovirus, and vectors derived from bacteria or a combination of bacterial sequences and sequences from other organisms, such as a cosmid or a plasmid. The vectors of the invention will generally

contain elements such as an origin of replication compatible with the intended host cells; transcription termination and RNA processing signals; one or more selectable markers compatible with the intended host cells; and one or more multiple cloning sites. Optionally, the vector will further contain sequences encoding tag sequences, such as GST tags, and/or a protease cleavage site, such as a Factor Xa site, which facilitate expression and purification of the encoded polypeptide.

The choice of particular elements to include in a vector will depend on factors such as the intended host cells; the insert size; whether expression of the inserted sequence is desired; the desired copy number of the vector; the desired selection system, and the like. The factors involved in ensuring compatibility between a host cell and a vector for different applications are well known in the art.

In applications in which the vectors are to be used for recombinant expression of the encoded polypeptide, the isolated nucleic acid molecules will generally be operatively linked to a promoter of gene expression, as described above, which may be present in the vector or in the inserted nucleic acid molecule. An exemplary vector suitable for fusion protein expression in bacterial cells is the pGEX-3X vector (Amersham Pharmacia Biotech, Piscataway, NJ).

Also provided are cells containing an isolated nucleic acid molecule encoding a prokineticin polypeptide. The isolated nucleic acid molecule will generally be contained within a vector. The isolated nucleic acid molecule can be maintained episomally, or incorporated into the host cell genome.

The cells of the invention can be used, for example, for molecular biology applications such as expansion, subcloning or modification of the isolated nucleic acid molecule. For such applications, bacterial cells, such as laboratory strains of *E. coli*, are useful, and expression of the encoded polypeptide is not required.

The cells of the invention can also advantageously be used to recombinantly express and isolate the encoded polypeptide. For such applications bacterial cells (e.g. *E. coli*), insect cells (e.g. *Drosophila*), yeast cells (e.g. *S. cerevisiae*, *S. pombe*, or *Pichia pastoris*), and vertebrate cells (e.g. mammalian primary cells and established cell lines; and amphibian cells, such as *Xenopus* embryos and oocytes). An exemplary cell suitable for recombinantly expressing prokineticin polypeptides is an *E. coli* BL21 cell.

The invention further provides isolated polynucleotides that contain at least 20 contiguous nucleotides from SEQ ID NOS:1 or 4, such as portions of SEQ ID NOS:1 or 4 that encode SEQ ID NOS:2, 3, 5 or 6, or from the complement thereof. The polynucleotides of the invention are thus of sufficient length to be useful as sequencing primers, PCR primers and hybridization probes to detect or isolate nucleic acid molecules encoding prokineticin polypeptides, and are also useful as therapeutic antisense reagents to inhibit prokineticin expression. The polynucleotides of the invention can, but need not, encode prokineticin polypeptides that are able to stimulate GI smooth muscle contraction. Those skilled in the art can determine the appropriate length and sequence of a polynucleotide of the invention for a particular application.

As used herein, the term "polynucleotide" refers to a nucleic acid molecule that contains at least 20 contiguous nucleotides from the reference sequence and which may, but need not, encode a functional polypeptide. Thus, a polynucleotide of the invention can contain at least 20, 22 or 25 contiguous nucleotides, such as at least, or not more than, 30, 40, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, or 300 contiguous nucleotides from SEQ ID NOS:1 or 4, or from their complement. A polynucleotide of the invention does not consist of the exact sequence of an EST present in publically available databases, including the sequences designated by GenBank Accession Nos. AI277349, AA883760, AQ426386, AC068519, AC026973, AL358215 and AL390797 or sequences which encode amino acid sequences having GenBank Accession Nos. AF182066, AF182064, AF182069 and AF182065.

For certain applications, such as for detecting prokineticin expression in a sample, it is desirable to use isolated polynucleotide molecules of the invention that specifically hybridize to a nucleic acid molecule encoding a prokineticin. The term "specifically hybridize" refers to the ability of a nucleic acid molecule to hybridize, under stringent hybridization conditions as described below, to a nucleic acid molecule that encodes a prokineticin, without hybridizing to a substantial extent under the same conditions with nucleic acid molecules that do not encode a prokineticin, such as unrelated molecules that fortuitously contain short regions of identity with a prokineticin. Thus, a nucleic acid molecule that "specifically hybridizes" is of a sufficient length and contains sufficient distinguishing sequence from a prokineticin for use in expression analysis, such as tissue blots and Northern blots (see Figure 2).

As used herein, the term "stringent conditions" refers to conditions equivalent to hybridization of a filter-bound nucleic acid molecule to a nucleic acid in a solution containing 50% formamide, 5X Denhart's solution, 5X SSC, 0.2% SDS at 42°C, followed by washing the filter in 0.1X SSC and 0.1% SDS at 65°C twice for 30 minutes. Equivalent conditions to the stringent conditions set forth above are well known in the art, and are described, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992).

The invention further provides a kit containing a pair of polynucleotides of the invention packaged together, either in a single container or separate containers. The pair of polynucleotides are preferably suitable for use in polymerase chain reaction (PCR) applications. Thus, the pair of polynucleotides can be used to detect or quantitate normal or abnormal expression of a nucleic acid molecule encoding a prokineticin. The pair of polynucleotides can also be used to amplify a nucleic acid molecule encoding a prokineticin, or any portion thereof, for sequencing, subcloning or for preparing sequence modifications. The kit can further contain written instructions for use of the pair of polynucleotides in PCR applications, or solutions and buffers suitable for such applications.

The isolated prokineticin nucleic acid molecules of the invention can be prepared by methods known in the art. An exemplary method for preparing an isolated prokineticin nucleic acid molecule involves amplification of the nucleic acid molecule using prokineticin-specific primers and the polymerase chain reaction (PCR). Using PCR, a prokineticin nucleic acid molecule having any desired boundaries can be amplified

exponentially starting from only a few DNA or RNA molecules, such as from a single cell. PCR methods, including methods of isolating homologs of a given nucleic acid molecule in other species using degenerate primers, are well known in the art.

Alternatively, an isolated prokineticin nucleic acid molecule can be prepared by screening a library, such as a genomic library, cDNA library or expression library, with a detectable prokineticin nucleic acid molecule or with an antibody. Human libraries, and libraries from a large variety of mammalian species, are commercially available or can be produced from species or cells of interest. The library clones identified as containing a prokineticin nucleic acid molecule can be isolated, subcloned or sequenced by routine methods.

Furthermore, an isolated prokineticin nucleic acid molecule can be prepared by direct synthetic methods. For example, a single stranded nucleic acid molecule can be chemically synthesized in one piece, or in several pieces, by automated synthesis methods known in the art. The complementary strand can likewise be synthesized in one or more pieces, and a double-stranded molecule made by annealing the complementary strands. Direct synthesis is particularly advantageous for producing relatively short molecules, such as probes and primers, and also for producing nucleic acid molecules containing modified nucleotides or linkages.

The invention also provides an antibody specific for a prokineticin polypeptide or peptide, such as an antibody specific for a polypeptide having the amino acid sequence of SEQ ID NOS:3 or 6. Also provided

is an antibody specific for an isolated immunogenic peptide that contains at least 10 contiguous amino acids of SEQ ID NOS:3 or 6.

The antibodies of the invention can be used, for example, to detect prokineticin expression in research and diagnostic applications. Such antibodies are also useful for identifying nucleic acid molecules that encode prokineticin polypeptides present in mammalian expression libraries, and for purifying prokineticin polypeptides by immunoaffinity methods. Furthermore, such antibodies can be administered therapeutically to bind to and block the activity of prokineticin, such as in applications in which it is desirable to inhibit GI smooth muscle contractions.

The term "antibody," as used herein, is intended to include molecules having specific binding activity for a prokineticin peptide or polypeptide of at least about 1×10^5 M^{-1}, preferably at least 1×10^7 M^{-1}, more preferably at least 1×10^9 M^{-1}. The term "antibody" includes both polyclonal and monoclonal antibodies, as well as antigen binding fragments of such antibodies (e.g. Fab, $F(ab')_2$, Fd and Fv fragments and the like). In addition, the term "antibody" is intended to encompass non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric antibodies, bifunctional antibodies, CDR-grafted antibodies and humanized antibodies, as well as antigen-binding fragments thereof.

Methods of preparing and isolating antibodies, including polyclonal and monoclonal antibodies, using peptide and polypeptide immunogens, are well known in the art and are described, for example, in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor

Laboratory Press (1988). Non-naturally occurring antibodies can be constructed using solid phase peptide synthesis, can be produced recombinantly or can be obtained, for example, by screening combinatorial libraries consisting of variable heavy chains and variable light chains. Such methods are described, for example, in Huse et al. Science 246:1275-1281 (1989); Winter and Harris, Immunol. Today 14:243-246 (1993); Ward et al., Nature 341:544-546 (1989); Hilyard et al., Protein Engineering: A practical approach (IRL Press 1992); and Borrabeck, Antibody Engineering, 2d ed. (Oxford University Press 1995).

The invention provides a method of identifying a prokineticin receptor ligand. The method is practiced by contacting a preparation containing prokineticin receptor with one or more candidate compounds, and identifying a candidate compound that specifically binds the receptor. Such a compound is characterized as a prokineticin receptor ligand.

The term "ligand," as used herein, includes compounds that bind to the prokineticin receptor at the same or different site as prokineticin.

