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(73) Proprietor: **Amarin Neuroscience Limited**
Stirling FK7 9JQ (GB)

(72) Inventors:
- **GLEN, Alastair, Campbell, Agnew**
  Glasgow G41 5LP (GB)
- **MCDONALD, Donald, John**
  Glasgow G73 6HA (GB)

(74) Representative: **Cockerton, Bruce Roger et al**
Carpmaels & Ransford,
43 Bloomsbury Square
London WC1A 2RA (GB)

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Description

[0001] The present invention is concerned with identification of proteins of the cytosolic phospholipase A_2 enzyme and the applications thereof, particularly in diagnosis, treatment monitoring and drug development.

[0002] Phospholipase A_2 (PLA_2) enzymes are generally characterised by their capacity to catalyse the hydrolysis of the Sn2 acyl-ester bond of glycerophosphate to release free fatty acid (Mayer R.J. and Marshall L., *FASEB J,* 7, 339-348; Dennis E.A., Ed., Phospholipase A_2 Methods in Enzymology, 1991, 197, 359-433). The scientific literature recognises different types of PLA_2 enzyme (Dennis E.A., *Trends Biochem. Sci.*, 22, 1-2). Types I, II and III are referred to as secretory phospholipase A_2 ($sPLA_2$) enzymes. Secretory phospholipase A_2 enzymes have a molecular weight of approximately 14 kDa, are found extracellularly and have been recognised in plasma.

[0003] The type IV PLA_2 enzyme is referred to as cytosolic phospholipase A_2 ($cPLA_2$). It features a calcium binding domain, termed the C2 domain, and its phospholipase activity is highly selective for highly-unsaturated fatty acids, particularly arachidonic acid, present in the Sn2 acyl-ester glycerophosphate bond. Its ability to release arachidonic acid from cell membrane phospholipids sets it in a key position for the regulation of the supply of arachidonate and subsequent action in cell messenger processes.

[0004] A further type of PLA_2 enzyme is the type VI enzyme which is also a cytosolic PLA_2 enzyme, but which does not possess the high selectivity for arachidonic acid in the Sn2 position of glycerophospate for phospholipid acyl ester hydrolysis (Tang J. *et al., J. Biol. Chem.*, 272, 1997, 8567-8575). It is termed $iPLA_2$ in recognition that it is independent of calcium level for its catalytic activity.

[0005] The type IV arachidonate-selective $cPLA_2$ enzyme has been identified in a number of tissues including human monocytes (Clark J.D. *et al., Cell,* 65, 1991, 1043-1051; Kramer R.M. *et al., J. Biol. Chem.,* 266, 1991, 5268-5272) and human platelets (Takayama K. *et al., FEBS,* 282, 1991, 326-330).

[0006] The amino acid sequence of type IV $cPLA_2$ has been determined for the enzyme purified from human monocyte (U937) cells (Clark J.D. *et al., Cell*, 65, 1991, 1043-1051). Type IV $cPLA_2$ found in U937 cells has an amino acid sequence which is 749 amino acids long. This sequence shows a region of homology with a limited number of other proteins including protein kinase C (PKC), GTPase activating protein (GAP), phospholipase C and synaptic vesicle protein p65. The points of homology occur towards the N-terminal end of the protein in the so-called calcium-binding portion which is at or around amino acids 36 to 81 of the sequence (counting from the NH_2 terminal end) (Nalefski *et al., J. Biol. Chem*., 269, 18239-18249). There are no known areas of sequence homology between other mammalian proteins and type IV $cPLA_2$ amino acids 82 to 749, apart from a sequence from amino acids 129 to 135 shared with pulmonary surfactant protein C (Clark *et al., J. Lipid Mediators Cell Signalling,* 12, 83-117) and two recently reported cytosolic phospholipase A_2 enzymes designated $cPLA_2\beta$ and $cPLA_2\gamma$ (R.T. Pickard *et al., J. Biol. Chem*., 1999, 274, 8823-8831). These are proteins different from the $cPLA_2$ enzyme isolated from human U937 monocytes (which now may be designated as $cPLA_2\alpha$). The $cPLA_2\alpha$, β and γ sequence homology is limited to five amino acids found in $cPLA_2\alpha$ at position serine 228 to tryptophan 232.

[0007] The catalytic active centre of type IV $cPLA_2$ has been reported as being located in a peptide sequence comprising amino acid 228 (Clark J.D. *et al., J. Lipid Mediators Cell Signalling,* 12, 83-117) and has recently been described as being dependent for its function on the proximity of two amino acids brought together in the folded tertiary structure of the molecule, serine 228 and aspartate 549 (Dessen A. *et al., Cell*, 1999, 97, 349-360).

[0008] Previous observations of PLA_2 enzymes in the circulation have been directed at measurements in serum or plasma by substrate assay methods and have not characterised the type of the enzyme involved (Thuren T. *et al., Clin. Chem*., 31, 1985, 714-717; Gattaz W.F. *et al., Biol. Psychiatry*, 22, 1987, 421-426; Gattaz W.F. *et al., Biol. Psychiatry,* 28, 1990, 495-501). Serum and plasma PLA_2 enzymes show increased activity relative to normal control subjects in the human disease schizophrenia, although again the PLA_2 type of the enzyme responsible has not been characterised. To date, there has been no identification of cytosolic phospholipase A_2 type IV protein either in or attached to red cells either physiologically or in pathology.

[0009] It has now been found that type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ can be detected in or on circulating red blood cells. The detection and assay of these $cPLA_2$ proteins has application in the diagnosis of disease, in the monitoring of the patient response to treatment and in the development of drugs which influence the activity or concentration of $cPLA_2$. Recognition that these $cPLA_2$ proteins may be detected in or on red blood cells provides a simple and convenient method of assaying for the $cPLA_2$ level.

[0010] According to a first aspect of the present invention, there is provided the use of red blood cells in an assay for detecting type IV $cPLA_2$ or a protein immunologically homologous to type IV $cPLA_2$. In a second aspect of the invention, there is provided the use of red blood cells in an assay for quantifying the level of type IV $cPLA_2$ or a protein immunologically homologous to type IV $cPLA_2$ in a sample of red blood cells. The assay may be performed on a sample of whole blood or on a sample of red blood cells after separation thereof from whole blood.

[0011] As used herein, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies that recognise an epitope or epitopes from any part of type IV $cPLA_2$ protein

from human monocyte (U937) cells, and preferably an epitope or epitopes from amino acids 82 to 749 of type IV $cPLA_2$ protein from human monocyte (U937) cells. In a preferred embodiment of the invention, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies that recognise an epitope or epitopes from a peptide sequence or sequences which comprise the catalytic active centre of type IV $cPLA_2$ protein from human monocyte (U937) cells. In a further preferred embodiment, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies that recognise an epitope or epitopes from the peptide sequence of amino acids 241 to 260 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

[0012] In an alternative embodiment, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies raised against an epitope or epitopes from any part of type IV $cPLA_2$ protein from human monocyte (U937) cells or a synthetic peptide matching the sequence of type IV $cPLA_2$ protein from human monocyte (U937) cells. In a preferred alternative embodiment, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies raised against an epitope or epitopes from amino acids 82 to 749, and preferably an epitope or epitopes from a peptide sequence or sequences which comprises the catalytic active centre, of type IV $cPLA_2$ protein from human monocyte (U937) cells or a synthetic peptide matching the sequence of amino acids 82 to 749, and preferably a sequence or sequences which comprises the catalytic active centre, of type IV $cPLA_2$ protein from human monocyte (U937) cells. In a further alternative embodiment, the term "a protein immunologically homologous to type IV $cPLA_2$" means a protein that binds specifically to an antibody or antibodies raised against an epitope or epitopes from the peptide sequence of amino acids 241 to 260 of type IV cPLA2 protein from human monocyte (U937) cells, or a synthetic peptide matching the sequence of amino acids from the peptide sequence of amino acids 241 to 260 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

[0013] According to a third aspect of the present invention, there is provided a method of diagnosis of a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the ex vivo detection of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells. The method may further comprise the step of determining the level of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells and, optimally, comparing this level with a control level.

[0014] As used herein, the term "highly unsaturated fatty acids" includes all fatty acids released by action of the type IV $cPLA_2$ enzyme. In an embodiment of the invention the term "highly unsaturated fatty acids" includes fatty acids having 3 or more carbon-carbon double bonds. In particular, the term includes the essential fatty acids, particularly the fatty acids of the group comprising dihomogammalinotenic acid (DGLA; 8,11,14- eicosatrienoic acid), arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid), adrenic acid (7,10,13,16-docosatetraenoic acid), 4,7,10,13,16-docosapentaenoic acid, stearidonic acid (SA; 6,9,12,15-octadecatetraenoic acid), 8,11,14,17-eicosatetraenoic acid, eicosapentaenoic acid (EPA; 5,8,11,14,17-eicosapentaenoic acid), docosapentaenoic acid (DPA; 7,10,13,16,19-docosapentaenoic acid) and docosahexaenoic acid (DHA; 4,7,10,13,16,19 docosahexaenoic acid).

