(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number
WO 01/16599 A1

(51) International Patent Classification[7]: G01N 33/53

(21) International Application Number: PCT/US00/24337

(22) International Filing Date: 25 August 2000 (25.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/384,356 27 August 1999 (27.08.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/384,356 (CIP)
Filed on 27 August 1999 (27.08.1999)

(71) Applicant *(for all designated States except US)*: **VIATECH IMAGING, LLC** [US/US]; 158 Main Street, P.O. Box 456, Ivoryton, CT 06442 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CORNELL-BELL, Ann** [US/US]; 55 Stevenstown Road, Westbrook, CT 06498 (US). **MADDEN, Kathleen, S.** [US/US]; 5101 River Road #1805, Bethesda, MD 20816 (US). **RIBLET, Leslie, A.** [US/US]; 145 Chestnut Hill Road, Killingworth, CT 06419 (US).

(74) Agent: **YANKWICH, Leon, R.**; Yankwich & Associates, 130 Bishop Allen Drive, Cambridge, MA 02139 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, 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, PL, PT, RO, RU, SD, SE, SG, SI, 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), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/16599 A1

(54) Title: ASSAY FOR DETECTING DAMAGE TO THE CENTRAL NERVOUS SYSTEM

(57) Abstract: A method is disclosed for the rapid diagnosis of disorders characterized by an ischemic event, such as stroke, transient ischemic attack, head trauma, myocardial infarction, or other insults resulting in interrupted cranial blood flow. The method involves detection of the presence of the gamma isoform of protein kinase C (PKCg) in peripheral blood, which signals damage to central nervous system tissue and at least transient breakdown of the blood brain barrier. The assay may be performed, e.g., by emergency medical personnel, in a time frame that allows treatment of the patient before permanent damage to the central nervous system occurs.

ASSAY FOR DETECTING DAMAGE TO THE CENTRAL NERVOUS SYSTEM

FIELD OF THE INVENTION

The present invention discloses a method for the rapid and accurate detection of damage to the central nervous system (CNS) caused by an ischemic event such as stroke or head trauma. The method focuses on detecting the release of the gamma isoform of protein kinase C (PKCg) into the bloodstream, e.g., as a result of the breakdown of the blood-brain barrier. The current invention provides a diagnostic method and a diagnostic kit that is useful for the early diagnosis and treatment of an ischemic event. Such methods and kits may be advantageously used, e.g., by emergency medical personnel, to obtain an early indication of an ischemic event within a time period following the event where permanent CNS damage may be avoided. The methods and kits may also be used to monitor patient progress and recovery following an ischemic event.

BACKGROUND OF THE INVENTION

Brain ischemia resulting from stroke, head trauma or other events that interfere with blood flow to the brain is a leading cause of death and disability in industrialized nations. Stroke, for example, affects 0.1-0.2% of the North American and European population. Approximately 700,000 people in the United States have a new or recurrent stroke each year, with a significant number resulting in death. An estimated 3,000,000 people in the United States have survived a stroke, however many of these survivors are considered to be at risk for recurrent episodes.

There are no specific neuroprotective drugs on the market to treat ischemic stroke, and consequently this condition represents a major clinical problem with 25-35% fatality for acute strokes within the first three weeks. Of the survivors, 25-50% will be totally dependent on family or institutional care for the rest of their lives.

One major impediment to the establishment of effective therapies for acute CNS injury has been the lack of definitive diagnostic procedures to permit proper and rigorous clinical trial design. Present diagnostic procedures are usually based on a sudden onset of neurologic signs such as hemiparesis, aphasia, hemianopia, altered consciousness, or gait disturbances.

The initial evaluation of a suspected stroke patient is time-consuming and usually occurs in the hospital after permanent neuronal damage has already occurred. Although the mechanisms involved in stroke are not fully understood, it has been proposed that the pathology arises from an

initial infarct, wherein perfusion pressure and blood flow to CNS tissues are reduced by intracellular and microvascular edema, followed by progressive compression and reduced perfusion in areas adjacent to the infarct. As this process evolves, a series of biochemical events takes place as cell damage progresses. These biochemical events, indicative of cell damage and cell death, may include oxidation of membrane components, free radical formation, altered fatty acid metabolism, activation of the gamma isoform of protein kinase C (PKCg), calcium entry into the cell, and disruption of the blood brain barrier.

If permanent brain damage from ischemic injury is to be avoided, appropriate treatment must be administered within two hours of the suspected insult. However, at the present time, definitive diagnostic procedures for these conditions are inaccurate, expensive, and not readily available to physicians or emergency medical personnel such that accurate diagnosis of stroke or other ischemic injury can be determined or measured within this important two-hour window. This absence of effective diagnostic procedures has also contributed to the lack of new therapies for the treatment or prevention of neuronal damage from stroke or other ischemic events.

Assay techniques have been suggested for a number of potential marker proteins associated with stroke, including neuron-specific enolase (NSE), myelin basic protein, glial fibrillary acidic protein, and S-100 protein (Missler et al., 1997). In most cases these substances are measured in cerebrospinal fluid (CSF), which is obtained by invasive and difficult procedures. Given the short diagnostic window for avoiding permanent injury, it is imperative that diagnostic methods be developed that can be performed using more easily obtainable samples, ideally peripheral blood samples. Even though NSE and S-100 are measurable in blood, peak levels are not found until approximately two days following infarction, which makes them impractical indicators of stroke, even though NSE and S-100 protein do correlate with infarct volume (Missler et al., 1997). Also, none of these markers are specific indicators of general brain damage, and S-100 has been shown to be a normal component of plasma (Shashoua et al., 1984).

As mentioned previously, the initial evaluation of a suspected stroke patient is time-consuming and usually occurs after permanent brain damage has occurred. Therefore, it would be advantageous to develop an assay to rapidly detect or quantitate a protein that is uniquely expressed at the early onset of stroke, and that appears in easily obtainable and rapidly assayed biological samples such as peripheral blood.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that a particular isoform of protein kinase C, namely the gamma isoform (abbreviated PKCγ or PKCg herein), appears in the peripheral blood very quickly after an ischemic event in the central nervous system (CNS).

The present invention describes a procedure for the rapid and accurate diagnosis of CNS and especially brain ischemia resulting from stroke, transient ischemia attacks (TIAs), head trauma, myocardial infarction, or other events resulting in interrupted spinal or cranial blood flow. The procedure can be easily performed by ambulance or emergency room personnel and can be performed with a venous (peripheral) blood sample and an assay kit as described herein.

The present invention provides an assay for the rapid detection of PKCg, a protein expressed only as a result of the type of cell damage that is characteristic of stroke, TIAs, head trauma, myocardial infarction, or other events or "insults" resulting in interrupted blood flow to structures of the CNS. Other events that might lead to such interrupted blood flow to the CNS include medical interventions, such as surgical procedures, surgical or physical errors, anesthesia, and therapeutic or pharmaceutical interventions.

According to the present invention, it can be determined whether a mammalian subject has suffered an ischemic event by detecting, in the subject or in a biological sample obtained from said the subject, the presence of PKCg outside (peripheral to) the CNS.

According to the preferred embodiments of the invention, a peripheral blood sample is drawn from the affected individual then analyzed for the presence of PKCg. Preferably, a venous blood sample is contacted with a PKCg binding partner, such as an anti-PKCg antibody specific for a PKCg epitope under conditions suitable for the formation of an anti-PKCg antibody/PKCg binding complex. Detection of the presence of PKCg in the blood sample, e.g., by detection of the antibody/PKCg complex, indicates a CNS ischemic injury.

In addition to determining whether or not damage to the CNS has occurred, i.e., by the presence or absence of PKCg in a blood sample, the present invention also provides for quantitation of the PKCg to determine the severity of the ischemic event. The quantity of PKCg in the original peripheral blood sample is directly correlated to the amount or severity of central nervous system damage.

Where immunosorbant methods are used, the anti-PKCg antibody most preferably includes a fluorescent tag or label for rapid detection using methods well known in the art. In a preferred antibody-based method, after the blood sample is contacted with the anti-PKCg antibody, the solution is passed over a column such as, for instance, a DEAE/Sepharose column in order to separate the binding complexes from cells and larger proteins in the sample. According to this preferred method, the unbound (free) labeled anti-PKCg antibody will bind to the column, and the

anti-PKCg antibody/PKCg complexes will flow through. Elimination of unbound labeled antibody from the sample improves the quantitative aspects of the method, making it possible to gauge progress or degree of ischemic insult to CNS tissues. The column may be washed one or more times to remove any of the larger complexes that may have bound. After collection of the flowthrough solution, the presence of any anti-PKCg antibody/PKCg complexes may be detected using methods well known in the art for detection of such complexes.