As used herein, the term "candidate compound" refers to any biological or chemical compound. For example, a candidate compound can be a naturally occurring macromolecule, such as a polypeptide, nucleic acid, carbohydrate, lipid, or any combination thereof. A candidate compound also can be a partially or completely synthetic derivative, analog or mimetic of such a macromolecule, or a small organic molecule prepared by combinatorial chemistry methods. If desired in a particular assay format, a candidate compound can be detectably labeled or attached to a solid support.

Methods for preparing large libraries of compounds, including simple or complex organic molecules, metal-containing compounds, carbohydrates, peptides, proteins, peptidomimetics, glycoproteins, lipoproteins, nucleic acids, antibodies, and the like, are well known in the art and are described, for example, in Huse, U.S. Patent No. 5,264,563; Francis et al., Curr. Opin. Chem. Biol. 2:422-428 (1998); Tietze et al., Curr. Biol., 2:363-371 (1998); Sofia, Mol. Divers. 3:75-94 (1998); Eichler et al., Med. Res. Rev. 15:481-496 (1995); and the like. Libraries containing large numbers of natural and synthetic compounds also can be obtained from commercial sources.

The number of different candidate compounds to test in the methods of the invention will depend on the application of the method. For example, one or a small number of candidate compounds can be advantageous in manual screening procedures, or when it is desired to compare efficacy among several predicted ligands, agonists or antagonists. However, it will be appreciated that the larger the number of candidate compounds, the greater the likelihood of identifying a compound having the desired activity in a screening assay. Additionally, large numbers of compounds can be processed in high-throughput automated screening assays. Therefore, "one or more candidate compounds" can be, for example, 2 or more, such as 5, 10, 15, 20, 50 or 100 or more different compounds, such as greater than about 10^3, 10^5 or 10^7 different compounds.

A suitable preparation for a identifying a prokineticin receptor ligand can employ a tissue, cell, cell membrane, or purified prokineticin receptor, so long as the preparation contains a prokineticin receptor in a suitable conformation for binding prokineticin with a

similar affinity and specificity as a prokineticin receptor expressed on GI smooth muscle tissues.

In one embodiment, the preparation is an intestinal smooth muscle preparation, such as a mammalian ileal, fundic muscle or proximal colon preparation, or membrane preparation thereof. A suitable intestinal smooth muscle preparation is a guinea pig ileal preparation prepared by the methods described in the Example.

In another embodiment, the preparation is a cell line that expresses prokineticin receptor, or membrane preparation thereof. A cell line that expresses prokineticin receptor can be identified by methods known in the art, such as the competitive binding assays described in the Example. An exemplary cell line that expresses prokineticin receptor is the melanoma cell line M2A7 (available from American Type Culture Collection as ATCC CRL-2500). Other cell lines that express prokineticin receptor include M2 melanoma cells (Cunningham et al., Science 255;325-327 (1992)) and RC-4B/C pituitary tumor cells (ATCC CRL-1903).

A suitable control cell line that does not express prokineticin receptor is HEK293 (available from American Type Culture Collection as CRL-1573). Other control cell include COS-7, COS-1, Ltk-, NIH3T3, C6, NS10Y and HT-29 cells.

Appropriate assays to identify receptor ligands are known in the art. Such assays can involve directly determining binding of the candidate compound to the receptor preparation. Direct assays are suitable when an appropriate control preparation is available that does not contain the prokineticin receptor. Such assays can

also involve determining the ability of the candidate compound to compete with a prokineticin polypeptide for binding to the receptor preparation. Competition assays can be performed by detectably labeling a candidate compound and competing the compound with an unlabeled prokineticin polypeptide, or competing an unlabeled candidate compound with a detectably labeled prokineticin polypeptide.

As used herein, the term "detectably labeled" refers to derivation with, or conjugation to, a moiety that is detectable by any analytical means. An exemplary detectable moiety is a radioisotope (e.g. ^{14}C, ^{131}I, ^{32}P or ^{3}H), fluorochrome (e.g. fluoroscein, green fluorescent protein), ferromagnetic substance, or luminescent substance. Methods of detectably labeling organic and inorganic compounds with such moieties are well known in the art.

An exemplary competitive binding assay suitable for detecting a prokineticin receptor ligand is described in the Example, below. Other suitable receptor binding assays, including high-throughput assays, are described, for example, in Mellentin-Micelotti et al., Anal. Biochem. 272:P182-190 (1999); Zuck et al., Proc. Natl. Acad. Sci. USA 96:11122-11127 (1999); and Zhang et al., Anal. Biochem. 268;134-142 (1999).

Other suitable assays for detecting binding include, for example, scintillation proximity assays (SPA) (Alouani, Methods Mol. Biol. 138:135-41 (2000)), UV or chemical cross-linking (Fancy, Curr. Opin. Chem. Biol. 4:28-33 (2000)), competition binding assays (Yamamura et al., Methods in Neurotransmitter Receptor Analysis, Raven Press, New York, 1990), biomolecular interaction analysis (BIA) such as surface plasmon resonance (SPR) (Weinberger

et al., Pharmacogenomics 1:395-416 (2000)), mass spectrometry (MS) (McLafferty et al., Science 284:1289-1290 (1999) and Degterev, et al., Nature Cell Biology 3:173-182 (2001)), nuclear magnetic resonance (NMR) (Shuker etal., Science 274:1531-1534 (1996), Hajduk et al., J. Med. Chem. 42:2315-2317 (1999), and Chen and Shapiro, Anal. Chem. 71:669A-675A (1999)), and fluorescence polarization assays (FPA) (Degterev et al., *supra*, 2001). An appropriate binding assay can be chosen depending on the nature and purity of the receptor preparation and the number and nature of the candidate compounds.

A compound that is determined to be a prokineticin receptor ligand can further be tested to determine whether it is an agonist or antagonist of prokineticin receptor. Likewise, a compound that is determined to be a prokineticin receptor ligand can further be tested to determine whether it modulates, either positively or negatively, GI smooth muscle contractility, using an *in vitro* or *in vivo* assay known in the art, such as the assays described herein.

The invention further provides a method of identifying a prokineticin receptor agonist. The method is practiced by contacting a preparation containing a prokineticin receptor with one or more candidate compounds, and identifying a compound that selectively promotes production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor agonist.

The invention also provides a method of identifying a prokineticin receptor antagonist. The method is practiced by contacting a preparation containing a prokineticin receptor with one or more

candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal. Such a compound is characterized as a prokineticin receptor antagonist. Using the invention method, prokineticin mutants designated SEQ ID NOS:16 and 18 were identified as prokineticin receptor antagonists.

The invention methods can be performed in the presence of a suitable concentration of a prokineticin, such as within 10-fold of its EC_{50}. Thus, an agonist that competes with prokineticin for signaling through the prokineticin receptor, or indirectly potentiates the signaling activity of prokineticin, can be readily identified. Likewise, an antagonist that prevents prokineticin from binding the prokineticin receptor, or indirectly decreases the signaling activity of prokineticin, can also be identified.

As used herein, the term "prokineticin receptor agonist" refers to a molecule that selectively activates or increases normal signal transduction through the prokineticin receptor. As used herein, the term "prokineticin receptor antagonist" refers to a compound that selectively inhibits or decreases normal signal transduction through the prokineticin receptor.

For therapeutic applications, a prokineticin receptor agonist preferably has an EC_{50}, and a prokineticin receptor antagonist preferably has an IC_{50}, of less than about 10^{-7} M, such as less than 10^{-8} M, and more preferably less than 10^{-9} or 10^{-10} M. However, depending on the stability, selectivity and toxicity of the compound, a prokineticin receptor agonist with a higher EC_{50}, or a prokineticin receptor antagonist with a higher IC_{50}, can also be useful therapeutically.

As described herein, the endogenous prokineticin receptor appears to be a G-protein coupled receptor. Signaling through the prokineticin receptor promotes intracellular calcium ion mobilization, suggesting that the prokineticin receptor normally couples to Gαq-containing G proteins. Therefore, signaling through the prokineticin receptor can be detected by any assay known in the art that detects intracellular calcium ion mobilization. Such an assay can be performed in the presence or absence of a prokineticin.

A suitable preparation for detecting calcium ion mobilization can be a tissue or cell line expressing the prokineticin receptor, such as an intestinal smooth muscle preparation, or the M2A7 cell line.

Calcium ion mobilization can conveniently be measured using detectably labeled Ca^{2+} ion indicators, such as fluorescently labeled or radiolabeled indicators, and suitable detection systems. Exemplary Ca^{2+} ion indicators include FLUO-3 AM, FLUO-4 AM, FURA-2, INDO-1, FURA RED, CALCIUM GREEN, CALCIUM ORANGE, CALCIUM CRIMSON, BTC, and OREGON GREEN BAPTA (see, for example, Grynkiewitz et al., J. Biol. Chem. 260:3440-3450 (1985); Sullivan et al., in Calcium Signal Protocol, Methods in Molecular Biology 114: 125-133, Edited by David G. Lambert, Human Press, Totowa, New Jersey (1999); Miyawaki et al., Proc. Natl. Acad. Sci. USA 96:2135-2140 (1999); and Coward et al., Analyt. Biochem. 270:242-248 (1999)). A suitable detection system for monitoring calcium ion mobilization is the FLIPR (Fluorometric Imaging Plate Reader) system available from Molecular Devices.