[0015] According to a fourth aspect of the invention, there is provided a method of monitoring the effectiveness of medication administered to a patient suffering from a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the ex vivo detection of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells. The method may, for example, comprise the steps of administering to a patient a compound to be tested, determining the level of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells and, optimally, comparing this level with a control level or with a level or levels determined at an earlier stage of the medication regime or before the medication regime commenced.

[0016] According to a fifth aspect of the invention, there is provided a method of drug development for a disease in which dysfunction of the cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the detection of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells. The method may, for example, be used in the screening of compounds for use in the treatment of a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, the method comprising the steps of administering to a patient or test animal a compound to be tested, determining the level of type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells and, optimally, comparing the level with a control level.

[0017] The invention is particularly concerned with diseases in which dysfunction of cell signalling systems involving arachidonic acid, dibomogammalinolenic acid, eicosapentaenoic acid, docosapentaenoic acid and/or docosahexaenoic acid is implicated. More particularly, the invention is concerned with diseases in which dysfunction of cell signalling systems involving arachidonic acid is implicated.

[0018] The disease conditions in which the identification of type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in or on red cells may be useful include:

1) schizophrenia, in which increased $cPLA_2$ expression and activity is a proposed mechanism in disease development;
2) bipolar or manic depressive illness, in which $cPLA_2$ abnormality may be present;
3) cachexia, in which tumour necrosis factor promotes $cPLA_2$ activity;
4) brain injury, including stroke and mechanical injury, in which $cPLA_2$ may be released from damaged membranes or as part of the process of apoptosis;
5) dyslexia, in which abnormal $cPLA_2$ activity may be present; and
6) any other disease or disease process in which type IV $cPLA_2$ activity or concentration is increased or decreased from normal levels, particularly a disease or disease process in which type IV $cPLA_2$ activity or concentration is increased.

[0019] The uses and methods of the invention may comprise the steps of collecting a sample of blood from a subject, and detecting the proteins *ex vivo.* Preferably, the uses and methods further comprise one or more of the steps of separating the red cells from the other blood components, disrupting the red cells by a method such as sonication, nitrogen cavitation, freezing or lysis, and detecting the proteins either directly or following a protein separation technique. Another application for the uses and methods of the invention include their employment on whole blood, with or without the requirement of prior separation of the red cells, and such applications would include, for example, a near-patient testing diagnostic strip, cartridge, or device.

[0020] In a preferred embodiment, the uses and methods of the invention comprise the detection in or on red blood cells of one or more proteins from one or more of three particular groups of proteins. The proteins are grouped by apparent molecular weight as measured using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) with western blot and can be designated as follows:

Group A, comprising one or more proteins of apparent molecular weight in the range of 80 to 110 kDa, particularly in the range of 90 to 105 kDa;
Group B, comprising more or more proteins of apparent molecular weight in the range of 70 to 80 kDa;
Group C, comprising one or more proteins of apparent molecular weight in the range of 50 to 60 kDa.

[0021] It is considered that the proteins in a given group may be structurally related, small variations in apparent molecular weight between the proteins in a given group possibly being due to the extent of phosphorylation. However, it is not intended that the scope of the invention be limited by this theory. Variations in apparent molecular weight among proteins of a given group may simply be due to variations in the length of the amino acid sequence of the protein or protein fragment.

[0022] These proteins react immunologically with antibodies to $cPLA_2$. The proteins can be identified by an antibody or antibodies which react with an epitope or epitopes present in a region of the type IV $cPLA_2$ protein which does not share sequence homology with other proteins, for example a region other than the calcium-binding portion of the protein. The proteins can also be identified by using a first antibody (a "capture antibody") which reacts with an epitope or epitopes present in a sequence having homology with other proteins, for example, the calcium binding portion of the type IV $cPLA_2$ protein, and a second antibody (a "detection antibody") which reacts with an epitope or epitopes in a region of the protein which does not have homology with other proteins in order to provide the necessary specificity. Protein detection and identification is discussed in more detail below.

[0023] It is considered that the proteins or protein fragments isolated from in or on red blood cells may be the same as the type IV $cPLA_2$ protein, or fragments thereof, found in human monocyte (U937) cells. Thus, it is considered that the proteins having apparent molecular weights in the range of 80 to 110 kDa (particularly 90 to 105 kDa), in the range 70 to 80 kDa and in the range 50 to 60 kDa may be either intact type IV $cPLA_2$ or major components of type IV $cPLA_2$. However, it is not intended that the scope of the invention be limited by this theory.

Protein Separation

[0024] The type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ proteins are typically separated from the other proteins of red blood cells by SDS PAGE but a variety of other protein separation techniques may be used, including native polyacrylamide gel electrophoresis and various column separation techniques such as fast protein liquid chromatography and variants of sepharose or other commercially available columns. Two dimensional electrophoretic techniques may also be used. As noted above, prior protein separation is not always necessary and the type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ may be detected in red cells by immunological means without the necessity of prior protein separation.

Protein Detection

[0025] The detection of type IV $cPLA_2$ proteins and/or proteins immunologically homologous to type IV $cPLA_2$ can be achieved by a wide range of protein detection procedures including:

1) enzyme linked immunoassay, radioimmunoassay, luminescence immunoassay, fluorescence immunoassay or any other variant of competitive or tagged antibody immunoassay, including biosensor technology (evanescent wave optical biosensor);
2) immunofluoroescence or other immunoassay on red blood cells in smears, droplets, films, fixed histological sections or tissue samples;
3) *in vitro* tests in which the protein is recognised by a specific polyclonal or monoclonal antibody and the specific antibody is itself recognised in a Coombs-type test; and
4) enzymic substrate assay of type IV $cPLA_2$ proteins utilising natural or artificial phospholipid substrates together with specific inhibitors of the other phospholipase enzymes.

[0026] In one embodiment of the invention, protein detection is achieved using diagnostic strips, cartridges or devices suitable for near-patient testing which detect the proteins using any of the above techniques or any other technique known in the art.

[0027] In a preferred embodiment, the proteins are detected using either a polyclonal or a monoclonal antibody or a combination thereof. Table 1 provides examples of antibodies suitable for use in the assays of the present invention for the detection of type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$.

[0028] Suitable antibodies or combinations of antibodies for use in the present invention can also include one or more of, for example, a polyclonal antibody raised against an epitope of the peptide chain comprising amino acids 730 to 749 of type IV $cPLA_2$ from U937 cells, or an extended or different amino acid sequence from the C-terminal end of the molecule; an antibody or antibodies raised against a mid-molecule peptide sequence or sequences which comprise the catalytic active site of type IV $cPLA_2$ from U937 cells; an antibody or antibodies raised against an epitope from the N-terminal part of the sequence (amino acids 1 to 216); and an antibody or antibodies raised against another epitope or epitopes from the amino acid sequence of type IV $cPLA_2$ protein from U937 cells between amino acids 82 and 749.

Table 1

ANTIGEN	TYPE	PRODUCTION METHOD	SOURCE	DETECTS $cPLA_2$-LIKE PROTEINS IN RED CELLS
Human type IV $cPLA_2$ synthetic peptide from mid molecule	Polyclonal IgG	Raised in Rabbits	Cayman Chemical	Yes
Human type IV $cPLA_2$ synthetic peptide of a 24 amino acid sequence from the C-terminal domain	Polyclonal IgG	Raised in Sheep	The Binding Site	Yes
Human $cPLA_2$ synthetic peptide to amino terminal (amino acids 1-216)	Monoclonal IgG	Mouse	Chemicon International Inc.	Yes

Table 1 (continued)

ANTIGEN	TYPE	PRODUCTION METHOD	SOURCE	DETECTS $cPLA_2$-LIKE PROTEINS IN RED CELLS
Human type IV $cPLA_2$ synthetic peptide mid molecule sequence (amino acids 241 to 260)[a]	Polyclonal IgG	Raised in Sheep	In-house production	Yes

a: This antibody is prepared using as antigen a synthetic peptide comprising amino acids 241 to 260 of type IV $cPLA_2$ (as found in human monocytes, U937 cells), coupled to ovalbumin. The antigen is injected into sheep with Freunds adjuvant combining the intra-muscular and subcutaneous routes in standard approved protocol. The resulting antiserum was purified initially using Protein A and further purified using an affmity matrix carrying the peptide 241 to 260 not coupled to ovalbumin, to provide an affinity purified IgG antibody.

[0029] The recognition of type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in an accessible component of blood opens the way to their use as follows:

a) Diagnostic use by direct immunoassay of their level.
b) Use in a diagnostic sense indirectly as part of a Coombs-type test.
c) Recognition by immunofluorescence on or in red blood cells on slide or smear or tissue preparation.
d) Use in an immune precipitation or other reaction to recognise type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in whole blood samples in near-patient testing.
e) Use in monitoring of treatments which are directed to suppress PLA_2 activity or concentration.
f) Use in the research for agents which suppress PLA_2 activity or concentration for drug discovery.