In another embodiment, the present invention provides a method for determining whether or not damage has occurred to the CNS as a result of a stroke or other event such as a transient ischemia attack, head trauma, myocardial infarction, or other event resulting in disruption of cranial blood flow through the use of a sandwich antibody assay. According to the present invention, a first anti-PKCg antibody (also referred to as the capture antibody), raised against a specific epitope on the PKCg protein, is immobilized on a solid substrate. A sample of venous blood, drawn from an individual suspected of having a stroke or other ischemic event involving damage to the CNS, is contacted with the first antibody immobilized on the solid substrate to form an antibody/PKCg binding complex. A subsequent wash step may be employed to remove any unbound PKCg protein from the rest of the sample mixture. A second anti-PKCg antibody specific for a different PKCg epitope than the first antibody and being detectably labeled, e.g., with a fluoresceinated tag, bioluminescent tag, colorimetric tag, or colored bead, is then contacted with the PKCg protein bound to the first antibody immobilized on the solid substrate under conditions suitable to form a binding complex with PKCg. A washing step may be included subsequent to this second binding step to remove any unbound labeled anti-PKCg antibody. Detection and/or quantitation of PKCg in the original sample may be conducted by methods well known in the art such as, for instance, fluorimetry, epifluorescence microscopy, confocal scanning laser microscopy, luminometer, or colorimetric assay.

In another embodiment of the sandwich antibody assay, the solid substrate on which the first anti-PKCg antibody is immobilized preferably is a magnetic bead, the surface of a well of a microtiter plate, or a piece or strip of solid substrate material that can be used as an assay strip. Assay strips of the invention are preferably flat rectangular strips of a plastic, nylon, or other solid material which contain an immobilized anti-PKCg antibody (capture antibody), which may be dipped into a sample and assay solutions, either manually or robotically, or to which a sample and assay solutions may be applied.

In still another embodiment of the sandwich antibody assay of the invention, one or both of the anti-PKCg antibodies used in the assays of the invention may be a full-length major class or subclass of immunoglobulin, a functional fragment thereof (such as Fab, $F(ab')_2$, Fv), a hybrid

antibody (such as a chimeric or humanized antibody), or a relatively small recombinant antibody molecule (such as a single chain antibody (scFv) or a diabody.

In another embodiment, a PKCg binding partner is a polypeptide other than an antibody and is used in place of an anti-PKCg antibody molecule in the assays, compositions, and kits of the invention.

In yet another embodiment, a kit is provided for detecting or measuring PKCg in a blood sample or other solution. A preferred kit of the invention comprises a first (capture) PKCg binding partner immobilized on an assay strip, the wells of a microtiter plate, or on beads or particles; a second PKCg binding partner that also contains a detectable label or component of a system to generate a detectable signal; and instructions that indicate how to use the kit to detect or measure PKCg in a blood sample or other solution.

DEFINITIONS

As used herein, the term "ischemic event" refers to any potentially harmful episode resulting from temporary or permanent decrease or elimination of blood flow to tissues, particularly, with respect to the present invention, any event or physiological occurrence that interrupts blood flow to the CNS, especially cranial blood flow, and leads, without treatment, to damage in the CNS. Specific types of ischemic events include stroke, transient ischemic attacks, head trauma, myocardial infarction or other events resulting in interrupted blood flow to the CNS. Events resulting in such interrupted blood flow may be naturally occurring or unexpected (i.e., as in the case of stroke or accidental trauma) or may be the result of interventionary procedures, such as surgical procedures, surgical or physical mishaps, anesthesia, and therapeutic and pharmaceutical interventions, causing (undesired) interruption of blood flow, as a side effect.

As used herein, a "PKCg binding partner" is any molecule that binds PKCg, including any polypeptide or immunoglobulin molecule, or fragment thereof, that specifically binds PKCg.

The term "specificity" refers to a binding partner or moiety having a higher binding affinity for one target over another. The term "PKCg specificity" and the phrase "specifically binds PKCg" refer to a PKCg binding partner having a higher affinity for PKCg as compared with another target, such as a serum protein (e.g., bovine serum albumin (BSA), human serum albumin (HSA)) or gelatin.

An "anti-PKCg antibody", or simply "PKCg antibody" or "PKCg immunoglobulin" is a PKCg binding partner that contains at least one antibody binding site for an epitope of PKCg or that contains one or more complementarity-determining regions (CDRs) which bind to PKCg. PKCg antibodies include any of the five full-length mammalian immunoglobulin classes (i.e., IgG, IgM, IgA, IgE, IgD) and subclasses thereof that bind PKCg. A PKCg antibody may also be a functional

fragment of a full-length immunoglobulin class that binds PKCg, such as a Fab, $F(ab')_2$, and Fv fragment, as well as PKCg binding molecules that may be produced by protein engineering or recombinant DNA, such as a chimeric immunoglobulin molecule, which comprises a binding domain or CDRs of one immunoglobulin fused to a portion of another immunoglobulin or other polypeptide; a humanized immunoglobulin molecule, which comprises the CDRs from a non-human antibody molecule inserted into the framework of a human antibody molecule; a single chain antibody (scFv); or a diabody (see, e.g., Holliger et al., *Proc. Natl. Acad. Sci. USA*, *90*: 6444-6448 (1993)). In addition, PKCg antibodies useful in the compositions and methods of the invention may be polyclonal antibodies (e.g., serum containing anti-PKCg antibodies from a mammal inoculated with PKCg antigen) or monoclonal antibodies produced by hybridoma technology (see, e.g., Milstein, *Scientific American*, *243*: 66-74 (1980).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows PKCg dose-dependent percent fluorescence increase, from a sandwich-type immunosorbent assay measuring PKCg captured on anti-PKCg beads, detected with anti-PKCg-FITC.

Figure 2 shows PKCg dose-dependent fluorescence in mean fluorescence units, from a sandwich-type immunosorbent assay measuring PKCg captured on anti-PKCg coated beads, detected with anti-PKCg-FITC.

Figure 3 shows a PKCg standard curve generated from the data depicted in Figure 2.

Figure 4 shows differences in detected level of PKCg in untreated (#1 - #3), sham treated (#4 - #6), and ischemic (#7 - #11) subjects from tested peripheral blood samples. Levels of PKCg in brain extract are also shown (#12, #13).

Figure 5 shows differences in PKCg levels among untreated, sham operated, and ischemic subjects detected in peripheral blood, and in brain extract, where PKCg amounts are read from the standard curve (see Figure 3).

Figure 6 shows PKCg levels (ng/ml) in blood samples collected from normal (no operation) rats, sham operated rats, and rats subjected to middle cerebral artery occlusion at 30 minutes, 90 minutes, and 3 hours after occlusion. Levels of PKCg are the mean value for three rats determined using a sandwich assay in microtiter plates.

Figure 7 shows PKCg levels (ng/ml) in blood collected from rats subjected to middle cerebral artery occlusion at 0 minutes, 15 minutes, 30 minutes, 120 minutes, 180 minutes, 6 hours, and 24 hours after occlusion. Blood collected approximately 30 minutes prior to occlusion served as a reference control. Levels of PKCg are the mean value for three rats determined using a sandwich assay in microtiter plates.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention describes a rapid and accurate method for detecting damage to the CNS caused by an ischemic event, which may result from stroke, TIAs, head trauma, myocardial infarction or other events resulting in interrupted cranial blood flow. Traumatic events, such as stroke, cause a series of biochemical events as cell damage progresses. One of these biochemical events is the production of the gamma isozyme of protein kinase C (PKCγ or PKCg), which is expressed in the CNS as a result of an ischemic event.

PKCg is a specific marker for CNS tissue that is activated by certain fatty acid moieties such as arachidonic acid (AA). In addition, AA is the fully competent precursor of the oxygenase pathways. In the CNS, noteworthy pathways are cyclooxygenase, which produces prostaglandins, thromboxanes, and prostacyclins, and lipoxygenases (including 5-HETE, 12-HETE, LTC_4, LxA), which are also activated during ischemia and CNS injury. PKCg has been shown to be activated during and immediately following ischemia and traumatic CNS injury. PKCg is specific for the CNS and is not normally found in peripheral blood. The discovery of the present invention follows the observation that the blood brain barrier is often compromised as a result of an ischemic event, leading to appearance of PKCg in peripheral blood. It has further been discovered that the presence of PKCg in peripheral blood occurs and can be detected in peripheral blood almost immediately following an ischemic event, and, most importantly, within the critical time window in which diagnosis and treatment of ischemic injury can prevent permanent damage to CNS tissue.