The specificity of Gα subunits for cell-surface receptors is determined by the C-terminal five amino acids of the Gα. Thus, a variety of signal transduction pathways can be assayed to determine transduction of a G-protein coupled signal by a prokineticin receptor, by recombinantly expressing a chimeric Gα containing the five C-terminal residues of a Gα known or predicted to couple to ADP-glucose receptor (such as Gαq or a promiscuous Gα such as Gα16), with the remainder of the protein corresponding to a Gα that couples to the signal transduction pathway to be assayed (e.g. Gαs, to assay increased cAMP production, or Gαq to assay intracellular Ca^{2+} mobilization). Based on the known sequences of Gα subunits, nucleic acid molecules encoding chimeric Gα can be constructed and expressed by methods known in the art and described, for example, in Conklin et al., Nature 363:274-276 (1993), and Komatsuzaki et al., FEBS Letters 406:165-170 (1995).

Thus, depending on the Gα subunit endogenously or recombinantly expressed in the assay system, prokineticin receptor signals that can be determined include, but are not limited to, calcium ion mobilization; increased or decreased production or liberation of arachidonic acid, acetylcholine, diacylglycerol, cGMP, cAMP, inositol phosphate and ions; altered cell membrane potential; GTP hydrolysis; influx or efflux of amino acids; increased or decreased phosphorylation of intracellular proteins; and activation of transcription of an endogenous gene or promoter-reporter construct downstream of any of the above-described second messenger pathways.

Suitable assays for detecting agonistic and antagonistic activity of G protein coupled receptors, including high-throughput signaling assays, are well

known in the art and reviewed, for example, in reviewed, for example, in Tate et al., Trends in Biotech. 14:426-430 (1996).

Assay methods for identifying compounds that selectively bind to or modulate signaling through a prokineticin receptor (e.g. ligands, agonists and antagonists) generally involve comparison to a control. One type of a "control" is a preparation that is treated identically to the test preparation, except the control is not exposed to the candidate compound. Another type of "control" is a preparation that is similar to the test preparation, except that the control preparation does not express the receptor, or has been modified so as not to respond selectively to prokineticin. In this situation, the response of the test preparation to a candidate compound is compared to the response (or lack of response) of the control preparation to the same compound under substantially the same reaction conditions.

A compound that is determined to be a prokineticin receptor agonist or antagonist can further be tested to determine whether it modulates, either positively or negatively, GI smooth muscle contractility, using an *in vitro* or *in vivo* assay known in the art, such as the assays described herein.

The invention also provides compositions suitable for use in assays to identify prokineticin ligands, agonists and antagonists. Suitable compositions contain a cell or tissue containing a prokineticin receptor and a prokineticin polypeptide, which optionally can be detectably labeled. An exemplary composition comprises a GI smooth muscle preparation, such as an ileal smooth muscle preparation. A further exemplary composition comprises a cell line, such as M2A7.

The prokineticin polypeptides described herein, as well as prokineticin ligands, agonists and antagonists identified by the described screening methods, are potential therapeutic compounds that can be administered to individuals with conditions associated with abnormal gastrointestinal motility, or other conditions associated with altered expression, or activity of a prokineticin or its receptor. The invention compounds can be formulated and administered in a manner and in an amount appropriate for the condition to be treated; the weight, gender, age and health of the individual; the biochemical nature, bioactivity, bioavailability and side effects of the particular compound; and in a manner compatible with concurrent treatment regimens. An appropriate amount and formulation for a particular therapeutic application in humans can be extrapolated based on the activity of the compound in the *in vitro* binding and signaling assays described herein, or from recognized animal models of the particular disorder.

The total amount of therapeutic compound can be administered as a single dose or by infusion over a relatively short period of time, or can be administered in multiple doses administered over a more prolonged period of time. Additionally, the compound can be administered in a slow-release matrice, which can be implanted for systemic delivery at or near the site of the target tissue. Contemplated matrices useful for controlled release of therapeutic compounds are well known in the art, and include materials such as DepoFoam™, biopolymers, micropumps, and the like.

The therapeutic compounds can be administered to a mammal by routes known in the art including, for example, intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally,

intracisternally, intra-articularly, intracerebrally, orally, intravaginally, rectally, topically, intranasally, or transdermally. Preferred routes for human administration are oral and intravenous administration, with oral routes particulary preferred.

Preferably, the therapeutic compounds are administered to a mammal as a pharmaceutical composition comprising the compound and a pharmaceutically acceptable carrier. The choice of pharmaceutically acceptable carrier depends on the route of administration of the compound and on its particular physical and chemical characteristics. Pharmaceutically acceptable carriers are well known in the art and include sterile aqueous solvents such as physiologically buffered saline, and other solvents or vehicles such as glycols, glycerol, oils such as olive oil and injectable organic esters. A pharmaceutically acceptable carrier can further contain physiologically acceptable compounds that stabilize the compound, increase its solubility, or increase its absorption. Such physiologically acceptable compounds include carbohydrates such as glucose, sucrose or dextrans; antioxidants, such as ascorbic acid or glutathione; chelating agents; and low molecular weight proteins.

For applications that require the compounds and compositions to cross the blood-brain barrier, or to cross cell membranes, formulations that increase the lipophilicity of the compound are particularly desirable. For example, the compounds of the invention can be incorporated into liposomes (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed. (CRC Press, Boca Raton FL (1993)). Liposomes, which consist of phospholipids or other lipids, are nontoxic,

physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

In one embodiment, a pharmaceutical composition containing a prokineticin polypeptide or a prokineticin agonist is administered to a mammal in an effective amount to stimulate gastrointestinal motility. Impaired GI motility is a common clinical manifestation of a variety of disorders, including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastrointestinal reflux disease, and the compositions of the invention can thus be used to ameliorate the symptoms of such disorders.

In another embodiment, a pharmaceutical composition containing a prokineticin antagonist is administered to a mammal in an effective amount to inhibit gastrointestinal motility. Enhanced GI motility is associated with diarrhea, which is a common symptom of infectious diseases, malabsorptive disorders, inflammatory bowel disorders, and intestinal cancers, and antagonistic compositions of the invention can thus be used to ameliorate the symptoms of such disorders.

Injection of Bv8 or MIT1 into the brain ventricles of rats leads to hyperalgesia (Mollay et al., Eur J Pharmacol. 374:189-196 (1999)). Therefore, prokineticin antagonists (e.g. prokineticin antibodies, mutant polypeptides comprising SEQ ID NOS:16 or 18, and other compounds determined by the methods described herein) can be administered to a mammal in an effective amount to act as an analgesic (pain killer).

Those skilled in the art can determine other conditions for which it is appropriate to administer a pharmaceutical composition of the invention, and can monitor the safety and efficacy of the therapy.

Preferably, the mammal administered a pharmaceutical composition of the invention is a human, but for certain applications the mammal can alternatively be a veterinary animal or a research animal. For example, in preclinical studies, the methods of the invention can be practiced with animals that serve as credible models of human disease, such as non-human primates, pigs, dogs, cats, and rodents (e.g. rats, mice and guinea pigs). Those skilled in the art understand which animals serve as appropriate models for a human disease of interest.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Identification, Preparation and Characterization of Prokineticins 1 and 2

This example shows the cloning, recombinant expression, purification and biological activities of human prokineticins 1 and 2, as well as modifications thereof.

Materials and methods

RNA blot

Human multiple tissue RNA blots containing normalized samples of polyA RNA were used as described by the manufacture's instructions (Clontech). The blots were probed with random primer-labeled probes (nucleotides

1-550 and 1-1178 for prokineticin 1 and prokineticin 2 cDNAs), and signals were visualized by exposing to Kodak XAR film.

Production, refolding and purification of recombinant prokineticins

The coding sequences for mature prokineticins were cloned into prokacyotic expression vector pGEX-3X (Pharmacia). The extra nucleotidcs between the factor Xa protease digestion site of GST (Glutathione-s-transferase) tag and mature prokineticins were removed by site-directed mutagenesis and confirmed by sequencing. To facilitate protein purification, a 6xHis-tag was added to the C-terminus so that the fusion proteins could be purified with Ni-NTA affinity chromatography (Qiagen).

The method for production of fusion proteins is as follows. The *E. coli* cells (BL21) were grown to OD 0.8 and induced with 600 nM IPTG for 2 hours at 37°C. The cells were then pelleted, washed, and lysed with buffer A (6 M guanidine hydrochloride, 100 mM NaH_2PO_4 and 1OmM Tris, pH 8.0). Fusion proteins were allowed to bind to Ni-NTA beads and then washed extensively with buffer C (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 6.3) and buffer D (8M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 5.9). Fusion protein-bound beads were equilibrated with factor Xa digestion buffer (50 mM Tris, 150 nM NaCl, and 1 mM CaCl, pH 7.5). Factor Xa digestion was performed overnight at room temperature with 10 ng/µ fusion protein. Cleaved GST tag was then washed away with buffer D. Mature prokineticins were then eluted with buffer E (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris, pH 4.5). Fractions were analyzed by SDS-PAGE. The pooled recombinant prokineticins were then refolded as follows. Proteins were diluted to 100 µg/ml with buffer E, and

dialyzed against renaturing buffer (4 M urea, 5 mM cysteine, 0.02% Tween-20, 10% glycerol, 10 mM Tris, 150 mM NaCI, 100 mM NaH_2PO_4, pH 8.3). New renaturing buffer (same component except 2 M urea) was then added, and dialysis was continued for four more days with at least one more change of renaturing buffer. The refolded protein was then desalted with a spin column (Qiagen) and analyzed by receptor binding or bioassay. The final purification was performed with reverse phase-HPLC (LKB). Functional proteins were eluted with 0.08% trifluoroacetic acid and 10-50% acetonitrile gradient. The elution of protein was monitored at 206 nm. Trifluoroacetic acid and acetonitrile were then evaporated by lyophilization.