[0030] The invention will now be described in detail with reference to the following examples. It will be appreciated that the invention is described by way of example and modification of detail may be made without departing from the scope of the invention.

EXAMPLES

I. Collection of Samples

[0031] The collection of samples for protein separation and detection were performed as follows:

1) Samples of venous blood (4 to 6 ml) were collected by venepuncture using EDTA at standard concentration for anticoagulation (Vacutainer ® or Starstedt Monovette ®). Other appropriate anticoagulants known to persons skilled in the art may also be used.

2) Within 3 minutes of the sample being withdrawn, protease inhibitors were added. To the 4 or 6 ml volume of blood were added 0.5 ml of a protease inhibitor cocktail, freshly prepared, as follows:
To 3 mls of Aprotinin (Trasylol ®) concentration 10,000 Kallikrein inactivator units per ml was added 2 mg phenyl-methylsulphonyl fluoride (PMSF) and 0.5 mg leupeptin. Other appropriate inhibitor preparations known to persons skilled in the art may also be used.

3) The whole blood sample was then centrifuged, at 3000 rpm (1,000 g), for 10 minutes. Plasma was removed and the layer of white cells and platelets (the buffy coat) removed by plastic pipette, to leave the red cells in the tube.

4) A volume of ice cold phosphate buffered saline PBS pH 7.4 or other appropriate buffer, equal in volume to the red cells was added and red cells and buffer were mixed by inversion.

5) The cells in buffer were then centrifuged, at 1,000 g, repeating steps 3 and 4 three times, providing washed red cells.

6) The washed red cells were frozen in 0.5 ml aliquots were suspended in 0.5 ml of buffer, prepared as follows: 0.37 g KCl, 0.74 g disodium EDTA, 3.0 g Tris, 9 g NaCl in 1 litre of water, adjusted to pH 7.4 with HCl. (Kramer *et*

al. J. Biol. Chem. 266, 5268-5272). The washed red cells may alternatively be frozen directly as packed cells. In a simplified procedure, blood is collected into an EDTA vacutainer, centrifuged at 3000 rpm (1000g) for 10 minutes, the plasma and the buffy coat removed by aspiration and the packed red cells stored frozen.

7) These samples were stored frozen at -80°C or used directly after brief freezing to provide lysis for SDS polyacrylamide gel electrophoresis or for immunoassay in a calibrated assay. Where packed cells are stored frozen prior to use they are thawed into the buffer described in step (6) above.
Where the simplified procedure is employed, protease inhibitor is included in the buffer added to the red cells for the thawing process in the form of one tablet of COMPLETE™ protease inhibitor (Boehringer Mannhein) per 200 ml of buffer.

II. Protein Separation and Detection using SDS PAGE

[0032] Protein separation and detection was achieved using SDS PAGE as described below.

[0033] In SDS PAGE, the sample was treated with a volume of sample buffer, comprising Tris buffer pH 6.8 containing 6% SDS, 0.5% dithiothreitol and 20 ml glycerol per 100 ml. (Alternative buffers known to persons skilled in the art may be used). Lysate was added to give a 4-fold or greater dilution of the lysate in sample buffer. The sample was transferred to the standard SDS 7.5% polyacrylamide gel for electrophoresis. After electrophoresis the proteins were transferred from the gel to a membrane by electroblotting.

[0034] The test membrane was reacted with polyclonal antibody to mid-molecule type IV $cPLA_2$ raised in rabbit and then anti rabbit IgG antibody raised in swine which has been coupled to horse raddish peroxidase to demonstrate the presence of the $cPLA_2$ antigen. The blank membrane was reacted with anti rabbit IgG antibody raised in swine which has been coupled to horse raddish peroxidase. Any bands observed on the blank membrane are due to the presence of antigens other than the $cPLA_2$ antigen.

[0035] Figure 1a and 1b show two western blot analyses (figure 1a is the test membrane; figure 1b is the blank membrane) on the following samples:

Lane 1:	Molecular weight markers
Lane 2:	Cell cytosol from insect cells infected with Baculovirus expressing type IV $cPLA_2$.
Lanes 3-12:	Patient samples. The samples were taken from schizophrenic patients on clozapine (lanes 4, 7, 9 and 10), schizophrenic patients on neuroleptic drugs (lanes 8 and 12) and from control patients (lanes 3, 5, 6 and 11).

[0036] The patient samples on the test membrane show three band types grouped by molecular weight as described above:

Group A: one band is observed above the 97 kDa molecular weight marker, having a molecular weight of about 100 to 105 kDa, and one band is observed below the 97 kDa molecular weight marker, having a molecular weight of about 90 to 95 kDa;

Group B: a single band is observed with molecular weight around 70 kDa;

Group C: three bands are observed with molecular weights around 60 kDa. Two of the bands in this molecular weight group are believed to be type IV $cPLA_2$ proteins. The most distinct of these also appears on the blank membrane, has a less specific immunological response and may not be a type IV $cPLA_2$ protein.

[0037] In summary, the red cell lysates contained five proteins, two in the range of about 90 to about 105 kDa, one in the range of about 70 to about 80 kDa, and two in the range of about 50 to about 60 kDa, which reacted specifically in SDS PAGE western blot analysis with polyclonal antibody prepared against mid-molecule sequences of type IV $cPLA_2$.

[0038] As noted above the bands grouped in Groups A, B and C may represent, respectively, the intact type IV $cPLA_2$ molecule in differing stages of phosphorylation and two groups of peptide sequences of type IV $cPLA_2$ proteins which have lost amino acids by protein degradation. However, as stated above, it is not intended that the scope of the invention be limited by this theory. The bands grouped in Groups A, B and C could also represent intact type IV $cPLA_2$ and fragments of type IV $cPLA_2$ differing in the length of amino acid sequence.

III. Protein Detection using Enzyme Linked Immunoassay (ELISA)

[0039] Immunoassay, specifically enzyme linked immunoassay (ELISA), was also used to detect red cell type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$. This method uses a luminescence end point for protein detection.

[0040] Samples are collected and prepared as hereinbefore described. Samples of red cells for analysis were stored at -80°C.

[0041] The following reagents are required for ELISA:

1) Coating buffer, pH 9.6
1.59 g sodium carbonate, 2.93 g sodium bicarbonate in 1 litre deionised water;
2) Wash buffer, pH 7.4
6.07 g Tris, 0.2 g potassium chloride, 29.2 g sodium chloride in 800 ml deionised water adjusted to pH 7.4 with hydrochloric acid and made up to 1 litre;
3) Buffer for standard/sample diluent, pH 7.4
0.37 g potassium chloride, 0.74 g disodium ethylenediaminetetra-acetate, 3g Tris, 8.58 g sodium chloride in 800 mls deionised water adjusted to pH 7.4 and made up to 1 litre;
4) Standard/sample diluent
200 ml of buffer (3) to which is added 0.2 g human albumin and 1 tablet of Complete® protease inhibitors (Boehringer Mannheim);
5) Citrate solution, pH 7.4
2.94 sodium citrate dissolved in 800 ml deionised water adjusted to pH7.4 with hydrochloric acid and made up to 1 litre;
6) Antibody conjugate reagent
5 mls reagent (2), 6 mls Roti-Block ® (Rotech Scientific, Milton Keynes, UK), 0.2 ml reagent (5), 0.012 g human serum albumin and 1 ml of the in-house anti-$cPLA_2$ IgG (as described herein) - alkaline phosphatase conjugate;
7) Luminescence reagent containing Lumi-Phos ® 530 (buffered solution containing dioxetane Lumigen ® PPD, fluorescer and surfactant) (obtained from Beckman Coulter, UK)

[0042] The assay is performed using 96 well microtitre plates according to the following procedure:

1) The capture antibody (obtained from The Binding Site, Birmingham, UK) was diluted 1:10 with saline and stored in 500 µl aliquots at -20°C. One aliquot of this antibody is added to 11.5 ml coating buffer, mixed and 100 µl of diluted capture antibody is pipetted into each well of a white microtitre plate to coat the wells of the plate with capture antibody. The plate is sealed and stored at 4°C overnight. Prior to use, the plate is then washed four times with wash buffer pH 7.4 (reagent (2) as hereinbefore described).
2) The plate is blocked by pipetting into each well 300 µl of Roti-Block® diluted 1:2 with reagent (2). Blocking takes 90 minutes at 25°C. Blocking reagent is removed by washing four times with reagent (2).
3) Duplicate aliquots (100 µl) of sample or standard are then pipetted into each microtitre plate well and the plate incubated for 90 minutes at 25°C. The incubation is stopped by washing the plate four times with reagent (2).
4) The second antibody (the conjugated antibody, reagent (6)) is then added. Antibody is conjugated to alkaline phosphatase by standard techniques (Duncan *et al., Analytical Biochemistry* 132, 68-73). 100 µl of the antibody conjugate, reagent (6), is added to each well and the plate incubated for 90 minutes at 25°C.
5) At the end of this incubation, the plate is washed 8 times with reagent (2).
6) Luminescence is developed in the wells of the plate by adding 100 µl luminescence reagent (7) to each well and incubating for 20 minutes with shaking in a luminometer (Luminoscan Labsystems, Helsinki).
7) Luminescence in relative light units for a given sample is measured and compared with that of standard type IV $cPLA_2$ preparations. The use of standard type IV $cPLA_2$ preparations can be used to derive a calibration curve for an assay for $cPLA_2$. Figure 2 shows a calibration curve derived from 10 separate assays with the standard error of the mean marked.
8) Sample preparation
Prior to analysis, the frozen red blood cells were thawed, centrifuged at 10,000 g for 5 minutes and diluted 1:40 with sample diluent (reagent (4)).
9) Standard preparation
A U937 cell cytosol was utilised as a standard. This was prepared from U937 human monocyte culture *(Clark et al., Proc. Nat. Acad. Sci. USA,* 87, 7708-7712), Cells were harvested from culture, resuspended in standard/sample diluent, omitting the albumin content and with the addition of 11.6 g/100 ml sucrose. The cells were disrupted by nitrogen cavitation in a Parr bomb and the resulting preparation centrifuged at 150,000 g for 1 hour at 4°C. The

supernatant, a U937 cell cytosol, was aliquoted and stored at -80°C for standardisation. Western blot analysis of dilutions of this material allowed allocation of a value for type IV $cPLA_2$ content from the point at which type IV $cPLA_2$ was just detected by Western blot. The standard is stable at -80°C. All assays included internal quality control samples of an aliquoted red cell haemolysate.

10) Units for the estimation of type IV $cPLA_2$ in red cells haemolysates are μg $cPLA_2$ per g haemoglobin. Haemoglobin amounts were estimated by diluting the sample to 0.04% ammonia and reading the optical density at 540 mm, standardised against haemoglobin of known concentration.
Within-batch percentage coefficient of variation (CV%), which is a measurement of relative variation, i.e. the standard deviation expressed as a percentage of the arithmetic mean, typically measured 8.6 % and between-batch CV% typically measured 10.8%. These were measured at 9.0 ng $cPLA_2$ per g of haemoglobin (ng/gHb) and 9.7 ng/gHb respectively.

IV. Measurement of Type N $cPLA_2$ Levels in Red Blood Cells in Various Patient Groups

[0043] As an illustration of the clinical application of the measurement in red cells of type IV $cPLA_2$ protein or protein immunologically homologous to type IV $cPLA_2$, various blood samples were collected from groups of patients each with a defined psychiatric illness and from a control group. The samples from each patient group in this study were analysed using the ELISA method hereinbefore described. The patients within each group met stated criteria for their clinical diagnosis, as detailed below. Samples were collected using steps 1 to 7 hereinbefore described. Specimens were also collected and prepared omitting washing steps 4 to 5. This alternative shortened cell preparation method, employing only steps 1 to 3 and 6 to 7 was examined for its influence on the assayed levels of $cPLA_2$. Comparisons were made of samples given the full wash procedure and samples using the shortened procedure. No significant difference was found either within groups or combining the results in all groups when the two red cell preparation procedures were compared. The patients groups were as follows:

Group 1: Schizophrenia

[0044] 49 Patients who met the DSM IV criteria for the diagnosis of schizophrenia
(Diagnostic and Statistical Manual of Mental Disorders 4th Edition, American Psychiatric Association 1994);
36 Males and 13 females, age range 17 to 66 with mean age 37.3 +/- 11.9 years.

Group 2: Dyslexia

[0045] 27 Volunteers with recognised criteria for identification of Dyslexia, i.e. a difference of 15 points or more between Wechsler Adult Intelligence Scale Revised (WAIS-R) pro-rated equivalent IQ (© The Psychological Corporation Limited 1986) and Wide Range Achievement Test score (WRAT) (Wilkinson © Wide Range Inc, 1993) or reaching a Bangor Dyslexia Test score (T.R. Miles in Dyslexia, The Pattern of Difficulties. Grenada, 1983) equal to or greater than 7;
17 Males and 10 females of age range 16 to 67 with mean age 35.4 +/- 13.2 years.

Group 3: Controls

[0046] 51 volunteers comprised the control group;
25 Males and 26 females, age range 16 to 57 with mean age 35.4 +/- 12.4 years.

[0047] The results of the study are displayed in Figures 3A, 3B and 3C which show the frequency histogram of red cell type IV $cPLA_2$ or proteins or proteins immunologically homologous to type IV $cPLA_2$ in μg $cPLA_2$ per g haemoglobin (Hb) as estimated by the ELISA method hereinbefore described. Figures 3A, 3B and 3C illustrate, respectively, the control group, the schizophrenic group and the dyslexic group.

[0048] The distribution of the results of the assays for red cell type IV $cPLA_2$ or proteins or proteins immunologically homologous to type IV $cPLA_2$ measured as μg per g haemoglobin (Hb) was positively skewed (Fig 3) and non-parametric tests were applied in analysis. Mann Whitney U test of the schizophrenia patient group and controls reveal a highly significant elevation of red cell $cPLA_2$ in the schizophrenia group compared with the control group ($P<0.0001$). There was a lesser elevation of red cell $cPLA_2$ in the dyslexic subjects compared with controls ($P<0.001$). Determination of a reference range encompassing 95% of the control group for red cell $cPLA_2$ permitted determination of an upper cut off point for the reference range for red cell $cPLA_2$ of 2.8 μg per g Hb. This is not an absolute value but is derived from the standardisation procedure used for these studies, as hereinbefore described.

[0049] Confirmation of the ELISA analysis of the type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in the red cell haemolysates may be obtained by using SDS PAGE and western blot analysis using the

techniques previously described. Samples from a patient in the control group and from a patient in the schizophrenic patient group (group 1) were subjected to further analysis using SDS PAGE and western blot. The group I sample was taken from a patient that had been shown by ELISA to have high levels of type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in the red cell haemolysates.

[0050] Figure 4 illustrates the type IV $cPLA_2$ proteins or proteins immunologically homologous to type IV $cPLA_2$ in the red cell haemolysates, and the proteins observed in a red cell haemolysate from a control patient, as observed by SDS PAGE/western blot. Protein of the molecular weight of $cPLA_2$ protein from human monocyte U937 cells is observed in the haemolysate from the schizophrenic patient; there was no protein detected which had this molecular weight in the haemolysate from the control subject. The various samples in the western blot analysis shown in Figure 4 are as follows:

Lane 1: Molecular weight markers
Lane 2: Cytosol from insect cells infected with baculovirus expressing type IV $cPLA_2$
Lane 3: Cytosol from human monocyte U937 cells containing a $cPLA_2$ protein
Lane 4: Red cell haemolysate from a control subject
Lane 5: Red cell haemolysate from a schizophrenia subject

Claims

1. Use of red blood cells in an ex vivo assay for the purpose of detecting type IV cytosolic phospholipase A_2 ($cPLA_2$) or a protein immunologically homologous to type IV $cPLA_2$.

2. Use according to claim 1 wherein said assay is for use in the diagnosis of a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated.

3. Use according to claim 1 wherein said assay is for use in monitoring the effectiveness of medication administered to a patient suffering from a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated

4. Use according to claim 1 wherein said assay is for use in drug development for a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated

5. A method of diagnosis of a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the ex vivo detection of type IV cytosolic phospholipase A_2 ($cPLA_2$) protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells.

6. A method of monitoring the effectiveness of medication administered to a patient suffering from a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the ex vivo detection of type IV cytosolic phospholipase A_2 ($cPLA_2$) protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells.

7. A method of drug development for a disease in which dysfunction of cell signalling systems involving highly unsaturated fatty acids is implicated, said method comprising the detection of type IV cytosolic phospholipase A_2 ($cPLA_2$) protein or a protein immunologically homologous to type IV $cPLA_2$ in or on red blood cells.

8. Use or method according to any one of claims 1 to 7 wherein the red blood cells are isolated from the human body.

9. Use or method according to any one of claims 1 to 7 wherein said assay or method comprises use of a whole blood sample without prior isolation of said red blood cells.

10. Use or method according to any one of claims 2 to 9 wherein said disease is a disease or disease process in which type IV $cPLA_2$ activity or concentration is altered from normal levels.

11. Use or method according to anyone of claims 2 to 9 wherein said disease is a disease or disease process in which type IV $cPLA_2$ activity or concentration is increased.

12. Use or method according to any one of claims 2 to 11 wherein the disease is schizophrenia, dyslexia, bipolar or

manic depressive illness, cachexia or brain injury.

13. Use or method according to claim 12 wherein the brain injury is stroke or mechanical brain injury.

14. Use or method according to any one of claims 1 to 13 wherein the type IV $cPLA_2$ protein or the protein immunologically homologous to type IV $cPLA_2$ has a molecular weight in the range 80 to 110 kDa or in the range 70 to 80 kDa or in the range 50 to 60 kDa.