Accordingly, the detection of PKCg in a peripheral blood sample is an early diagnostic indicator of an ischemic event such as stroke, TIA, head trauma, or myocardial infarction, making early and effective treatment possible. Moreover, the amount of PKCg detected in a sample is proportional to the degree of the damage or insult to normal tissues, and therefore quantitative assay of the PKCg in a sample also is indicative of the extent of the trauma to the CNS.

Insult to brain tissue from stroke, TIAs, head trauma, myocardial infarction, or other events resulting in the disruption of cranial blood flow share a common pathway of cell-mediated damage which originates with the activation and eventual release of PKCg from neural tissues. PKCg is normally found only in the CNS and is not known to be localized to any other tissues. In the event of ischemic damage, there is an accompanying breakdown of the blood brain barrier, which results in the release of PKCg from its normal location in the brain into venous blood.

The methods of the present invention are especially contemplated to benefit human subjects, but they are of course suitable for any mammalian subject in which the presence of a PKCg isoform signals CNS damage.

Any means of detection for the PKCg marker is suitable, and any known means of detecting a specific protein in a sample may be employed. Preferably, PKCg is detected in a sample of blood from a mammalian subject, by contacting the sample with a binding partner for PKCg, that is, a peptide, immunoglobulin, small molecule, or other moiety capable of forming an association complex with PKCg. Most preferably, the PKCg in a sample is detected using antibodies specific for PKCg. Numerous PKCg antibodies are commercially available that can be used in the methods, compositions, and kits of the invention to detect or measure PKCg from humans and other mammals. For example, monoclonal antibodies that bind to epitopes of human PKCg are produced by a number of hybridomas on deposit with the American Type Culture Collection (ATCC), including ATCC accession no. 1021866 (commercial source: Transduction Laboratories, Lexington, KY), ATCC accession no. 1022645 (commercial source: Fitzgerald Industries International, Inc., Concord, MA), ATCC accession no. 1022853 (commercial sources: Calbiochem Novabiochem International, La Jolla, CA; Oncogene Research Products, Cambridge, MA). The anti-PKCg monoclonal antibody produced by ATCC accession no. 1025435 (commercial source: Sigma Chemical Co., St. Louis, MO) reacts with the carboxy terminal region of PKCg and may be used in the methods, compositions, and kits of the invention to detect PKCg in samples from various species. Other anti-PKCg antibodies, which react with PKCg from one or more sources, are also commercially available, for example, the polyclonal rabbit antiserum (commercial source: Calbiochem) and the monoclonal antibody (commercial source; Transduction Laboratories) used the Examples (*infra*). In addition, PKCg and fragments thereof may be isolated from tissues from humans and other species, produced by recombinant DNA techniques (e.g., commercial source: A. Ullrich, Milwaukee, WI), or chemically synthesized by standard methods known in the art. Such PKCg or fragment thereof may then be used as an immunogen in any of a variety of methods known and available in the art for producing polyclonal, monoclonal, or recombinant forms of antibodies which bind to PKCg and which may also be used in the various methods (e.g., sandwich assays, *infra*), compositions, and kits described herein.

For detection or measurement of PKCg levels in a sample, fluorescently labeled antibodies are most preferred. Many other means of detecting PKCg directly or detecting a complex of PKCg with another moiety are known, including gas chromatography mass spectroscopy, thin layer chromatography, hydroxyl apatite chromatography, high pressure liquid chromatography, colloidal gold immunolabeling read by electron microscopy, enzyme-linked immunosorbent assays (ELISAs), radioactively labeled tags or antibodies specific for PKCg read using a scintillation counter, bioluminescently labeled antibodies read on a colorimeter, etc.; however, most of these methods require several hours or even days for sample preparation and/or measurement of the signal, making them inferior to sensitive fluorescence-based assays such as described in the

examples (*infra*). Also, the apparatus necessary for detection in some cases (e.g., mass spectrophotometer, electron microscope) would not fit inside an ambulance, making performance of the assays by emergency medical personnel impossible before the patient suspected of suffering an ischemic event is brought to a hospital.

PKCg binding partners used to detect or measure (quantitate) PKCg in a blood sample according to the invention may be used in solution or immobilized on the surface of any of a variety of solid substrates. Solid substrates to which a PKCg binding partner may be immobilized for use in the methods and compositions of the invention include, but are not limited to, magnetic or chromatographic matrix particles, the surface of a well of an assay plate (such as a microtiter assay plate), and pieces of a solid substrate material, such as pieces or strips of plastic, nylon, wood, paper, or other solid material, which may be dipped into or otherwise placed in contact with a sample or assay solution.

The PKCg binding partner may be immobilized to the surface of a solid substrate by adsorption (non-covalent adherence) or by covalently linking the binding partner directly to the solid surface or to a linker molecule that permits the binding partner to be tethered to the solid substrate. Adsorption of a PKCg binding partner to the surface of the wells of a microtiter plate permits the detection or assay for PKCg to be carried out using the various semi-automatic or automatic microtiter plate readers used in standard ELISA procedures. Such devices are particularly useful for assaying large numbers of samples for PKCg.

Preferably, PKCg in a blood sample from an individual is detected using a sandwich assay in which a first PKCg binding partner (or capture molecule) serves to capture (bind) PKCg in a sample, and a second binding partner detects the complex formed by the PKCg bound to the first capture binding partner. Preferably, such a sandwich assay of the invention may be carried out in solution, in wells of a microtiter plate as used in standard ELISA protocols, or on assay strips as described herein. In the ELISA-type sandwich assay protocol, a first PKCg binding partner is adsorbed to the wells of a microtiter plate. Unbound PKCg binding partner may be removed from the well by inverting the plate and tapping out the solution and/or by washing the well with a buffer solution. To prevent non-specific binding by potentially interfering substances, any of the various blocking proteins or agents used in ELISA protocols, such as bovine serum albumin, may also be applied to the well to block any areas on the surface of the well that are not occupied by the first PKCg binding partner. After the PKCg binding partner is immobilized on the surface of the well and unbound material removed, a sample known or suspected of containing PKCg is added to the well. Unbound material may then be removed or washed away. A second PKCg binding partner that binds PKCg at a different site than the immobilized first PKCg binding partner is then added to the well to detect the presence of PKCg bound in the well. This second (detection and/or

quantitation) PKCg binding partner preferably carries a detectable label that provides or is capable of producing a detectable signal. Such detectable labels include any of a variety of labels known in the art that may be used to detect specific binding, for instance an antibody binding to an antigen. Frequently used detectable labels include but are not limited to fluorescent labels, radioactive labels, biotin, streptavidin, bioluminescent or chemiluminescent labels, and enzymes that are capable of reacting with colorigenic substrates. The signal generated by such systems may be readily detected or measured by an appropriate instrument that reads microtiter plates. Colorigenic labels may also be detected visually where detection of PKCg, alone, is sufficient as in an emergency situation to simply detect that an ischemic event has occurred.

In another method of the invention, PKCg is detected using an assay strip to which a PKCg binding partner is adsorbed or covalently linked. Such assay strips provide a convenient means to detect or measure PKCg in a sample. As with the microtiter plates described above, a first PKCg binding partner is immobilized on the strip and unbound material removed, e.g., by rinsing. The strips containing immobilized PKCg binding partner may also be rinsed to remove unbound material and/or incubated with a blocking agent, such as bovine serum albumin, to reduce nonspecific binding by potentially interfering molecules. According to the invention, an assay strip containing the immobilized PKCg binding partner is contacted with a sample suspected of containing PKCg, for example, by manually or robotically dipping the strip into the sample. The assay strip then may optionally be rinsed to remove unbound material, and then dipped or otherwise contacted with a second PKCg binding partner that carries any of the types of detectable labels described above. If necessary, the assay strip is further dipped or contacted with any reagents that are necessary to develop or generate a measurable signal on the strip, such as a colorigenic substrate. The assay strip is then observed visually or read by an appropriate signal detection instrument, as described above, for evidence of PKCg in the sample.

In an alternative procedure for the sandwich assays described above, a sample known or suspected of containing PKCg may be first mixed with the quantitation PKCg binding partner to form a complex between PKCg in the sample and the quantitation antibody, and the mixture then added to a well or other solid substrate containing the capture antibody for detection (see, Example 5, *infra*).