Mass spectrometry

The electrospray ionization mass spectrometry was pwformed with a 6.5 T HiResESI Fourier Transform mass spectrometer (IonSpec, Irvine, CA) as previously described (Li et al., Anal. Chem. 66:2077-2083 (1994)). Protein eluted from RP-HPLC was lyophilized and dissolved in nanopure water and then diluted to a concentration of 1nM with methanol-water- acetic acid (49.5%:49.5%:1%, v/v/v). 100 µl of sample was infused.

Measurement of smooth muscle contraction in isolated organ preparations

Guinea pigs were euthanized with CO_2, and a section of ileum (2-3 cm) approximately 10 cm rostral to the cecum was removed. The ileum was washed clean with Krebs-Ringer bicarbonate (KRB) buffer (124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 26 nM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.8 mM CaCl, and 10 mM glucose) and mounted longitudinally in an organ bath containing KRB buffer. Isometric contractions were measured with a force-displacement transducer and polygraph as described previously (Thomas et al.,

Biochem. Pharmacol. 51:779-788 (1993)). The ileum was allowed to incubate for 1 hr, and then three test doses of the muscarinic agonist, oxotremorine-M, were added to ensure that the contractions were reproducible and of sufficient magnitude. The ileum was washed and allowed to rest for 5 min between each test dose. The longitudinal fundic strip and the zig-zag tracheal preparation were prepared as described previously (15). Isolated colon (proximal and distal) was prepared as described (Sawyer et al., J. Pharmacol. Exp. Ther. 284:269-277 (1998)). Aorta and femoral artery were taken from adult rats. A 10 ml bath was used for aorta and femoral artery experiments. Tension was recorded on a Grass polygraph with initial preloads of 0.5 g for intestinal tissues and tracheal preparations and 2 g for aorta and femoral artery.

Iodination

Prokineticin 1 was iodinated by the iodogen method as described (Fraker and Speck, Biochem. Biophys. Res. Commun. 80:849-857 (1978)). Briefly, refolded prokineticin 1 (7.5 µg) was incubated with 50 µg of iodogen in 50 µL of 0.5 M PBS buffer, pH 7.2 for 15 minutes at room temperature. The reaction was stopped by removal of the mixture from the iodogen tube and placing it in a microfuge tube with 100 µL of PBS containing 1 mM NaI. Following the addition of 100 µL of PBS with 1mM NaI and 0.1% BSA, the free iodine was removed by gel filtration on Bio-Gel P2 and the radioactivity was counted. Assuming all the radioactivity was incorporated into 6.0 µg prokineticin 1 recovered (80% recovery rate), specific radioactivity was calculated as 819 cpm/fmol or 372 Ci/mole.

Receptor binding

Membranes were prepared from guinea pig ileum as described (Li et al., Mol. Pharmacol. 57:446-452 (2000)), except additional steps of differential centrifugation (800g, 10,000g, 100,000g, 4°C, 20 min each) were applied to reduce the background binding. Incubation was performed in 4 ml in 20 mM Tris-HCl pH 7.4 buffer containing 0.1% BSA at room temperature. For saturation binding, 1.5-200 pM of labeled prokineticin 1 was used. Non-specific binding was defined in the presence of 20nM unlabeled prokineticin 1. For displacement experiments, unlabeled protein was pre-incubated with membrane in 3ml total reaction volume for 1hr, then ^{125}I-prokineticin 1 (20pM) was added. The membrane was incubated for an additional 3 hrs at room temperature. The binding mixture was filtered through GF-C glass filters and washed with 10 ml of 20 mM Tris-HCI, pH 7.4. Radioactivity retained on filters was counted in gamma counter. The data were analyzed with the LIGAND program.

Results

Identification and analysis of two mammalian homologues for frog Bv8 and snake MIT1

In an effort to identify mammalian homologues of frog Bv8 and snake MIT1, multiple databases (EST and HGTS) were searched using the BLAST 2.1 algorithm (Altschul et al. Nucleic Acids Res. 25:3389-3400 (1997)), with their protein sequences as queries. A search of the EST database revealed the presence of two human EST sequences (ai277349 and aa883760). Sequence analysis of these two EST clones revealed that aa 883760 encodes a predicted protein (Heijne Nucleic Acids Res. 14:4683-4690 (1986)) with a signal peptide of 19 amino acids and a mature protein of 86 amino acids. Clone ai277349 was

found to be a partial cDNA. Full-length sequence for EST clone ai277349, cloned by 5' RACE with human brain cDNA as template, was found to contain a signal peptide of 27 amino acids and a mature protein of 81 amino acids (Figure 1). These proteins were respectively named as prokineticin 1 and prokineticin 2 (see below).

Sequence analysis reveals that prokineticin 1 and 2 have about 44% amino acid identity, including ten conserved cysteines. Both prokineticins possess about 43% identity with frog Bv8 and snake MIT1. Interestingly, the N-terminal sequences before the first cysteine (AVITGA) is completely conserved among all species (Figure 1), suggesting the functional significance of this region. Preliminary analysis of the mouse prokineticin 1 gene indicates that the N-terminal sequence AVITG is derived from the first exon that also contains the signal peptide sequence, whereas the cysteine-rich sequences are from other exon(s).

Prokineticins are expressed in various adult and embryonic tissues

As an initial survey of prokineticin expression, a human masterr blot was probed using fragments of human prokineticin cDNAS. Both prokineticins were widely expressed in various adult tissues, with a generally higher expression level of prokineticin 1 compared to prokineticin 2 (Figure 2A, 2B). The exception was found in GI tract, liver and spleen, whereas prokineticin 2 expression seemed comparable to that of prokineticin 1. The highest level of prokineticin 1 expression is found in testis and placenta. Among human fetal tissues, all showed a similar level of expression, again with an expression level of prokineticin 1 higher than that of prokineticin 2.

The expression of prokineticins in human brain was further examined by Northern blot analysis. Figure 2D showed that prokineticin 1 mRNA size is about 1.5kb with the highest expression in the putamen, thalamus, temporal lobe, and corpus callosum. Prokineticin 2 expression in human brain was undetectable (data not shown).

Production, refolding and purification of human prokineticins

As the N-terminal sequences were completely conserved (Figure 1), recombinant proteins with authentic N-terminal residue were produced first as GST-fusion proteins, followed by digestion with protease factor Xa to remove the GST tag. Figure 3 shows that a protein with correct molecular weight was produced by factor Xa digestion.

Bioassay with guinea-pig ileum preparations revealed the unfolded recombinant proteins were inactive. As NMR examination indicated that 10 cysteines of MIT1 are formed into 5 disulfide bonds (Boisbouvier et al., J. Mol. Biol. 283:205-219 (1998)) and these 10 cysteines are all conserved in human prokineticins, it was considered that these disulfide bonds were probably essential for protein bioactivities. Thus considerable effort was devoted to ensure proper disulfide bond formation (out of 945 possible combinations).

Initial refolding in a single dilution into refolding buffer was unsuccessful, as almost all recombinant proteins were precipitated, probably due to the formation of inter-molecular disulfide bonds. A series of modifications to control protein aggregation and to slow disulfide bond formation were then adopted. These modifications included: 1) reduction of protein concentration to 100 µg/ml or less to favor forming

intra- but not inter-molecular disulfide bonds; 2) refolding proteins by dialysis method instead of direct dilution; 3) using higher levels of urea (4 M and then 2 M) in all dialysis buffers; 4) omitting oxidants cystine or oxidized glutathione from redox pairs, leaving only 5 mM cysteine or 3 mM reduced glutathione; 5) adding glycerol to further reduce protein aggregation; 6) cooling proteins and buffers to 4°C before initiating the refolding process. These carefully controlled steps allowed the successful refolding of recombinant prokineticins with minimal protein aggregarion.

The refolded proteins were finally purified by RP-HPLC (Figure 3A, lane 5). Mass spectrometry confirmed the formation of five disulfide bonds in refolded recombinant prokineticin 1. The molecular weight of 6xHis-tagged prokineticin 1, determined with a Fourier transform mass spectrometer, was found to be 10480.30 Da (Figure 3C). As the calculated molecular weight with all ten cysteines present in reduced form was 10490.20, five pairs of disulfide bonds were clearly formed.

Refolded recombinant prokineticins potently contract gastrointestinal smooth muscle

The refolded recombinant prokineticins were then tested on isolated smooth muscle preparations. Figure 4 shows that both recombinant prokineticin 1 and prokineticin 2 potently stimulated the contraction of guinea-pig ileum longitudinal muscle with ED5O values of about 0.46 and 0.90 nM, respectively. Prokineticin 1 (5 nM) also stimulated the contraction of fundic muscle strip and proximal colon, but had no effect on distal colon (25 nM, data not shown). Recombinant prokineticin 1 (25 nM) also had no effect on other smooth muscle tissues, including aorta and femoral artery, trachea and gallbladder. Thus, the contractile effect of

prokineticins appears to be specific for GI smooth muscle.