15. Use or method according to any one of claims 1 to 13 wherein the type IV $cPLA_2$ protein or the protein immunologically homologous to type IV $cPLA_2$ has a molecular weight in the range 90 to 105 kDa or in the range 70 to 80 kDa or in the range 50 to 60 kDa.

16. Use or method according to any preceding claim wherein said assay or method comprises the steps of collecting a sample of blood from a subject and detecting the proteins *ex vivo.*

17. Use or method according to claim 16 wherein said assay or method comprises one or more of the steps of separating the red cells from the other blood components, disrupting the red cells, detecting the proteins either directly or following a protein separation technique.

18. Use or method according to claim 17 wherein the red cells are disrupted by sonication, freezing, nitrogen cavitation or lysis.

19. Use or method according to any preceding claim wherein said proteins are detected by immunoassay.

20. Use or method according to any preceding claim wherein said proteins are detected using an antibody or antibodies that recognise an epitope or epitopes from amino acids 82 to 749 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

21. Use or method according to any preceding claim wherein said proteins are detected using an antibody or antibodies raised against an epitope or epitopes from amino acids 82 to 749 of type IV $cPLA_2$ protein from human monocyte (U937 cells) or raised against an epitope or epitopes of a synthetic peptide matching amino acids 82 to 749 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

22. Use or method according to claim 20 or 21 wherein said epitope or epitopes are from a peptide sequence or sequences which comprise the catalytic centre of type IV $cPLA_2$ protein from human monocyte (U937) cells.

23. Use or method according to claim 20 or 21 wherein said epitope or epitopes are from the peptide sequence of amino acids 241 to 260 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

24. Use or method according to claim 19 wherein said proteins are detected using an antibody or antibodies raised against an epitope or epitopes from amino acids 1 to 216 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

25. Use or method according to claims 20, 21, 22, 23 or 24 wherein two or more of the antibodies are used in combination or in sequence to detect the said proteins with the required specificity.

26. Use or method according to any of claims 1 to 18 for detecting type IV $cPLA_2$ wherein said proteins are detected by substrate assay.

27. A diagnostic kit comprising means for disrupting red blood cells and further comprising an antibody or antibodies to a protein obtainable by isolation from red blood cells, said protein being type IV $cPLA_2$ protein or a protein immunologically homologous to type IV $cPLA_2$.

28. A diagnostic kit according to claim 27 wherein said antibody or antibodies is/are raised against an epitope or epitopes from amino acids 82 to 749 of type IV $cPLA_2$ protein from human monocyte (U937) cells.

29. A diagnostic kit according to claim 27 wherein said antibody or antibodies is/are raised against an epitope or epitopes from a peptide sequence or sequences which comprise the catalytic active centre of type IV $cPLA_2$ protein

from human monocyte (U937) cells.

30. A diagnostic kit according to claim 27, 28 or 29 wherein said means for disrupting red blood cells is a means for lysing red blood cells.

31. A diagnostic kit according to claim 27, 28 or 29 which is suitable for near-patient testing.

Patentansprüche

1. Verwendung von roten Blutkörperchen in einem ex vivo-Test zum Zwecke des Detektierens von Typ IV cytosolischer A_2(cPLA$_2$)-Phospholipase oder einem Protein, das immunologisch homolog zu Typ IV cPLA$_2$ ist.

2. Verwendung nach Anspruch 1, worin der Test zur Verwendung bei der Diagnose einer Krankheit ist, in welcher eine Fehlfunktion von Zellsignalsystemen impliziert ist, welche hochgradig ungesättigte Fettsäuren involviert.

3. Verwendung nach Anspruch 1, worin der Test zur Verwendung beim Überwachen der Effizienz von einer an einen Patienten verabreichten Medikation ist, welcher unter einer Krankheit leidet, in welcher Zellsignalsystem-Fehlfunktion impliziert ist, die hochgradig ungesättigte Fettsäuren involviert.

4. Verwendung nach Anspruch 1, worin der Test zur Verwendung bei der Medikamentenentwicklung für eine Krankheit ist, in welcher Zellsignalsystem-Fehlfunktionen impliziert sind, die hochgradig ungesättigte Fettsäuren involvieren.

5. Verfahren zur Diagnose einer Krankheit, in welcher Zellsignalsystemfehlfunktion impliziert ist, die hochgradig ungesättigte Fettsäuren involviert, wobei das Verfahren die ex vivo-Detektion von Typ IV cytosolischem A_2(cPLA$_2$)-Phospholipaseprotein oder einem Protein umfasst, das immunologisch homolog zu Typ IV cPLA$_2$ in oder an roten Blutkörperchen ist.

6. Verfahren zum Überwachen der Effizienz von einer an einen Patienten verabreichten Medikation, welcher unter einer Krankheit leidet, in welcher Zellsignalsystem-Fehlfunktion impliziert ist, die hochgradig ungesättigte Fettsäuren involviert, wobei das Verfahren die ex vivo-Detektion von Typ IV cytosolischem A_2(cPLA$_2$)-Phospholipaseprotein oder einem Protein umfasst, das immunologisch homolog zu Typ IV cPLA$_2$ in oder an roten Blutkörperchen ist.

7. Verfahren zur Medikamentenentwicklung für eine Krankheit, in welcher Zellsignalsystemfehlfunktion impliziert ist, die hochgradig ungesättigte Fettsäuren involviert, wobei das Verfahren die ex vivo-Detektion von Typ IV cytosolischem A_2(cPLA$_2$)-Phospholipaseprotein oder einem Protein umfasst, das immunologisch homolog zu Typ IV cPLA$_2$ in oder an roten Blutkörperchen ist.

8. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 7, worin die roten Blutkörperchen aus dem menschlichen Körper isoliert werden.

9. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 7, worin der Test oder das Verfahren die Verwendung einer vollständigen Blutprobe ohne vorherige Isolierung der roten Blutkörperchen umfasst.

10. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 9, worin die Krankheit eine Krankheit oder ein Krankheitsprozess ist, in welchem (in welcher) Typ IV cPLA$_2$-Aktivität oder Konzentration von normalen Gehalten verändert ist.

11. Verwendung oder Verfahren nach einem der Ansprüche 2 bis 9, worin die Krankheit eine Krankheit oder ein Krankheitsprozess ist, in welcher (in welchem) Typ IV cPLA$_2$-Aktivität oder -Konzentration erhöht ist.

12. Verwendung oder Verfahren nach einem der Ansprüche 2 bis 11, worin die Krankheit Schizophrenie, Dyslexie, bipolar- oder manisch-depressive Krankheit, Kachexie oder Hirnverletzung ist.

13. Verwendung oder Verfahren nach Anspruch 12, worin die Hirnverletzung Schock oder mechanische Hirnverletzung ist.

14. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 13, worin Typ IV $cPLA_2$-Protein oder das immunologisch zu Typ IV $cPLA_2$ homologe Protein ein Molekulargewicht im Bereich von 80 bis 110 kDa hat oder im Bereich von 70 bis 80 kDa oder im Bereich von 50 bis 60 kDa hat.

15. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 13, worin das Typ IV $cPLA_2$-Protein oder das immunologisch zu Typ IV $cPLA_2$-Protein homologe Protein ein Molekulargewicht im Bereich von 90 bis 105 kDa oder im Bereich von 70 bis 80 kDa oder im Bereich von 50 bis 60 kDa hat.

16. Verwendung oder Verfahren nach einem der vorhergehenden Ansprüche, worin der Test oder das Verfahren die Schritte des Sammelns einer Blutprobe von einem Subjekt und Detektierens des Proteins ex *vivo* umfasst.

17. Verwendung oder Verfahren nach Anspruch 16, worin der Test oder das Verfahren einen oder mehrere der Schritte des Trennens roter Blutkörperchen von anderen Blutbestandteilen, Aufbrechen der roten Blutkörperchen, Detektieren der Proteine entweder direkt oder einer Proteintrennungstechnik folgend umfasst.

18. Verwendung oder Verfahren nach Anspruch 17, worin die roten Blutkörperchen durch Beschallung, Gefrieren, Stickstoffaushöhlung oder Zersetzung unterbrochen werden.

19. Verwendung oder Verfahren nach einem der vorhergehenden Ansprüche, worin die Proteine durch Immunotest detektiert werden.

20. Verwendung oder Verfahren nach einem der vorhergehenden Ansprüche, worin die Proteine unter Verwendung eines Antikörpers oder Antikörpern detektiert werden, die ein Epitop oder Epitope von Aminosäuren 82 bis 749 von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) erkennen.

21. Verwendung oder Verfahren nach einem der vorhergehenden Ansprüche, worin die Proteine detektiert werden unter Verwendung eines Antikörpers oder Antikörpern, die gegen ein Epitop oder Epitope von den Aminosäuren 82 bis 749 von Typ IV $cPLA_2$-Protein aus humanen Monocyten (U937-Zellen) gezogen worden sind oder gegen ein Epitop oder Epitope von einem synthetischen Protein gezogen sind, das den Aminosäuren 82 bis 749 von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) angepasst ist.