The assays for PKCg according to the invention may be carried out on large and small scales depending on the volume of the sample or number of samples to be tested for the presence of PKCg. Preferably, a single assay of the invention requires no more than about one-half of a milliliter (ml) of peripheral or venous blood, more preferably about 50 to about 400 microliters (μl), and even more preferably less than about 50 microliters (μl) of peripheral blood to detect or measure PKCg in a blood sample. Most preferably, an assay of the invention requires only one to

several drops of blood, for example as obtained from a pin puncture of a digit or an ear, to detect or measure PKCg in the blood sample of mammalian subject.

It is understood that the assays described herein may be used to detect or measure PKCg in any liquid sample containing PKCg, including, but not limited to, blood samples such as samples of whole blood or blood fractions such as blood plasma, as well as aqueous solutions containing PKCg.

To accurately measure (quantitate) the amount of PKCg in a blood sample, a standard curve may be generated using an assay of the invention. For example, an assay described herein may be carried out on a blood sample and on a series of solutions containing known concentrations of PKCg. The signal obtained for each solution of known concentration of PKCg is then used to construct a standard curve that correlates the magnitude or intensity of a signal with the amount or concentration of PKCg (such as in Example 1, *infra*). The signal from a sample of unknown PKCg content may then be read on the standard curve to determine the amount of PKCg present in the sample.

The materials necessary for detection of PKCg in a sample of venous blood are conveniently assembled into a kit, so that personnel treating or transporting a trauma victim can determine quickly whether an ischemic event has been suffered by their patient and then begin treatment immediately to prevent further damage to the brain and/or central nervous system. One kit useful for such diagnoses is based on PKCg binding and is capable of providing multiple levels of detection and quantitation. The level of detection provides quantitative assessment of ischemic damage based on calibration of fluorescently tagged antibodies to PKCg detected in venous blood. A preferred kit of the invention comprises a first (capture) PKCg binding partner immobilized on a solid substrate material, such as an anti-PKCg antibody immobilized on an assay strip, the wells of a microtiter plate, or on beads or particles; a second PKCg binding partner containing a detectable label or component to produce a detectable signal; and instructions that indicate how to use the kit to carry out the PKCg assay. Beads, assay strips, or microtiter plates with immobilized PKCg binding partner in kits of the invention may be packaged in a variety conditions, including a dry, unhydrated state; a freeze-dried or dehydrated state; or a hydrated state in a physiological buffer solution. Other solutions for washing or signal generation may also be included in the kits of the invention. In another preferred embodiment, the kits of the invention comprise a capture PKCg binding partner immobilized on a solid substrate, such as a bead, an assay strip or a microtiter plate, which has also been pre-treated to prevent interfering signals by non-specific binding to the substrate.

In addition to providing rapid diagnosis of an ischemic event by emergency and medical personnel, the methods and kits described herein also may be used to monitor PKCg levels as part

of a routine checkup procedure or to monitor recovery from ischemic injury to CNS tissues. The nature of the methods and kits described herein makes it possible to perform diagnosis and monitoring of ischemic events in many environments, for instance ambulances or other mobile medical facilities, laboratories, hospitals, emergency rooms, or even homes, sanitoria or other private facilities.

Examples illustrating the detection of PKCg as a means of detecting an ischemic event in accordance with this invention will be set forth below. The specific materials and parameters included in the following examples are intended to illustrate the practice of the invention, and they are not presented to in any way limit the scope of the invention.

EXAMPLES

Example 1: Measurement of PKCg in Ischemic Rats

The animal model of middle cerebral artery occlusion (MCA-O) used in this example relies on an intraluminal filament technique in the rat (see, Zhao et al., 1994a,b). Rats were anesthetized with halothane in a nitrous oxide/oxygen mixture and the carotid artery exposed. A chamfered monofilament suture (3/0) was introduced into the ligated carotid artery, past the bifurcations of the external and common carotid, and the internal carotid and the pterygopalatine artery, into the intracranial circulation, where it lodged in the narrow proximal anterior carotid occluding the middle cerebral artery. The wound was then sutured and the animal allowed to recover from anesthesia. Sham operations were also performed on some animals (sham operated controls) in which the animals underwent all of the surgical procedure except for the step used to occlude the artery. After 90 minutes of occlusion, neurologic function was assessed. The animal was then decapitated and trunk blood collected into a container containing 1.5 mg/ml EDTA which was frozen until analysis.

Blood samples were collected from three groups of animals: (1) untreated controls (n=3); (2) sham operated controls (n=3); and (3) ischemic animals (n=5). Neurologic function was assessed and graded from normal to severe impairment. The assessment scale is set forth in Table 1.

Table 1 – Neurological Impairment in Control and Induced Rats		
Neurological Impairment Assessment	Severity	Score
No observable deficit	Normal	0
Forelimb flexion; grip reflex present in both forepaws	Moderate	1
Forelimb flexion; grip reflex present in contralateral forepaw only	Severe	1.5
Forelimb flexion; grip reflex absent in both forepaws	Severe	2
Forelimb flexion; grip reflex absent in both forepaws, plus circling toward paretic side	Severe	3

The neurological impairment scores of the control groups were 0 in both cases; the average impairment score of the ischemic group was 1.9.

These results demonstrate that the middle cerebral artery occlusion (MCA-O) procedure resulted in severe neurological damage, whereas neither the untreated nor sham operated control groups showed any signs of neurological damage. It was then demonstrated that the neurological impairment scores positively correlated with the amount of PKCg measured from peripheral blood samples of ischemic animals but not with sham operated or untreated control animals.

The assay of PKCg in samples of venous blood used an immobilized "capture" antibody specific for one epitope on the PKCg protein and a second "quantifying" antibody, which included a fluorescent marker attached thereto and which recognized a different epitope of PKCg than the capture antibody.

In the present example, the capture antibody was a polyclonal rabbit antiserum (Calbiochem; Catalog No. 539529, used at 1:500 dilution) recognizing the amino acid sequence extending from amino acids 306-318 of the PKCg protein. The quantifying antibody was a monoclonal antibody (Transduction Laboratories; Catalog No. P20420, used at 1:200 dilution) reactive with amino acids 499-697 at the carboxyl end of the PKCg protein. The quantifying antibody was fluoresceinated using a FluoroTag kit (Sigma Chemical). A brain extract sample was used as a brain-derived PKCg positive control as supplied by the antibody manufacturer (Transduction Laboratories; Catalog No. B30900).

The capture antibody was immobilized according to the manufacturer's instructions on tosyl-activated magnetic Dynabeads (Dynal Catalog No. 142.03,1402.4). These materials allowed ease of imaging and quantification of the fluorescence using a confocal scanning laser microscope (Nikon PCM200 equipped with Fluorescein filters).

Paramagnetic beads coated with capture antibody and contained in a small test tube were placed in a holder containing a magnet, which attracted and pelleted the beads. Binding of the

capture antibody to the magnetic beads was tested, after rinsing the beads in buffer, by resuspending in goat anti-rabbit IgG-fluorescein antibody solution which binds selectively to rabbit antibodies. Bright fluorescence observed by laser microscope confirmed binding of the capture antibody to the magnetic beads.

Once the capture antibody binding to the magnetic beads was confirmed, a standard curve was generated using known concentrations of PKCg (obtained from Calbiochem; Catalog No. 539627): 0 pg/ml, 1 pg/ml, 10 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, and brain extract. The various concentrations of PKCg were incubated with the beads and complexed with the capture antibody. Then the quantifying antibody with the fluoresceinated label was added, and complexed fluorescence measured using intensity-measuring software on the confocal microscope. Fluorescent intensity measurements from 3 separate bead preparations are shown in Figure 1. Measurement bias was minimized or eliminated by writing a macro for the measurement software, whereby a fixed-diameter circle was used to delineate an area for intensity measurement in at least 200 beads per PKCg concentration (i.e., n=200). Only beads that were in focus within the image plane were measured for fluorescence intensity. As seen in Figure 1, the fluorescence intensity, taken as a mean of >100 beads per dose of PKCg, increases in a dose-dependent fashion. The data in Figure 1 are expressed as percent (%) increase in specific fluorescence, which was derived from subtracting the baseline fluorescence from the increase in fluorescence due to anti-PKCg-FITC antibody binding to the PKCg.