To probe the possible signaling mechanisms of prokineticins, a number of kinase and ion channel inhibitors were tested. Tetrodotoxin (TTX), which is known to block nerve action potential propagation, had no effect on prokineticin 1-stimulated ileum longitudinal muscle contraction (Figure 4B), indicating that prokineticin 1 acts directly on the smooth muscle. The contractile mechanism of prokineticin was further investigated with a number of compounds, including the protein kinase C inhibitor calphostin C (1 μM), the phospholipase A2 inhibitor 7, 7-dimethyl-(5Z,8Z)-eicosa-dienoic acid (10 μM), the tyrosine kinase inhibitor genistein (5 μM), the MEK inhibitor PD 098059 (10 μM) and L-type calcium channel blocker verapamil. Only verapamil was effective, with 1 μM completely inhibiting the contractile effect of 2 nM prokineticin I (Figure 4C). The same concentration of verapamil also completely blocked the contractile action of 100 nM oxotremorine-M (Figure 4F). This result indicates that, like muscarinic M3 receptor mediated contraction of the ileum (Eglen et al., Pharmacol. Rev. 48:531-565 (1996) and Ehlert et al., Muscurinic Receptors and Gastrointestinal Smooth Muscle, ed. Eglen, CRC Press, pgs 92-147 (1997)), calcium entry via the voltage-gated calcium channel is an essential component of prokineticin signaling.

Bioactivities of prokineticins are mediated by membrane receptors

The potent contractile action of recombinant prokineticins on guinea-pig GI smooth muscle and the inhibitory effect of the calcium channel blocker verapamil suggest a receptor-mediated mechanism for

prokineticins. To provide direct evidence that prokineticins are interacting with selective membrane receptors, recombinant prokineticin was labeled with ^{125}I and receptor binding experiments were carried out.

Scatchard analysis indicated that the specific binding of prokineticin 1 was best fitted with two-site model (F=38.78, P<0.001 versus one site model; Figure 5A). The high- and low-affinity constants (K_d) were 5.0 ± 0.8 pM and 227 ± 63 pM (n = 3), respectively. The B_{max} for high- and low-affinity sites were 7.8 ± 1.2 and 26.4 ± 8.4 fmol/mg of protein, respectively (n = 3). Competition experiments revealed that the specific binding was displaced by recombinant prokineticin 1. The displacement curves were also best fitted with two-site model (with K_i of 8.0 ± 3.9 pM, and 1.50 ± 0.9 nM, n = 3 for high- and low-affinity sites, respectively) (Figure 5B). Figure 5B also shows that prokineticin 2 displaced labeled prokineticin 1 with similar affinity (K_i of 4.2 pM for high affinity and 1.22 nM for low affinity site, average of two experiments).

Because agonist binding to many G protein-coupled receptors is inhibited by GTP, it was investigated whether GTPγS had any effect on specific ^{125}I-labeled prokineticin 1 binding. As shown in Figure 5B, GTPγS caused a concentration-dependent inhibition of ^{125}I-prokineticin 1 binding. At the highest concentration tested (10 μM), GTPγS displaced 85% of the specific prokineticin binding to ileal membranes. These results suggest that prokineticin receptor(s) belong to the G protein-coupled receptor family.

Stability of prokineticins

Experiments were also performed to determine the half-life of prokineticins. The half-life of intravenously injected iodinated human prokineticin 1 was approximately 3 hours, compared to 10 min for motilin, a small peptide that also increases GI motility. A reasonably long half-life in the blood circulation is crucial for achieving therapeutic effect. Therefore, prokineticins are likely to be effective as therapeutics.

Structure/activity relationship studies of prokineticins

Sequence analysis indicated that prokineticins may contain two functional domains, namely the short N-terminus and the cysteine-rich C-terminus. As the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Figure 1), it was predicted that this region has functional importance.

In addition to prokineticins, the ten-cysteine motif is also found in a number of secreted proteins, including colipase, a cofactor for intestinal lipid digestive enzyme lipase, and dickkopfs, a family of proteins that have an important role in early embryonic development.

A number of N-terminal substitution, deletion, and insertion mutants were constructed, and recombinant, refolded proteins produced. Bioassays with ileal smooth muscle preparations revealed that these mutant proteins at concentrations up to 250 nM are not able to elicit contractions (Table 1). However, an N-terminal deletion mutant (SEQ ID NO:16) and an N-terminal insertion mutant (SEQ ID NO:18) were able to weakly antagonize the contractile effect of prokineticin 1. Therefore,

N-terminal variants of prokineticins, such as SEQ ID NOS:16 and 18, are potential therapeutics for inhibiting GI contractility.

Table 1

	Polypeptide	Contractile Activity	Antagonistic Activity
Wild Type (SEQ ID NO:3)	AVITGA[Prokineticin 1]	+	-
Insertion (SEQ ID NO:15)	GILAVITGA[Prokineticin 1]	-	ND
Deletion (SEQ ID NO:16)	VITGA[Prokineticin 1]	-	+
Substitution (SEQ ID NO:17)	AAAAAA[Prokineticin 1]	-	ND
Insertion (SEQ ID NO:18)	MAVITGA[Prokineticin 1]	-	+
Chimera	AVITGA[Co-lipase]	-	-
Chimera	AVITGA[dickkopf4]	-	ND
peptide (SEQ ID NO:19)	AVITGACERDVQCG	-	-
Cys mutation	AVITGA[Prokineticin 1]18S	-	-
Cys mutation	AVITGA[Prokineticin 1]60R	-	-

Chimeric recombinant proteins containing N-terminal sequences from prokineticin 1 and the C-terminal ten-cysteine domain from either colipase or Dickkopf 4 were also constructed. These two chimeric recombinant proteins were non-functional when tested with ileal smooth muscle preparation at concentrations up to 250 nM. Also tested was an N-terminal peptide (SEQ ID NO:19), which also was non-functional.

These results indicate that both N-terminal conserved sequence and C-terminal cysteine-rich domain are essential for the contractile activity of prokineticins.

Chimeric prokineticins

A search of the draft human genome database with prokineticin cDNAs as queries revealed that genes encoding prokineticin 1 and 2 are composed of three exons. The signaling peptide and N-terminal conserved AVITG sequence are encoded in the first exon, while the cysteine-rich domain is encoded by exons 2 and 3. The 21 amino acid insertion of prokineticin 2 is encoded by an alternatively spliced mini-exon. To explore the functional difference of prokineticin 1 and 2, chimeric polypeptides were made with their exons 3 swapped (see Figure 6). The chimeric polypeptides were designated chimera 12 (SEQ ID NO:13) and chimera 21 (SEQ ID NO:14), designating the swapped exons, as shown in Figure 6.

Functional assays of refolded chimeric prokineticins 12 and 21 indicated that both of these chimeric polypeptides are active in contracting GI smooth muscle (Figure 7A) . However, the EC_{50} for the chimeric prokineticin 21 (SEQ ID NO:14) was about 8-fold higher than prokineticin 1 or prokineticin 2. Additionally, although the peak contractions were not affected, chimeric prokineticin polypeptides resulted in prolonged contraction of ileal strips (Figure 7B). For wild type prokineticins, the time constants to midway contraction (half way from peak contraction to sustained plateau) were about 15 mins. In contrast, for the chimeric polypeptides, these time constants were prolonged to about 40 mins.

These results suggest that the chimeric prokineticins interact slightly differently with the receptor than wild type prokineticins, and cause less pronounced tachyphylaxis. Thus, the chimeric prokineticins (SEQ ID NOS:13 and 14) may have more potent pharmacological activity *in vivo* than wild-type prokineticins.

Effects of prokineticin on guinea pig ileum smooth muscle in vivo.

To monitor the effects of prokineticin on the contraction of ileal smooth muscle *in vivo*, extraluminal force transducers were implanted on the serosal surface of the guinea pig ileum. Recombinant prokineticin 1 was then administered as a bolus into the jugular vein over a 10-second period. As shown in Figure 8, an intravenous bolus of prokineticin 1 contracts guinea pig ileal smooth muscle in a dose-dependent manner. The threshold dose of prokineticin 1 is about 0.03 µg/kg, and a dose of 30 µg/kg produces the maximum effect.

Therefore, prokineticins, demonstrated above to be able to contract ileal smooth muscle in *ex vivo* preparations, are also effective *in vivo*.

Prokineticin signal transduction

To probe the potential signaling mechanisms of prokineticins, cell lines were identified that express prokineticin receptor endogenously. Over twenty cell lines were screened for binding to iodinated prokineticin 1. One cell line, M2A7 melanoma cells (ATCC CRL-2500; Cunningham et al., Science 255;325-327 (1992)), clearly displayed specific binding, with a receptor level of about 150 fmole/mg protein. Other cell lines that specifically bound prokineticin included M2 melanoma

cells (Cunningham et al., Science 255;325-327 (1992)) and RC-4B/C pituitary tumor cells (ATCC CRL-1903). Cell lines that did not bind prokineticin included HEK293, COS-7, COS-1, Ltk-, NIH3T3, C6, NS10Y and HT-29 cells.