22. Verwendung oder Verfahren nach Anspruch 20 oder 21, worin das Epitop oder Epitope aus einer Peptidsequenz oder -sequenzen ist, welche das katalytische Zentrum von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) umfasst.

23. Verwendung oder Verfahren nach Anspruch 20 oder 21, worin das Epitop oder Epitope von der Peptidsequenz von Aminosäuren 241 bis 260 von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) sind.

24. Verwendung oder Verfahren nach Anspruch 19, worin die Proteine unter Verwendung eines Antikörpers oder von Antikörpern detektiert werden, die gegen ein Epitop oder Epitope von den Aminosäuren 1 bis 216 von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) gezogen sind.

25. Verwendung oder Verfahren nach den Ansprüchen 20, 21, 22, 23 oder 24, worin zwei oder mehr der Antikörper in Kombination oder in Abfolge verwendet werden, um die Proteine mit der erforderlichen Spezifität zu detektieren.

26. Verwendung oder Verfahren nach einem der Ansprüche 1 bis 18 zum Detektieren von Typ IV $cPLA_2$-Protein, worin die Proteine durch Substrattest detektiert werden.

27. Diagnostischer Kit, welcher Mittel zum Unterbrechen roter Blutkörperchen umfasst und weiterhin einen Antikörper oder Antikörper gegen ein Protein umfasst, das durch Isolierung aus roten Blutkörperchen erhältlich ist, wobei das Typ IV $cPLA_2$-Protein oder ein immunologisch zu Typ IV $cPLA_2$ homologes Protein ist.

28. Diagnostischer Kit nach Anspruch 27, worin der Antikörper oder die Antikörper gegen ein Epitop oder Epitope von den Aminosäuren 82 bis 749 von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) gezogen ist/sind.

29. Diagnostischer Kit nach Anspruch 27, worin der Antikörper oder die Antikörper gegen ein Epitop oder Epitope einer Peptidsequenz oder -sequenzen gezogen ist/sind, welche das katalytisch aktive Zentrum von Typ IV $cPLA_2$-Protein aus humanen Monocytenzellen (U937) umfasst.

30. Diagnostischer Kit nach Anspruch 27, 28 oder 29, worin das Mittel zum Aufbrechen roter Blutkörperzellen ein Mittel zur Lyse roter Blutkörperzellen ist.

31. Diagnostischer Kit nach Anspruch 27, 28 oder 29, welcher für das Testen am Patienten geeignet ist.

Revendications

1. Utilisation de globules rouges dans un dosage *ex vivo* dans le but de détecter la phospholipase cytosolique A_2 ($cPLA_2$) de type IV ou une protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV.

2. Utilisation selon la revendication 1 dans laquelle ledit dosage est destiné au diagnostic d'une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturés est impliqué.

3. Utilisation selon la revendication 1 dans laquelle ledit dosage est destiné à être utilisé pour surveiller l'efficacité du médicament administré à un patient souffrant d'une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturés est impliqué.

4. Utilisation selon la revendication 1 dans laquelle ledit dosage est destiné à être utilisé pour l'élaboration d'un médicament pour une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturés est impliqué.

5. Procédé de diagnostic d'une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturés est impliqué, ledit procédé comprenant la détection *ex vivo* de la protéine phospholipase cytosolique A_2 ($cPLA_2$) de type IV ou d'une protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV dans ou sur des globules rouges.

6. Procédé de surveillance de l'efficacité d'un médicament administré à un patient souffrant d'une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturés est impliqué, ledit procédé comprenant la détection *ex vivo* de la protéine phospholipase cytosolique A_2 ($cPLA_2$) de type IV ou d'une protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV dans ou sur des globules rouges.

7. Procédé d'élaboration d'un médicament pour une maladie dans laquelle un dysfonctionnement des systèmes de signalisation cellulaire impliquant des acides gras hautement insaturé est impliqué, ledit procédé comprenant la détection de la protéine phospholipase cytosolique A_2 ($cPLA_2$) de type IV ou d'une protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV dans ou sur des globules rouges.

8. Utilisation ou procédé selon l'une quelconque des revendications 1 à 7 dans lesquels les globules rouges sont isolés du corps humain.

9. Utilisation ou procédé selon l'une quelconque des revendications 1 à 7 dans lesquels ledit dosage ou procédé comprennent l'utilisation d'un échantillon de sang complet sans isolement préalable desdits globules rouges.

10. Utilisation ou procédé selon l'une quelconque des revendications 2 à 9 dans lesquels ladite maladie est une maladie ou un processus morbide dans lesquels l'activité ou la concentration de $cPLA_2$ de type IV sont modifiées relativement à des niveaux normaux.

11. Utilisation ou procédé selon l'une quelconque des revendications 2 à 9 dans lesquels ladite maladie est une maladie ou un processus morbide dans lesquels l'activité ou la concentration de $cPLA_2$ de type IV sont accrues.

12. Utilisation ou procédé selon l'une quelconque des revendications 2 à 11 dans lesquels la maladie est la schizophrénie, la dyslexie, la dépression bipolaire ou maniaque, la cachexie ou une lésion cérébrale.

13. Utilisation ou procédé selon la revendication 12, dans lesquels la lésion cérébrale est un AVC ou une lésion cérébrale mécanique.

14. Utilisation ou procédé selon l'une quelconque des revendications 1 à 13 dans lesquels la protéine $cPLA_2$ de type IV ou la protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV ont un poids moléculaire dans le domaine de 80 à 110 kDa ou dans le domaine de 70 à 80 kDa ou dans le domaine de 50 à 60 kDa.

15. Utilisation ou procédé selon l'une quelconque des revendications 1 à 13 dans lesquels la protéine $cPLA_2$ de type IV ou la protéine homologue sur le plan immunologique à la $cPLA_2$ de type IV ont un poids moléculaire dans le domaine de 90 à 105 kDa ou dans le domaine de 70 à 80 kDa ou dans le domaine de 50 à 60 kDa.

16. Utilisation ou procédé selon l'une quelconque des revendications précédentes dans lesquels ledit dosage ou ledit procédé comprennent les étapes consistant à prélever un échantillon de sang sur un sujet et à détecter les protéines *ex vivo.*

17. Utilisation ou procédé selon la revendication 16 dans lesquels ledit dosage ou ledit procédé comprennent une ou plusieurs des étapes consistant à séparer les globules rouges des autres constituants du sang, dégrader les globules rouges, détecter les protéines directement ou après une technique de séparation des protéines.

18. Utilisation ou procédé selon la revendication 17, dans lesquels les globules rouges sont dégradés par sonication, congélation, cavitation à l'azote ou lyse.

19. Utilisation ou procédé selon l'une quelconque des revendications précédentes dans lesquels lesdites protéines sont détectées par immunodosage.

20. Utilisation ou procédé selon l'une quelconque des revendications précédentes dans lesquels lesdites protéines sont détectées en utilisant un anticorps ou plusieurs anticorps qui reconnaissent un épitope ou plusieurs épitopes des acides aminés 82 à 749 de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

21. Utilisation ou procédé selon l'une quelconque des revendications précédentes dans laquelle lesdites protéines sont détectées en utilisant un anticorps ou plusieurs anticorps dirigés contre un épitope ou plusieurs épitopes des acides aminés 82 à 749 de la protéine $cPLA_2$ de type IV de monocytes humains (U937) ou dirigés contre un épitope ou plusieurs épitopes d'un peptide synthétique correspondant aux acides aminés 82 à 749 de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

22. Utilisation ou procédé selon la revendication 20 ou 21 dans lesquels ledit épitope ou lesdits épitopes sont ceux d'une séquence peptidique ou de plusieurs séquences peptidiques qui comprennent le centre catalytique de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

23. Utilisation ou procédé selon la revendication 20 ou 21 dans lesquels ledit ou lesdits épitopes sont ceux de la séquence peptidique des acides aminés 241 à 260 de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

24. Utilisation ou procédé selon la revendication 19 dans lesquels lesdites protéines sont détectées en utilisant un anticorps ou plusieurs anticorps dirigés contre un épitope ou plusieurs épitopes des acides aminés 1 à 216 de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

25. Utilisation ou procédé selon les revendications 20, 21, 22, 23 ou 24 dans lesquels deux des anticorps ou plus sont utilisés en association ou en séquence pour détecter lesdites protéines avec la spécificité requise.

26. Utilisation ou procédé selon l'une quelconque des revendications 1 à 18 pour détecter la protéine $cPLA_2$ de type IV, dans lesquels lesdites protéines sont détectées par dosage de substrat.

27. Trousse de diagnostic comprenant un moyen pour dégrader les globules rouges et comprenant en outre un anticorps ou plusieurs anticorps dirigés contre une protéine pouvant être obtenue par isolement à partir de globules rouges, ladite protéine étant la protéine $cPLA_2$ de type IV ou une protéine homologue sur le plan immunologique à la protéine $cPLA_2$ de type IV.