In order to establish a standard curve from the dose response curves derived from increasing concentrations of PKCg, the fluorescence intensity was again measured from beads using the confocal microscope, however the fluorescence intensity measurements were left as fluorescence units, as seen in Figure 2. A simple curve fit was used to establish the standard curve of fluorescence intensity due to increasing concentrations of PKCg. The standard curve is shown in Figure 3. The standard curve of PKCg-induced fluorescence was used to establish the levels of PKCg found in trunk blood of surgically treated rats. The PKCg concentrations were read directly from the standard curve.

Results from the blood samples demonstrate that the sandwich technique is sensitive enough to detect significant increases in the PKCg in peripheral blood samples following the MCA-O procedure. As seen in Figure 4, blood from the untreated and sham operated control animals showed significantly less fluorescence in every case when compared to the ischemic animals. In addition, brain extract controls also showed significantly higher fluorescence levels than the untreated and sham controls.

When fluorescence levels are converted to picograms (pg) of PKCg per ml using the standard curve shown in Figure 3, the difference in surgical treatments on PKCg levels becomes

even more pronounced, as illustrated in Figure 5. Figure 5 shows a dramatic increase in the detectable levels of PKCg following the ischemic event. Levels of PKCg detected in blood collected outside of the brain following MCA-O are more than 50% higher than levels of PKCg derived from brain extract.

PKCg is activated and mobilized at the time of the ischemic event. PKCg is released from damaged cells where it initiates several damage cascades including the arachidonic acid and lipoxygenase cycles. PKCg also initiates glutamate-induced excitoxicity cascades. One of the events which rapidly follows the ischemic episode is at least a transient breakdown of the blood-brain barrier. At 90 minutes following the MCA-O procedure, the trunk blood samples contained levels of PKCg that are approaching 6 ng/ml as determined from the antibody sandwich technique using the magnetic beads.

This ischemic level is at least ten times the limit of PKCg detection (600-1000 pg/ml) that has been shown using this method. This is an acceptable ratio of detection to actual measurements, which confirms the utility of the sandwich assay format to monitor ischemic events measured from a peripheral blood sample. The PKCg detection can also be performed visually from confocal images of beads bound with a fluorescent quantifying antibody.

Example 2: Detection and Quantitation of PKCg in a Venous Blood Sample Using Multi-well Microtiter Plate Format

Experience with the sandwich-type assay described above has led to a general protocol for the assay of this invention using a 96-well microtiter plate format.

1. Dilute capture antibody to 1:500 using a 0.1M Na-phosphate buffer (pH 7.4) containing 1% BSA (Fraction V, Sigma Chemical, St. Louis, MO) and 0.1% Tween-20 detergent (Sigma Chemical). To make Na-Phosphate buffer use 2.62g NaH_2PO_4 (MW= 137.99) and 14.42 grams $Na_2HPO4{\cdot}2H_2O$) (MW=177.99) in 1 liter distilled water and adjusted pH to 7.4. Add capture antibody to plastic 96-well microtiter plate (or other vessel) and incubate at 37°C for at least 2 hr.

2. Rinse plate with the PBS/Tween/BSA 2-3 times to remove unbound antibody from plate wells. Minimal changes to this procedure will not affect antibody binding.

3. To prevent non-specific binding, block wells with 200 µl of 10% fetal calf serum in PBS for at least 2 hr. It is possible to substitute 10% horse serum or 3% BSA (Fraction V, Sigma Chemical). Rinse 2-3 times with the PBS/Tween/BSA solution mentioned above.

4. Add standards or samples to the well(s), dilute in PBS/10%serum (50-100 µl sample size) and let stand for 30 min.-1 hr. at 37°C. Wash 4 times using PBS/Tween/BSA.

5. Add quantifying antibody and incubate for 30 min. Wash 4 times using PBS/Tween/BSA.

6. Read fluorescence on a fluorimeter, such as a CytoFluor II from Perseptives Instruments. Fluorescein optics were used with an excitation of 450 nm, and emission at 515 nm.

An alternate and more preferred protocol for the sandwich-type assay using a multi-well plate format is also provided in Example 5, which was particularly useful for assaying blood samples from the middle cerebral artery occlusion (MCA-O) studies in rats described in Examples 3 and 4.

Example 3: Time Course of Detection of PKCg in Rats Beginning 30 Minutes After Ischemic Event

Plasma PKCg levels were determined at various times between 30 minutes and three hours after blocking middle cerebral artery blood flow in rats.

The middle cerebral artery of rats weighing 300-325 grams each were occluded by insertion of a catheter with a blunt end into the left carotid artery and threading it up the point of bifurcation so that it blocked (occluded) blood flow to the portion of the brain supplied by the middle cerebral artery. Sham operations were performed on some of the animals in which the animals underwent all of the surgical procedure except that the blocking catheter was not placed in the artery (i.e., surgery with no arterial occlusion). Blood (2 ml) was collected before occlusion and at 30, 90, and 180 minutes after occlusion was begun, with three rats per time point. The blood samples were centrifuged at 2000 rpm for 15 minutes in a refrigerated centrifuge to obtain plasma, which was then frozen and stored at -70°C. Plasma was also prepared for analysis from three sham-operated rats and three untreated rats, which were included as controls. Plasma PKCg levels were measured by following the immunochemical sandwich method using the multi-well microtiter plate format as described in Example 5 (*infra*).

Figure 6 shows that middle cerebral artery occlusion causes an increase in plasma PKCg levels at each of the collection times. PKCg levels in the untreated and sham operated samples were on the order of 1-3 ng/ml, whereas PKCg levels increased to 40-50 ng/ml for each of the time points for samples from animals subjected to the artery occlusion (Figure 6 data presented as mean and standard error for an n of 3).

Example 4: Time Course of Detection of PKCg in Rats Beginning 15 Minutes After Ischemic Event

Plasma PKCg levels were determined at various times between 15 minutes and 24 hours after permanently blocking middle cerebral artery blood flow in rats.

This experiment was conducted as described for Example 3 (see above), except that the maximum post occlusion blood collection time was increased from 3 to 24 hours. Blood collected approximately 30 minutes before occlusion (baseline) served as a reference control. Plasma PKCg levels were measured in samples by following the immunochemical sandwich method using the multi-well microtiter plate format as described in Example 5 (*infra*).

Figure 7 shows that middle cerebral artery occlusion causes a sharp increase in plasma PKCg levels and that PKCg levels remained elevated over baseline at each of the collection times. The PKCg level in the baseline sample was on the order of 1ng/ml, increasing to a maximum of 67 ng/ml at 30 minutes post-occlusion. The PKCg level at 15 minutes was only one-fifth (12 ng/ml) the 30 minute level. The levels decreased gradually to 15 ng/ml by 24 hours. (Figure 7 data presented as mean and standard error for an n of 3).

Example 5. An Alternate, Preferred Protocol for Detection and Quantitation of PKCg in Venous Blood Samples Using Multi-well Plate Format

This Example demonstrates an alternate protocol to that in Example 2 for using a sandwich-type assay in a multi-well plate format for the detection and quantitation of PKCg in a venous blood sample. This protocol was particularly useful for assaying PKCg in blood samples from the middle cerebral artery occlusion studies in rats described in Examples 3 and 4.

1. Preparation of Multi-well Plate

A 0.1 M Borate buffer (pH 9.5) was prepared by diluting 6.183 g H_3BO_3 in 1 liter of distilled H_2O and adjusting the pH with 5 M NaOH. A high protein binding plate (Nunc Maxisorb, Nalge Nunc, Denmark) was rinsed 3 times (5 minutes per rinse) with the 0.1 M Borate buffer (pH 9.5) using a fluid handling robot (Quadra 96 fluid handling robot, supplied from Tomtec, Inc., Hamden, CT).

2. Immobilization of Capture Antibody on Multi-well Plate

A polyclonal anti-PKCg capture antibody (Calbiochem, Catalog No. 539529) was diluted 1:1000 using 0.1 M Borate buffer (pH 9.5). This polyclonal anti-PKCg reacts with (binds) the region of amino acids 306-318 of PKCg. Cross reactivity of this antibody with other PKCg isozymes was determined to be less than 0.1% by ELISA. Plates were incubated at 37°C for 2-4 hours to insure stable attachment of the capture antibody to the plastic wells. This method provided immobilized capture antibody that was stable enough after incubation to withstand rinses of 10 mM

Phosphate Buffered Saline (PBS, pH 7.4) using the fluid handling robot (200 µl into the well, 200 µl removed from the well).