To assess signaling in M2A7 cells, cytosolic calcium was measured by fura-3 fluorescence using a FLIPR system (Fluorometric Imaging Plate Reader; Molecular Devices). Cells were suspended in HEPES medium and incubated with 2 μM of fura-3 AM for 20 min at 31°C. The cells were then centrifuged, washed, resuspended in fura-3-free medium and seeded into 96 wells at 4 x 10^4 cells per well. The cells were loaded with Fluo-3 AM (Molecular Probes) in standard buffer solution (130 mM NaCl,2 mM $CaCl_2$, 5 mM KCl, 10 mM glucose, 0.45 mM KH_2PO_4, 0.4 mM Na_2HPO_4, 8 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 20 mM HEPES and 10 μM probenecid) with 0.1% fetal bovine serum for 1 h at 37°C, then washed with standard buffer solution. Transient changes in $[Ca_{2+}]_i$ evoked by prokineticin (0.01, 0.1, 0.3, 1, 3, 10, 100 nM) were monitored using the FLIPR system in 96-well plates at 488 nm for 210 s.

As shown in Figure 9, prokineticins can mobilize calcium in M2A7 melanoma cells, with EC_{50} of about 12 and 21 nM for recombinant prokineticin 1 and prokineticin 2, respectively. The signaling is specific, as there was no response in HEK 293 cells. The calcium signaling mobilized by prokineticins is comparable to calcium signal activated by control MCH (melanin-concentrating hormone) receptor SLC1 (Saito et al., Nature 400:265-269 (1999)). The calcium signals elicited by prokineticins are more much robust than the modest calcium signal induced by activation of a typical receptor tyrosine kinase. This result is consistent with

the observation, described above, that the tyrosine protein kinase inhibitor genistein (5mM) had no effect on the contractile activity of prokineticin on ileal smooth muscle.

These results indicate that the prokineticin receptor(s) is/are likely to be GPCR(s), and to signal through Gαq.

Discussion

The results described above establish the existence of mammalian homologues of frog BV8 and snake MIT1. To reflect their potent and specific effects on GI smooth muscle, these proteins have been named prokineticins. Their high potency in specifically stimulating the contraction of guinea-pig ileum smooth muscle but not other smooth muscles including aorta, femoral artery, trachea, and gallbladder indicate that prokineticins may be important endogenous regulators of GI motility. Prokineticins may regulate GI smooth muscle as neurocrine signaling molecules, or circulating hormones, or paracrine humoral agents. Since prokineticins are also widely expressed outside the GI system, it is possible that prokineticins may be released from remote organs and regulate GI activity. In this respect, it has also been determined that prokineticins are resistant to protease treatment, which supports their potential long-range and long-term effects.

The molecular size and the processing of prokineticins distinguish them from typical neuropeptides, and indicate they are more similar to cytokines. As one mechanism for eliminating pathogenic organisms is to enhance motility and push the offending

organisms out of the GI tract, prokineticins may also be part of defending immune response, i.e. functioning as inflammatory cytokines that increase the GI motility.

The high potency of recombinant prokineticins on GI contractility suggests that prokineticins probably interact with cell surface receptor(s). This conclusion is reinforced by the receptor binding experiments described above, which demonstrate a saturably high affinity for the iodinated recombinant prokineticin. Moreover, the observation that 10 μM GTPγS can displace almost all of the specific binding indicates the involvement of G protein in prokineticin receptor signaling. Furthermore, the inhibitory effect of the calcium channel blocker verapamil on the contractile effect of prokineticin is consistent with a receptor-mediated mechanism for prokineticins, and also suggests a similar signaling mechanism of prokineticins as those of the M3 muscarinic and motilin receptor in contracting GI smooth muscle: calcium entry via voltage-gated calcium channel is an essential component. Thus, prokineticin receptor most likely is a G protein coupled receptor.

However, alternative interpretations are possible. For instance, prokineticins may cause smooth muscle contraction by directly activating non-selective cation ion channels, or blocking inhibitory potassium channels on GI smooth muscle cells.

Sequence analysis indicates that prokineticins may contain two functional domains: the short N-terminus and the cysteine-rich C-terminus. Since the N-terminal sequences preceding the first cysteine are completely conserved among prokineticins (Figure 1), this region is likely to have functional importance. In addition to

prokineticins and their isoforms from other species, a similar ten-cysteine motif is also found in a number of other secreted proteins, including colipase, a cofactor for intestinal lipid digestive enzyme lipase (van Tilbeurgh et al., Nature 359:159-162 (1992)) and dikkopfs, a family of proteins that have important roles in early embryonic development (Glinka et al., Nature 391:357-362 (1998) and Aravind et al., Curr. Biol. 8:R477-478 (1998)). Interestingly, dickkopfs actually possess two ten-cysteine domains that have mirror symmetry. X-ray crystallography and solution structural analysis have demonstrated that MIT1 is formed of five pairs of disulfide bonds and folded into a structure similar to colipase (Boisbouvier et al., J. Mol. Biol. 283:205-219 (1998)).

Successful refolding of proteins with five pairs of disulfide bonds has not hitherto been accomplished *in vitro*. Refolding of proteins with more than three pairs of disulfide bonds is still regarded as challenging and difficult (Georgiou et al., Curr. Opin. Biotechnol. 7:190-197 (1996) and Lihe et al., Curr. Opin. Biotechnol. 9:497-501 (1998)). The expression of such disulfide bond-rich proteins in *E. coli* often results in no formation of disulfide bonds, or more probably the formation of incorrect intramolecular or intermolecular disulfide bonds. These events routinely lead to production of inactive recombinant proteins and their aggregation in bacterial inclusion bodies.

In this study, a slow exchange method was utilized to refold prokineticins that have five pairs of disulfide bonds. A number of factors eventually contributed to the successful refolding of prokineticins: 1) a slow rate of removal of denaturing agent; 2) using only reducing agents in the redox refolding mixture,

allowing slow formation of disulfide bonds; 3) low temperature; 4) high concentration of urea and glycerol in dialyzing buffer to prevent protein aggregation; 5) low concentration of recombinant protein to favor forming intra- but not inter-molecular disulfide bonds. These refolding conditions can be used to design protocols for refolding other recombinant proteins that possess multiple disulfide bonds.

In summary, cDNAs encoding two prokineticins have been described. Refolded recombinant prokineticins potently and specifically stimulate the contraction of GI smooth muscle. As impaired GI motility is a very common clinical manifestation in many common disorders including irritable bowel syndrome, diabetic gastroparesis, postoperational ileus, chronic constipation, and gastroesophageal reflux disease (Longo et al., Dis Colon Rectum 36:696-708 (1993); Tonini, Pharmacol. Res. 33:217-226 (1996); Samsom and Smout, Dig Dis. 15:263-274 (1998); Achem and Robinson, Dig Dis. 16:38-46 (1998) and Briejer et al., Trends Pharmacol Sci. 20:1-3 (1999)), the discovery of endogenous regulators of GI smooth muscle should facilitate the development of novel therapeutics for such disorders that will benefit from altered GI motility.

All journal article, reference and patent citations provided above, in parentheses or otherwise, whether previously stated or not, are incorporated herein by reference in their entirety.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. An isolated polypeptide that stimulates gastrointestinal smooth muscle contraction, comprising an amino acid sequence at least 80% identical to the sequence of human prokineticin 1 (SEQ ID NO:3), said sequence comprising the N-terminal 6 amino acids of SEQ ID NO:3, the 10 conserved cysteine residues of SEQ ID NO:3, and from 0 to 9 of the 9 C-terminal amino acids of SEQ ID NO:3.

2. The isolated polypeptide of claim 1, wherein amino acid residues that differ from residues in SEQ ID NO:3 are conservative substitutions thereof.

3. The isolated polypeptide of claim 1, wherein amino acid residues that differ from residues in SEQ ID NO:3 consist of the corresponding residues from SEQ ID NO:6.

4. The isolated polypeptide of claim 3, comprising SEQ ID NO:13.

5. The isolated polypeptide of claim 1, comprising amino acids 1-77 of SEQ ID NO:3.

6. The isolated polypeptide of claim 1, comprising SEQ ID NO:3.

7. The isolated polypeptide of claim 1, comprising a 6XHis tag.

8. The isolated polypeptide of claim 1, which is detectably labeled.

9. An isolated peptide comprising at least 10 contiguous amino acids of SEQ ID NO:3, wherein said peptide is immunogenic.

10. A pharmaceutical composition, comprising the isolated polypeptide of claim 1 and a pharmaceutically acceptable carrier.

11. A method of stimulating gastrointestinal smooth muscle contraction in a mammal, comprising administering to said mammal an effective amount of the polypeptide of claim 1.

12. A nucleic acid molecule encoding the polypeptide of claim 1.

13. An expression vector containing the nucleic acid molecule of claim 12 operatively linked to a promoter of gene expression.

14. A host cell comprising the expression vector of claim 13.

15. A method of preparing the isolated polypeptide of claim 1, comprising culturing the host cell of claim 14 so as to express said polypeptide, substantially purifying said polypeptide, and refolding said polypeptide.

16. An antibody that selectively binds the polypeptide of claim 1.

17. An isolated polypeptide that stimulates gastrointestinal smooth muscle contraction, comprising an amino acid sequence at least 80% identical to the sequence of human prokineticin 2 (SEQ ID NO:6), said sequence comprising the N-terminal 6 amino acids of SEQ ID NO:6, the 10 conserved cysteine residues of SEQ ID NO:6, and from 0 to 4 of the 4 C-terminal amino acids of SEQ ID NO:6.

18. The isolated polypeptide of claim 17, wherein amino acid residues that differ from residues in SEQ ID NO:6 are conservative substitutions thereof.

19. The isolated polypeptide of claim 17, wherein amino acid residues that differ from residues in SEQ ID NO:6 consist of the corresponding residues from SEQ ID NO:3.