28. Trousse de diagnostic selon la revendication 27 dans laquelle ledit anticorps ou lesdits anticorps sont dirigés contre un épitopes ou plusieurs épitopes des acides aminés 82 à 749 de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

29. Trousse de diagnostic selon la revendication 27 dans laquelle ledit anticorps ou lesdits anticorps sont dirigés contre un épitope ou plusieurs épitopes d'une séquence peptidique ou de plusieurs séquences qui comprennent le centre actif catalytique de la protéine $cPLA_2$ de type IV de monocytes humains (U937).

30. Trousse de diagnostic selon la revendication 27, 28 ou 29 dans laquelle ledit moyen pour dégrader les globules rouges est un moyen pour lyser les globules rouges.

31. Trousse de diagnostic selon la revendication 27, 28 ou 29 qui est adaptée pour le dosage au chevet du patient.

FIG. 1a

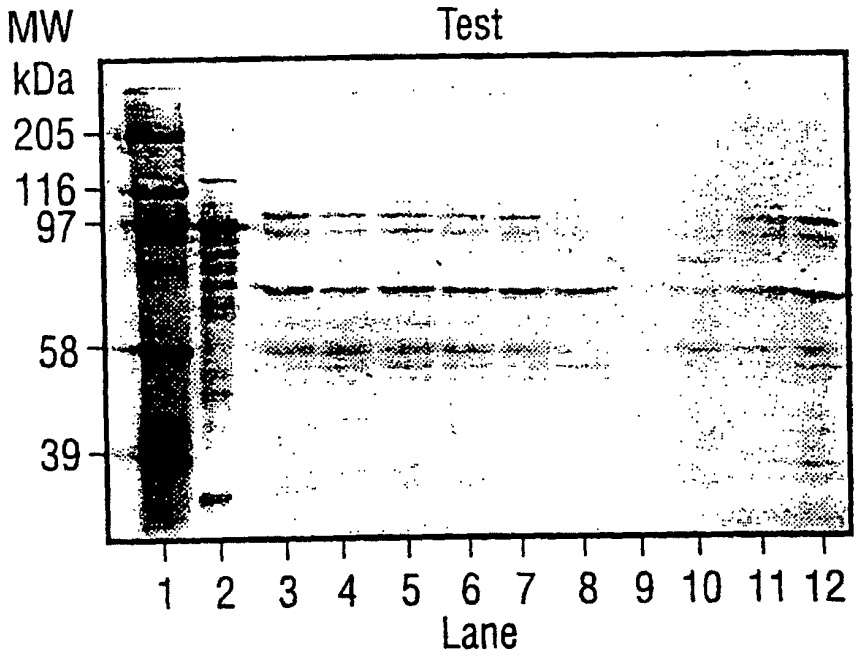

MW
kDa
205
116
97
58
39
Test
1 2 3 4 5 6 7 8 9 10 11 12
Lane

FIG. 1b

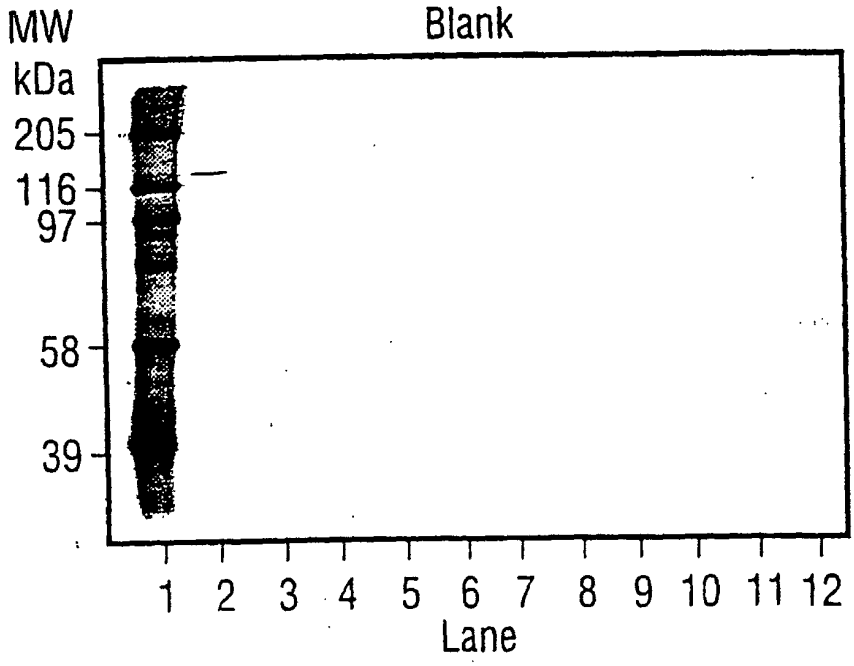

MW
kDa
205
116
97
58
39
Blank
1 2 3 4 5 6 7 8 9 10 11 12
Lane

FIG. 2

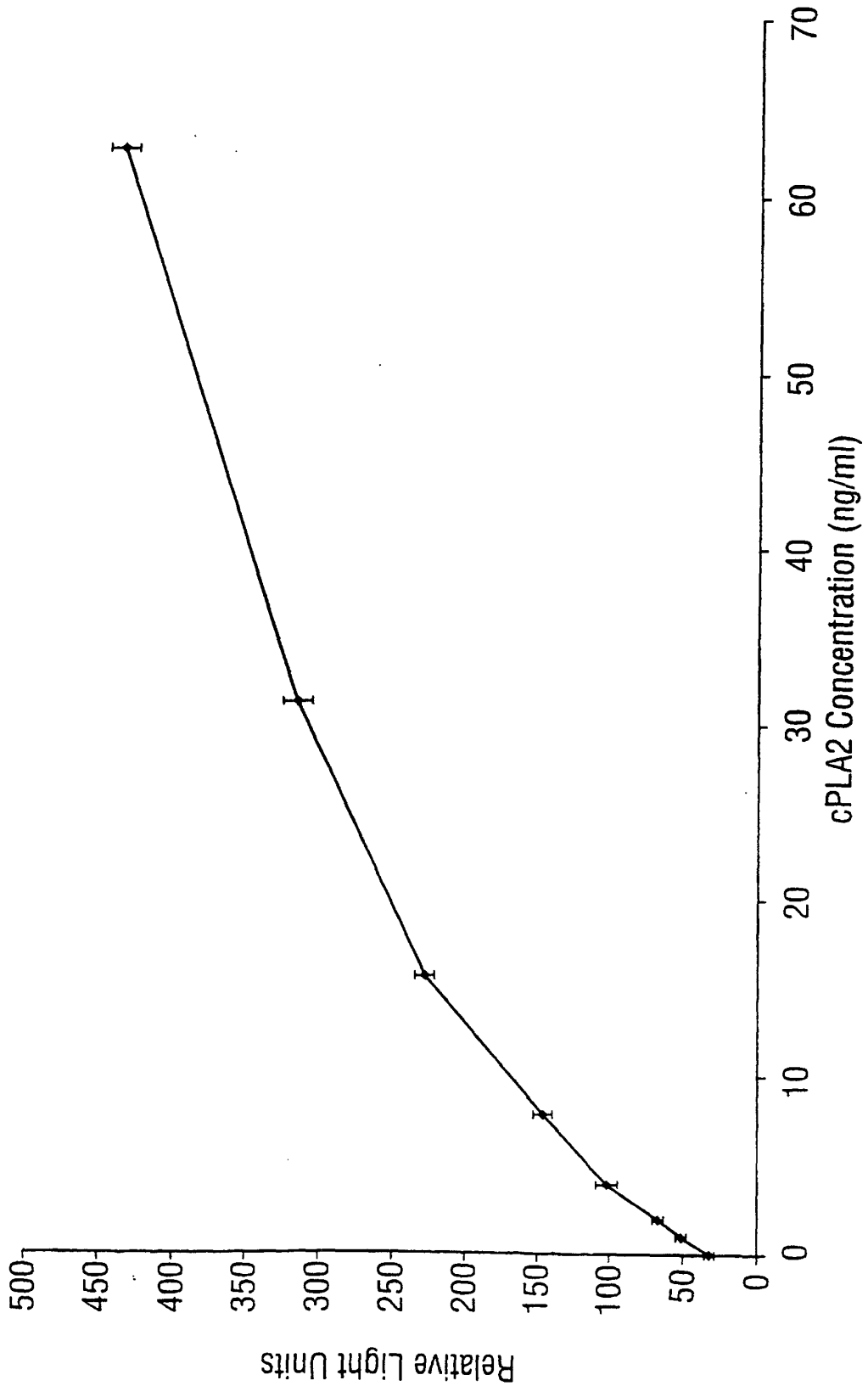

Relative Light Units
500
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
cPLA2 Concentration (ng/ml)