3. Fluoresceinate Labeling (Quantitation) Antibody

A monoclonal anti-PKCg antibody, which binds PKCg in the region of amino acids 676-689 of the carboxy terminal substrate binding region of PKCg was used as the labeling (quantitation) antibody (Transduction Laboratories, Louisville, KY; P82820). This anti-PKCg was fluorescein-labeled using a Fluoro Tag FITC conjugation kit (Sigma Chemical, St. Louis, MO; Catalog No. FITC-1). An excess of fluorescein isothiocyanate (FITC) was dissolved in 0.1 M Na carbonate (pH 9.0) buffer according to manufacturer's specifications. The antibody was conjugated to the fluorescein in a reaction volume of 0.25 ml for 2 hours at room temperature with constant mixing. The resulting FITC-anti-PKCg antibody conjugate and unbound FITC mixture was applied to a Sephadex G30 chromatographic column. The column was eluted with 2.5-3.0 ml of PBS/TWEEN/BSA (pH 7.4) buffer, which is PBS (pH 7.4) containing 0.1 % polyoxyethylenesorbitan monostearate (TWEEN 60, Sigma Chemical) and 0.1% bovine serum albumin Fraction V (BSA, Sigma). Fractions of 300 µl were collected from the G30 column into a 96 well plate and then read on a CytoFluor II fluorimeter (Perseptives Instruments, Framingham, MA) using an excitation wavelength of 480 nm and an emission wavelength of 515 nm. The FITC-anti-PKCg antibody eluted in the void volume and constituted a first fluorescence peak. The unbound FITC was retained and eluted as a later second peak of fluorescence. The FITC-anti-PKCg antibody was diluted to a volume that represented approximately 1500 fluorescence units in 300 µl using the PBS/TWEEN/BSA buffer (pH 7.4), described above. The final dilution for this FITC-anti-PKCg preparation was to 30 ml in the PBS/TWEEN/BSA buffer (pH 7.4).

4. PKCg Standard Curve

A recombinant human PKCg (Calbiochem, Catalog No. 539637) was used to construct a standard curve of PKCg concentrations extending from 0 to 600 ng PKCg/ml. PKCg was diluted in PBS/TWEEN/BSA buffer (pH 7.4). The conditions for maximal sensitivity of the assay were determined experimentally. The standard sample protocol combined 100 µl of a PKCg standard sample, 250 µl of FITC-anti-PKCg antibody, and 650 µl of PBS/TWEEN/BSA buffer (pH 7.4). Each mixture was incubated for 30-60 minutes at 37°C with constant mixing. Following incubation of the standards with the FITC-anti-PKCg, a volume of 125 µl of each mixture was placed in 8 wells of a multi-well plate containing capture antibody and incubated for 30 minutes at 37°C. Fluorescence readings from the 8 sample repeats were then read in the fluorimeter using filters for FITC, excitation wavelength of 450 nm, and emission wavelength of 515 nm. Standard curves were constructed from the readings for each standard PKCg sample.

5. Venous Blood Samples from MCA-O-Treated Rats

Samples of plasma from the peripheral blood of rats subjected to the middle cerebral artery occlusion (MCA-O) procedure (see, Examples 3 and 4 above) were prepared using the same protocol described above for preparing the PKCg standard samples: 100 µl of each plasma sample was added to 250 µl of FITC-anti-PKCg antibody and 650 µl of PBS/TWEEN/BSA (pH 7.4). Each sample mixture was then incubated for 30-60 minutes at 37°C as above. A volume of 125 µl of each sample mixture was placed in 8 wells of a multi-well plate containing immobilized capture antibody as described above and incubated for 30 minutes at 37°C. Fluorescence readings of each sample were collected in the fluorimeter using filters for FITC, excitation wavelength of 450 nm, and emission wavelength of 515 nm. The levels of PKCg in each sample were established from reading levels of PKCg from standard curves as described above. Mean PKCg levels (n=8 wells) and the standard errors were derived for each treatment sample. As mentioned in Example 4, samples from MCA-O-treated animals showed significantly higher levels of PKCg when compared to sham-operated and untreated control samples. Levels reached significance at times as early as 15 minutes following the ischemic event and remained at a significant level for at least 24 hours after the event (see, e.g., Figure 7).

Although a number of embodiments have been described above, it will be understood by those skilled in the art that modifications and variations of the described compositions and methods may be made without departing from either the spirit of the invention or the scope of the appended claims. The publications cited herein are incorporated by reference.

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The following publications pertain to the state of the art and/or illustrate techniques that may be utilized in practicing the present invention.

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Zhao et al., Delayed treatment with the spin trap alpha-phenyl-N-tert-butyl nitrone (PBN) reduces infarct size following transient middle cerebral artery occlusion in rats, *Acta Physiol. Scand.*, 152: 349-350 (1994b).

The publications mentioned herein are incorporated by reference in their entireties.

CLAIMS

1. A method for determining whether a mammal has suffered an ischemic event, comprising detecting the presence of the gamma isoform of protein kinase C (PKCg) in the mammal peripheral to the central nervous system.

2. A method for detecting an ischemic event in a mammalian subject comprising:
   (a) contacting a peripheral blood sample obtained from said subject with a detectably labeled binding partner capable of forming a binding complex with PKCg;
   (b) detecting the presence of a PKCg/binding partner complex formed in step (a).

3. The method according to Claim 2, wherein said binding partner is in the form of a finely divided solid substrate dispersible in a fluid sample, which substrate exhibits a surface moiety capable of complexing PKCg.

4. The method according to Claim 2, wherein said binding partner is in the form of an anti-PKCg antibody.

5. The method according to Claim 4, wherein said anti-PKCg antibody is selected from the group consisting of a major immunoglobulin class, an immunoglobulin subclass, a Fab fragment, a $F(ab')_2$ fragment, an Fv fragment, a single chain antibody (scFv), a chimeric antibody, a humanized antibody, a polyclonal antibody, a monoclonal antibody, and a diabody.

6. The method according to Claim 4, wherein said anti-PKCg antibody is fluorescently labeled.

7. The method according to Claim 1 or 2, wherein the ischemic event is the result of stroke, transient ischemic attack, head trauma, myocardial infarction, or trauma resulting in interrupted cranial blood flow.

8. A method for detecting a PKCg in the blood of a mammalian subject comprising the steps of:
   (a) contacting a peripheral blood sample obtained from said subject with a solid substrate having a first anti-PKCg antibody immobilized thereon under conditions suitable for forming a binding complex with PKCg in said sample;
   (b) contacting the substrate with a second antibody comprising a detectably labeled anti-PKCg antibody under conditions suitable for the second antibody to react with PKCg, wherein said second antibody recognizes a different PKCg epitope than said first antibody; and

(c) measuring the presence of the detectable label.

9. A method for detecting an ischemic event in a mammalian subject comprising the steps of:

(a) contacting a peripheral blood sample obtained from said subject with a solid substrate having a first anti-PKCg antibody immobilized thereon, under conditions suitable for forming a binding complex with PKCg in said sample;

(b) contacting the substrate with a second antibody comprising a detectably labeled anti-PKCg antibody under conditions suitable for the second antibody to react with PKCg, wherein said second antibody recognizes a different PKCg epitope than said first anti-PKCg antibody; and

(c) measuring the presence of the detectable label.

10. The method according to Claim 9, which further comprises the step, after step (a):

(a-1) separating the solid substrate from the rest of said sample.

11. The method according to Claim 10, which further comprises the step, after step (b):

(b-1) removing any unbound second antibody.

12. The method according to Claim 11, which further comprises the step of quantitating the level of PKCg in the blood sample based on the amount or intensity of the detectable label measured in step (c).

13. The method according to Claim 9, wherein the substrate is a well of a microtiter plate.

14. The method according to Claim 9, wherein the substrate is a magnetic bead.

15. The method according to Claim 9, wherein the substrate is a piece of plastic, nylon, wood or paper that is manually or robotically placed in contact with the blood sample.

16. The method according to Claim 9, wherein the second antibody is fluorescently labeled.

17. The method according to Claim 9, wherein the ischemic event is the result of stroke, transient ischemic attack, head trauma, myocardial infarction or trauma resulting in interrupted cranial blood flow.

18. The method according to Claim 8 or Claim 9, wherein the first and second anti-PKCg antibodies are independently selected from the group consisting of a major immunoglobulin class, an

immunoglobulin subclass, a Fab fragment, a $F(ab')_2$ fragment, an Fv fragment, a single chain antibody (scFv), a chimeric antibody, a humanized antibody, a monoclonal antibody, a polyclonal antibody, and a diabody.