20. The isolated polypeptide of claim 19, comprising SEQ ID NO:14.

21. The isolated polypeptide of claim 17, comprising amino acids 1-77 of SEQ ID NO:6.

22. The isolated polypeptide of claim 17, comprising SEQ ID NO:6.

23. The isolated polypeptide of claim 17, comprising a 6XHis tag.

24. The isolated polypeptide of claim 17, which is detectably labeled.

25. An isolated peptide comprising at least 10 contiguous amino acids of SEQ ID NO:6, wherein said peptide is immunogenic.

26. A pharmaceutical composition, comprising the isolated polypeptide of claim 17 and a pharmaceutically acceptable carrier.

27. A method of stimulating gastrointestinal smooth muscle contraction in a mammal, comprising administering to said mammal an effective amount of the polypeptide of claim 17.

28. A nucleic acid molecule encoding the polypeptide of claim 17.

29. An expression vector containing the nucleic acid molecule of claim 17 operatively linked to a promoter of gene expression.

30. A host cell comprising the expression vector of claim 29.

31. A method of preparing the isolated polypeptide of claim 17, comprising culturing the host cell of claim 30 so as to express said polypeptide, substantially purifying said polypeptide, and refolding said polypeptide.

32. An antibody that selectively binds the polypeptide of claim 17.

33. A method of identifying a prokineticin receptor ligand, comprising contacting a preparation comprising prokineticin receptor with one or more candidate compounds, and identifying a compound that specifically binds to said receptor, said compound being characterized as a prokineticin receptor ligand.

34. The method of claim 33, wherein said preparation is an intestinal smooth muscle preparation or membrane preparation thereof.

35. The method of claim 33, wherein said preparation is a cell line or membrane preparation thereof.

36. The method of claim 35, wherein said cell line is M2A7 (ATCC CRL-2500).

37. The method of claim 33, wherein the ability of said ligand to selectively agonize or antagonize prokineticin receptor signaling is further determined.

38. The method of claim 37, wherein said signaling is determined in a cell line.

39. The method of claim 38, wherein said cell line is M2A7 (ATCC CRL-2500).

40. The method of claim 37, wherein said signaling is determined by monitoring calcium mobilization.

41. The method of claim 33, wherein the ability of said ligand to modulate smooth muscle contractility is further determined.

42. A method of identifying a prokineticin receptor agonist, comprising contacting a preparation comprising a prokineticin receptor with one or more candidate compounds, and identifying a compound that

selectively promotes production of a prokineticin receptor signal, said compound being characterized as a prokineticin receptor agonist.

43. The method of claim 42, wherein said preparation is a cell line.

44. The method of claim 43, wherein said cell line is M2A7 (ATCC CRL-2500).

45. The method of claim 42, wherein said signaling is determined by monitoring calcium mobilization.

46. The method of claim 42, wherein the ability of said agonist to modulate smooth muscle contractility is further determined.

47. A method of identifying a prokineticin receptor antagonist, comprising contacting a preparation comprising a prokineticin receptor with one or more candidate compounds in the presence of a prokineticin, and identifying a compound that selectively inhibits production of a prokineticin receptor signal, said compound being characterized as a prokineticin receptor antagonist.

48. The method of claim 47, wherein said prokineticin comprises an amino acid sequence selected from the group consisting of amino acids 1-77 of SEQ ID NOS:3 and amino acids 1-77 of SEQ ID NO:6.

49. The method of claim 47, wherein said preparation is a cell line.

50. The method of claim 49, wherein said cell line is M2A7 (ATCC CRL-2500).

51. The method of claim 47, wherein said signaling is determined by monitoring calcium mobilization.

52. The method of claim 47, wherein the ability of said antagonist to modulate smooth muscle contractility is further determined.

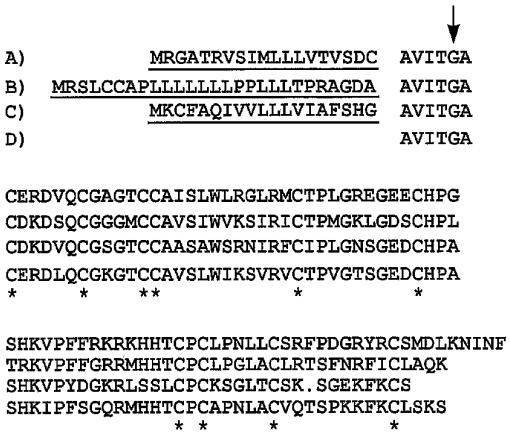
A) MRGATRVSIMLLLVTVSDC AVITGA
B) MRSLCCAPLLLLLLLPPLLLTPRAGDA AVITGA
C) MKCFAQIVVLLLVIAFSHG AVITGA
D) AVITGA
CERDVQCGAGTCCAISLWLRGLRMCTPLGREGEECHPG
CDKDSQCGGGMCCAVSIWVKSIRICTPMGKLGDSCHPL
CDKDVQCGSGTCCAASAWSRNIRFCIPLGNSGEDCHPA
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Figure 1

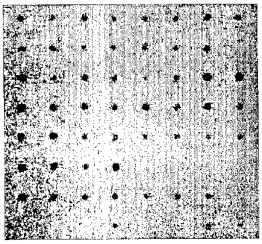


Figure 2A

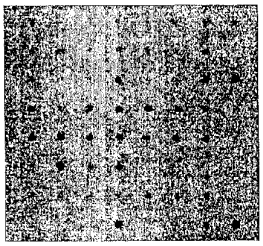


Figure 2B

	1	2	3	4	5	6	7	8
A	WHOLE BRAIN	AMYGDALA	CAUDATE NUCLEUS	CEREBELLUM	CEREBRAL CORTEX	FRONTAL LOBE	HIPPO-CAMPUS	MEDULLA OBLONGATA
B	OCCIPITAL LOBE	PUTAMEN	SUBSTANTIA NIGRA	TEMPORAL LOBE	THALAMUS	NUCLEUS ACCUMBEUS	SPINAL CORD	
C	HEART	AORTA	SKELETAL MUSCLE	COLON	BLADDER	UTERUS	PROSTATE	STOMACH
D	TESTIS	OVARY	PANCREAS	PITUITARY GLAND	ADRENAL GLAND	THYROID GLAND	SALIVARY GLAND	MAMMARY GLAND
E	KIDNEY	LIVER	SMALL INTESTINE	SPLEEN	THYMUS	PERIPHERAL LEUKOCYTE	LYMPH NODE	BONE MARROW
F	APPENDIX	LUNG	TRACHEA	PLACENTA				
G	FETAL BRAIN	FETAL HEART	FETAL KIDNEY	FETAL LIVER	FETAL SPLEEN	FETAL THYMUS	FETAL LUNG	
H	YEAST TOTAL RNA 100ng	YEAST tRNA 100ng	E.COLI TRNA 100ng	E.COLI DNA 100ng	POLY r(A) 100ng	HUMAN COT1DNA 100ng	HUMAN DNA 100ng	HUMAN DNA 500ng

Figure 2C

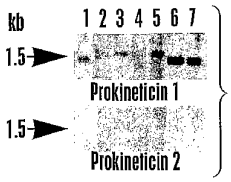


Figure 2D

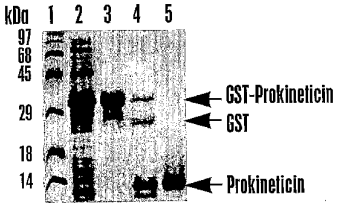


Figure 3A

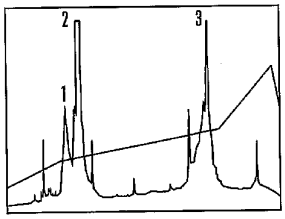


Figure 3B

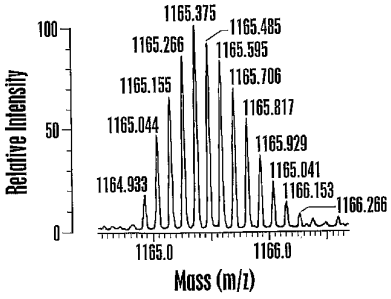


Figure 3C

Figure 4A

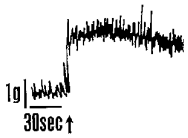


Figure 4B

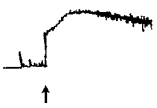


Figure 4C

Figure 4D

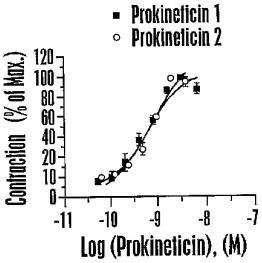


Figure 4E

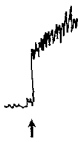


Figure 4F

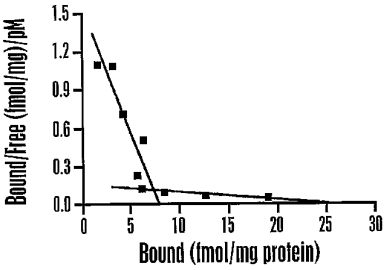


Figure 5A

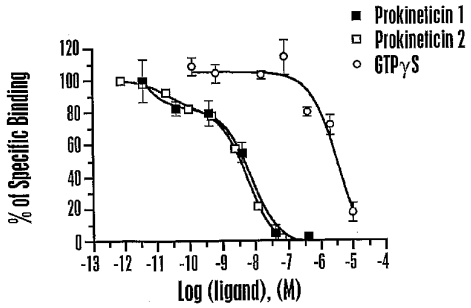


Figure 5B

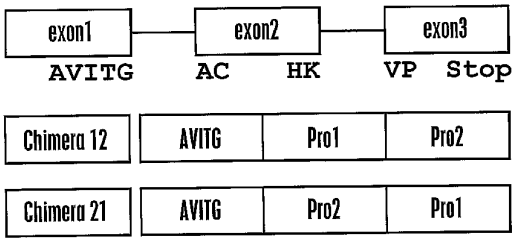

exon1
exon2
exon3
AVITG
AC
HK
VP
Stop
Chimera 12
AVITG
Pro1
Pro2
Chimera 21
AVITG
Pro2
Pro1