FIG. 3A

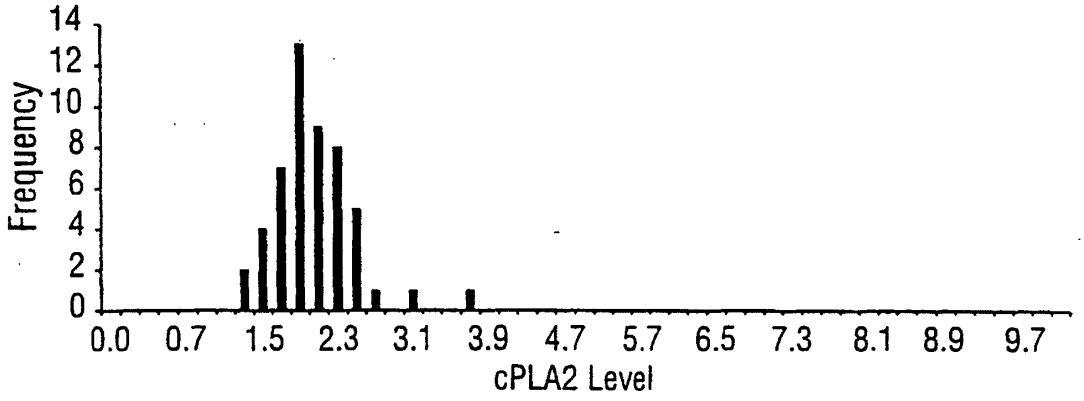


FIG. 3B

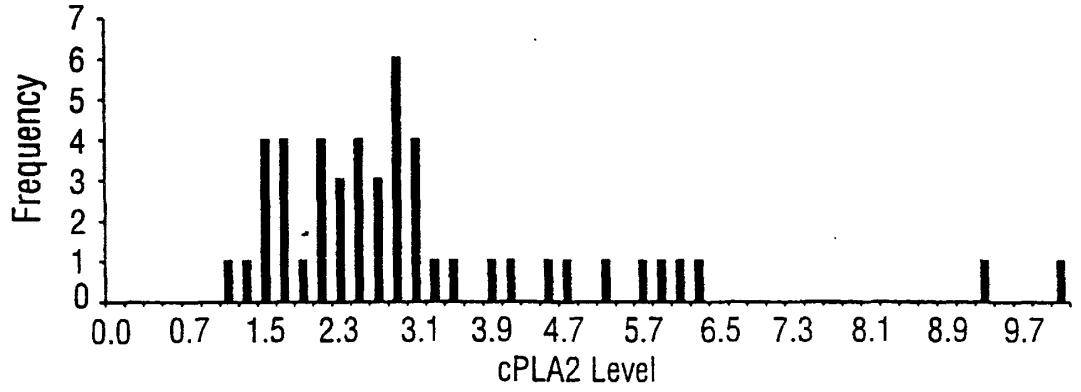


FIG. 3C

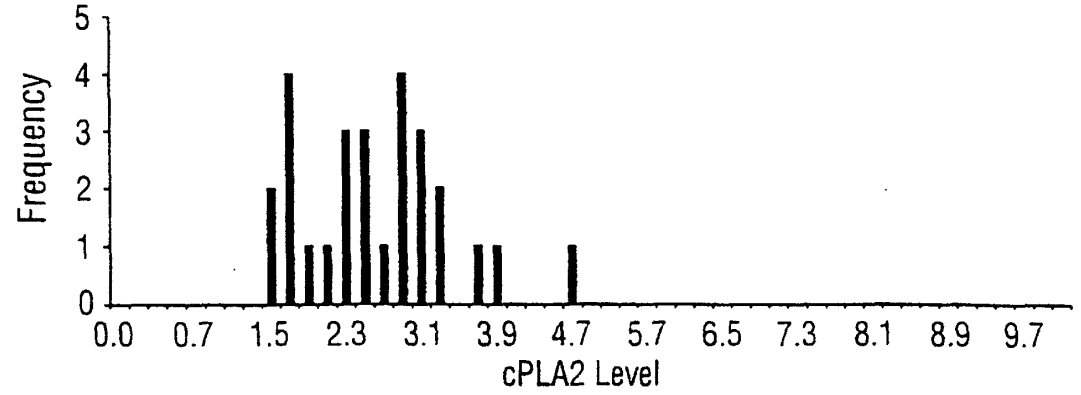
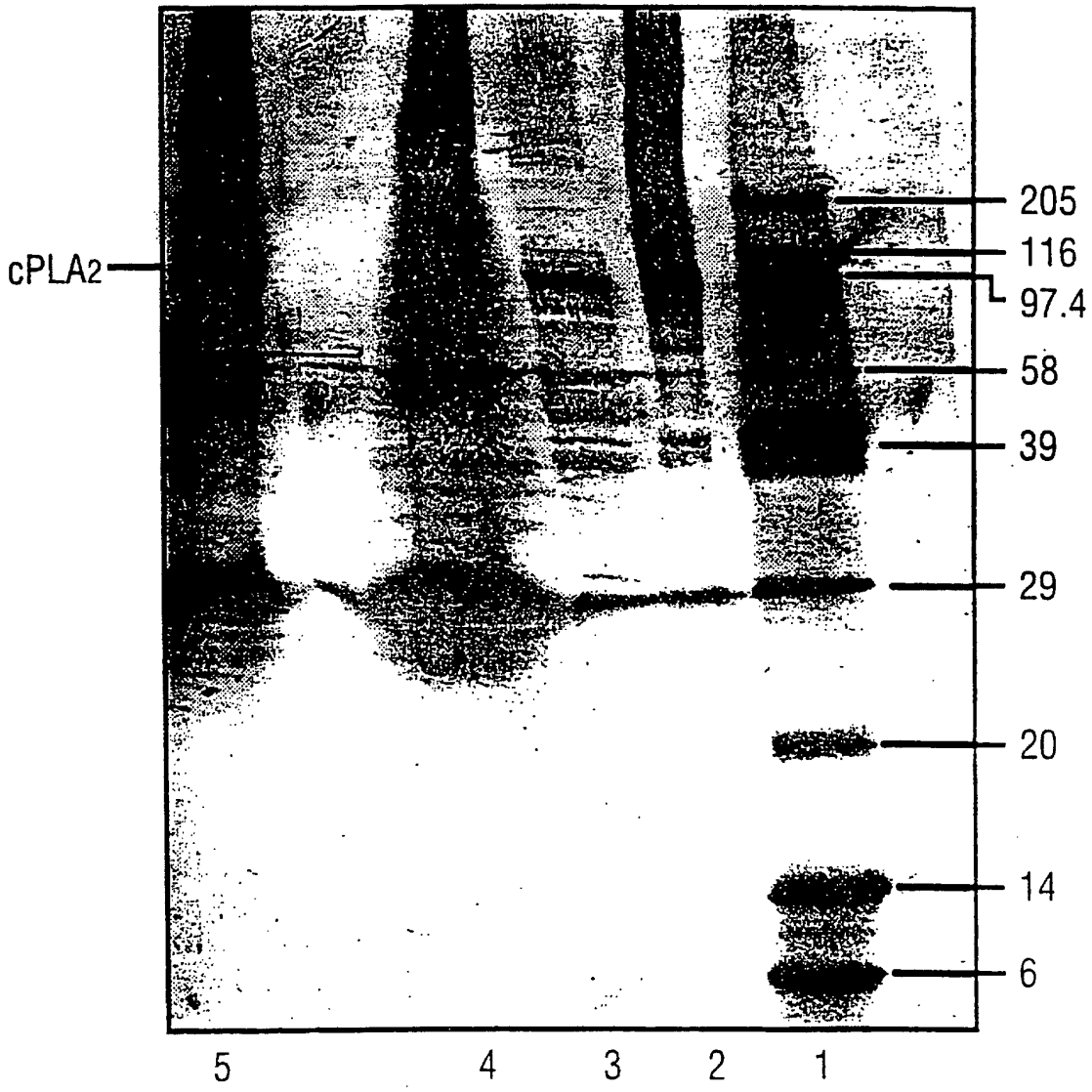


FIG. 4



专利名称(译)	诊断测试		
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申请号	EP2000907846	申请日	2000-03-08
申请(专利权)人(译)	LAXDALE有限公司		
当前申请(专利权)人(译)	AMARIN神经科学有限公司		
[标]发明人	GLEN ALASTAIR CAMPBELL AGNEW MCDONALD DONALD JOHN		
发明人	GLEN, ALASTAIR, CAMPBELL, AGNEW MCDONALD, DONALD, JOHN		
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其他公开文献	EP1159616A2		
外部链接	Espacenet		

摘要(译)

用于检测IV型胞质磷脂酶Aβ2（cPLA2？）或与IV型cPLA2β免疫学同源的蛋白质的试验，该试验包括使用红细胞，特别是用于诊断疾病。涉及涉及高度不饱和脂肪酸的细胞信号系统的功能障碍。

FIG. 1a

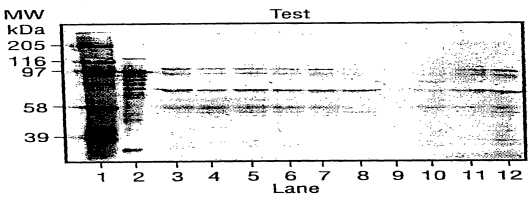

FIG. 1b