19. The method according to Claim 8 or 9, wherein the peripheral blood sample is mixed with the second antibody comprising a detectably labeled anti-PKCg antibody, and the mixture is then contacted with the solid substrate having the first anti-PKCg antibody immobilized thereon.

20. A method for diagnosing stroke, transient ischemic attack, head trauma, myocardial infarction, or trauma resulting in interrupted cranial blood flow in a mammalian subject comprising: detecting the presence of PKCg in the subject's peripheral blood.

21. The method according to Claim 20, wherein PKCg is detected in a sample of venous blood taken from the subject.

22. The method according to Claim 20, wherein PKCg is detected using a detectably labeled anti-PKCg antibody.

23. A kit for rapid diagnosis of injury resulting from an ischemic event in a mammalian subject, comprising:

(a) a solid substrate having immobilized thereon a first PKCg binding partner,

(b) a second PKCg binding partner reactive with a different epitope of PKCg than is recognized by said first PKCg binding partner, which second PKCg binding partner is detectably labeled, and

(c) instructions and reagents for performing a sandwich-type assay for the presence of PKCg in a sample of peripheral blood obtained from said subject, wherein detection of PKCg is indicative of an injury from an ischemic event.

24. The kit according to Claim 23, wherein said first PKCg binding partner, said second PKCg binding partner, or both of said PKCg binding partners are anti-PKCg antibodies.

25. A kit according to Claim 23 or 24, wherein said first binding partner is immobilized on magnetic beads.

26. A kit according to Claim 23 or 24, wherein said first binding partner is immobilized in wells of a multi-well microtiter plate.

27. A kit according to Claim 23 or 24, wherein said first binding partner is immobilized on a piece of plastic, nylon, paper or wood.

28. A kit according to Claim 23 or 24, wherein said second PKCg binding partner is fluorescently labeled.

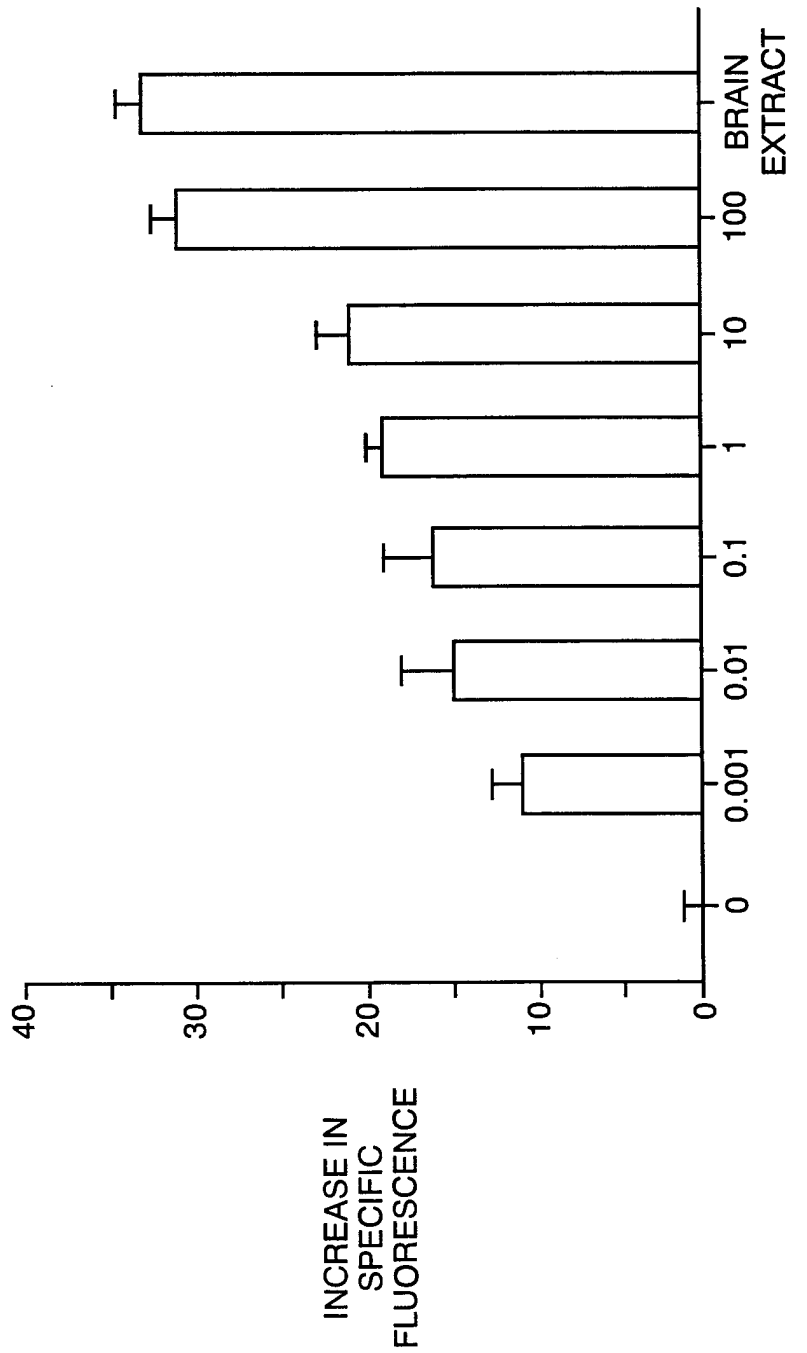
INCREASE IN SPECIFIC FLUORESCENCE
40
30
20
10
0
0
0.001
0.01
0.1
1
10
100
BRAIN EXTRACT
PKCg CONCENTRATION (ng/ml)

FIG. 1

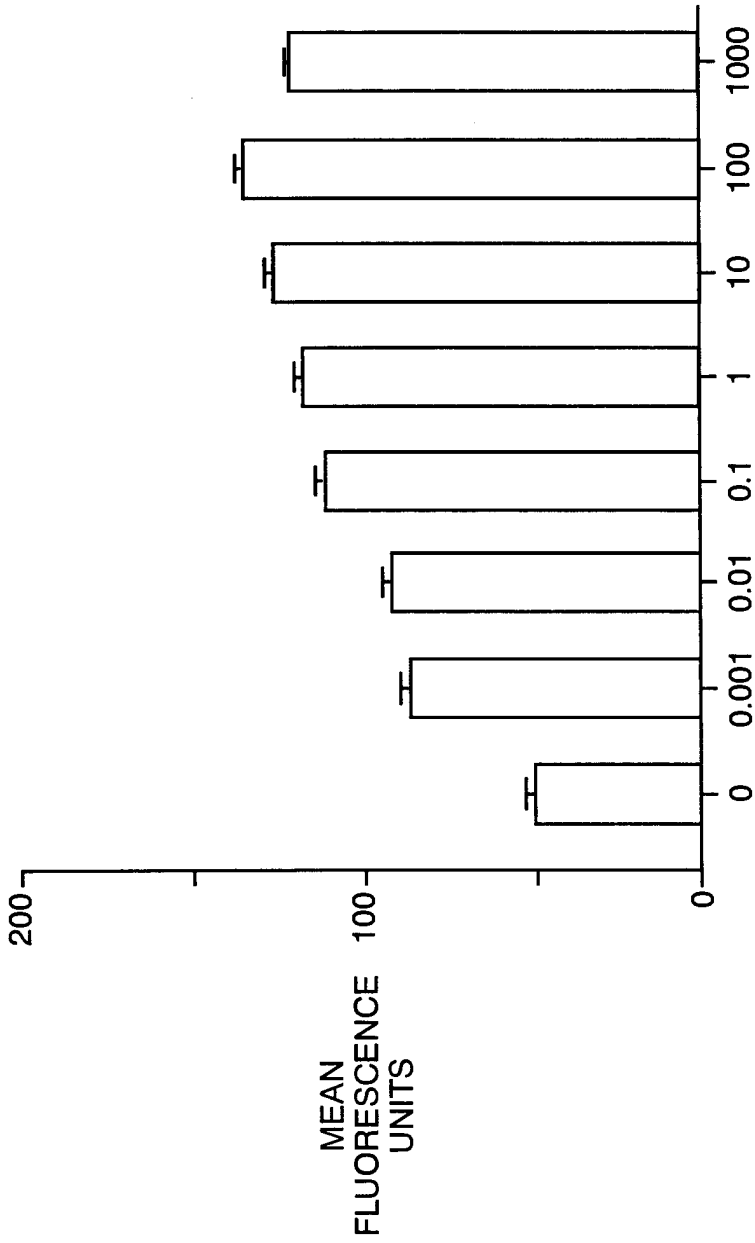
MEAN FLUORESCENCE UNITS
200
100
0
0
0.001
0.01
0.1
1
10
100
1000
PKCg CONCENTRATION (ng/ml)