Figure 6

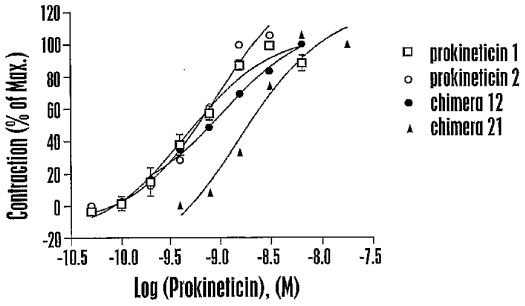


Figure 7A

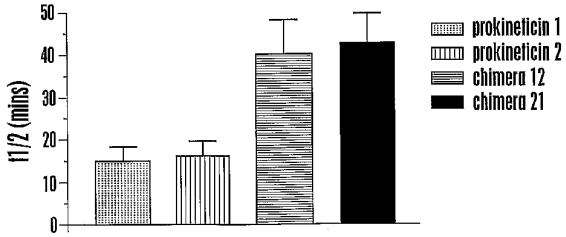


Figure 7B

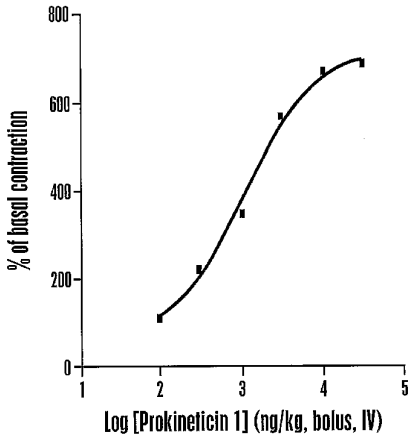


Figure 8A

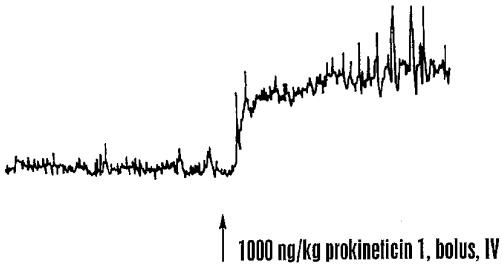
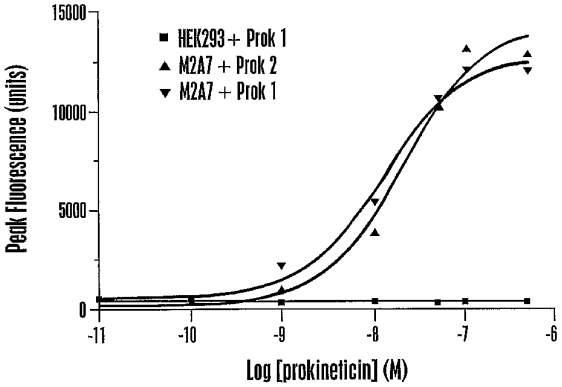


Figure 8B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5000
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M2A7 + Prok 2
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-8
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Figure 9

SEQUENCE LISTING

<110> The Regents of the University of California

<120> Prokineticin Polypeptides, Related
Compositions and Methods

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    50                  55                  60
His Lys Val Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys
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35 40 45
Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
50 55 60
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35 40 45
His Lys Val Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys
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Pro Phe Phe Arg Lys Arg Lys His His Thr Cys Pro Cys Leu Pro Asn
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        35                  40                  45
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【国際調査報告】

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/47969

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/12 C07K16/18 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, SEQUENCE SEARCH, MEDLINE, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 63088 A (BAKER KEVIN ;CHEN JIAN (US); GENENTECH INC (US); YUAN JEAN (US); G) 9 December 1999 (1999-12-09) PRO1186 page 35, paragraph 3; figures 265,266 ---	1-16, 33-52
X	WO 00 52022 A (MILLENNIUM PHARM INC) 8 September 2000 (2000-09-08) TANGO 266; SEQ ID N°:10-12 figures 14-16 ---	1-16, 33-52
X	WECHSELBERGER C. ET AL.: "The mammalian homologues of frog BV8 are mainly expressed in spermatocytes" FEBS LETTERS, vol. 462, 1999, pages 177-181, XP002206667 the whole document ---	17-32
	-/--	

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 2002	08. 08. 2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Piret, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/47969

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LI M ET AL: "IDENTIFICATION OF TWO PROKINETICIN CDNAS: RECOMBINANT PROTEINS POTENTLY CONTRACT GASTROINTESTINAL SMOOTH MUSCLE" MOLECULAR PHARMACOLOGY, BALTIMORE, MD, US, vol. 59, no. 4, April 2001 (2001-04), pages 692-698, XP001010437 ISSN: 0026-895X the whole document ---	1-52
P,X	WO 01 36465 A (ZYMOGENETICS INC) 25 May 2001 (2001-05-25) SEQ ID N°:1-6 page 56 -page 58 page 74, paragraph 2 ---	1-52
P,X	WO 01 16309 A (TERAO YASUKO; WATANABE TAKUYA; SHINTANI YASUSHI) 8 March 2001 (2001-03-08) SEQ ID N°:20-31 figure 9; examples 4-6 & EP 1 207 198 A (TAKEDA CHEMICAL INDUSTRIES LTD) 22 May 2002 (2002-05-22) ---	1-16, 33-52
A	SCHWEITZ H ET AL: "MIT1, a black mamba toxin with a new and highly potent activity on intestinal contraction" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 461, no. 3, 19 November 1999 (1999-11-19), pages 183-188, XP004260546 ISSN: 0014-5793 cited in the application the whole document -----	1-52

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/47969

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 11 and 27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

International Application No. PCT/US 01/47969

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 33-35, 37, 38, 40-43, 45-49, 51, 52 relate to methods involving preparations "comprising a prokineticin receptor", which represents an extremely large number of possible preparations. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the methods claimed, i.e. those originating from smooth muscle cell or from the cell lines M2A7, M2 and RC-4B/C. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the methods involving preparations obtained from smooth muscle cell, or from the cell lines M2A7, M2 and RC-4B/C.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

International Application No. PCT/US 01/47969

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-32 (entirely) and 47-52 (partially)

   Polypeptides comprising any of the sequences SEQ ID N°:3, 6, 13 or 14, reagents derived therefrom, and methods using these.

2. Claims: 33-46 (entirely) and 47-52 (partially)

   Methods for identifying ligands of a "prokineticin receptor".

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/47969

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9963088	A	09-12-1999	AU 4328699	A	20-12-1999
			CA 2328895	A1	09-12-1999
			WO 9963088	A2	09-12-1999
			US 2002072496	A1	13-06-2002
			US 2002072067	A1	13-06-2002
			US 2002072092	A1	13-06-2002
			US 2002072497	A1	13-06-2002
			AU 2212299	A	26-07-1999
			WO 9935170	A2	15-07-1999
WO 0052022	A	08-09-2000	AU 3386800	A	21-09-2000
			EP 1173456	A1	23-01-2002
			WO 0052022	A1	08-09-2000
WO 0136465	A	25-05-2001	AU 1608201	A	30-05-2001
			WO 0136465	A2	25-05-2001
WO 0116309	A	08-03-2001	AU 6728000	A	26-03-2001
			EP 1207198	A1	22-05-2002
			WO 0116309	A1	08-03-2001
			JP 2002095474	A	02-04-2002

Form PCT/ISA/210 (patent family annex) (July 1992)

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摘要(译)

本发明提供了刺激胃肠道平滑肌收缩的分离的多肽，包括人促动蛋白1和人促动蛋白2多肽，及其功能片段和修饰。 还提供了通过向哺乳动物给药有效量的促动蛋白原来刺激哺乳动物的胃肠道平滑肌收缩的方法。 本发明还提供了编码促动蛋白多肽的核酸分子，以及选择性结合促动蛋白多肽的抗体。 还提供了鉴定促动蛋白受体配体，激动剂或拮抗剂的方法。

	ポリペプチド	収縮活性	拮抗活性
野生型 (SEQ ID NO:3)	AVITGA[ブロキネチシン 1]	+	-
挿入 (SEQ ID NO:15)	GILAVITGA[ブロキネチシン 1]	-	ND
欠失 (SEQ ID NO:16)	VITGA[ブロキネチシン 1]	-	+
置換 (SEQ ID NO:17)	AAAAAA[ブロキネチシン 1]	-	ND
挿入 (SEQ ID NO:18)	MAVITGA[ブロキネチシン 1]	-	+
キメラ	AVITGA[コリバーゼ]	-	-
キメラ	AVITGA[dickkopf4]	-	ND
ペプチド (SEQ ID NO:19)	AVITGACERDVQCG	-	-
Cys **変異体**	AVITGA[ブロキネチシン 1]18S	-	-
Cys **変異体**	AVITGA[ブロキネチシン 1]60R	-	-