FIG. 2

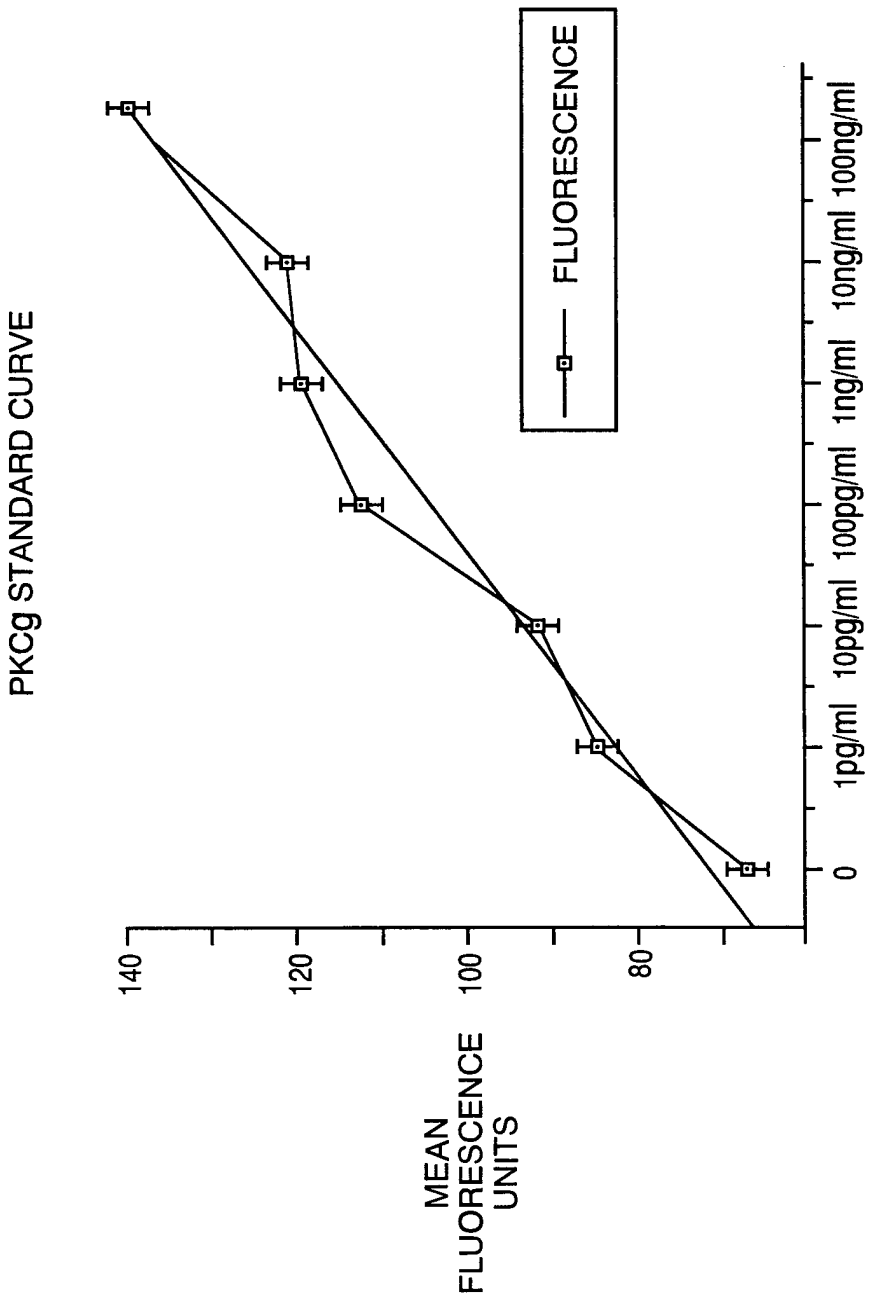
PKCg STANDARD CURVE
FLUORESCENCE
MEAN FLUORESCENCE UNITS
140
120
100
80
0
1pg/ml
10pg/ml
100pg/ml
1ng/ml
10ng/ml
100ng/ml
PKCg CONCENTRATION (ng/ml)

FIG. 3

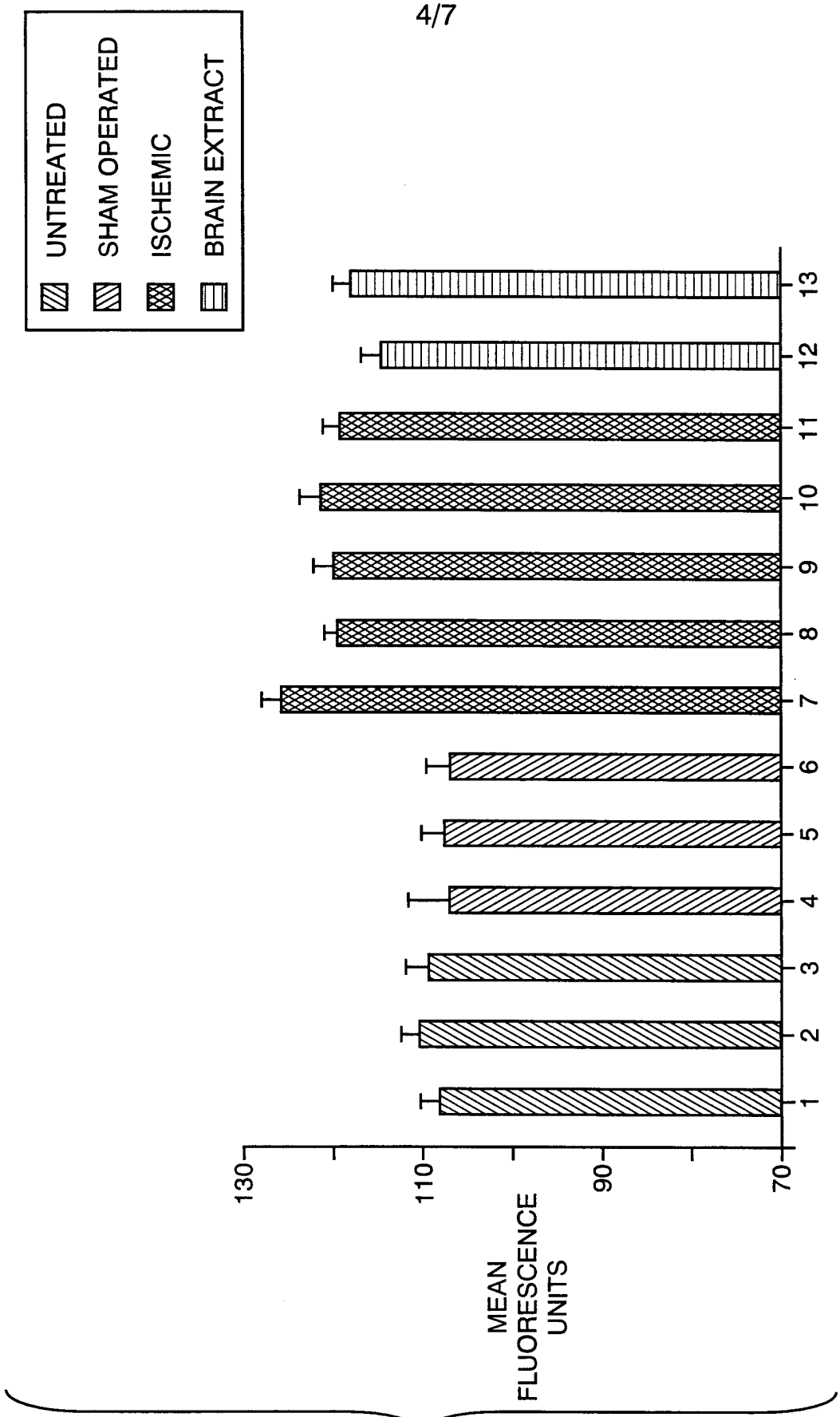
UNTREATED
SHAM OPERATED
ISCHEMIC
BRAIN EXTRACT
MEAN FLUORESCENCE UNITS
130
110
90
70
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 4


UNTREATED
SHAM
MCA-O TREATED
BRAIN EXTRACT
PKCg LEVELS FROM STANDARD CURVE
AVERAGE LEVELS PKCg IN pg/ml
7000
6000
5000
4000
3000
2000
1000
0

FIG. 5

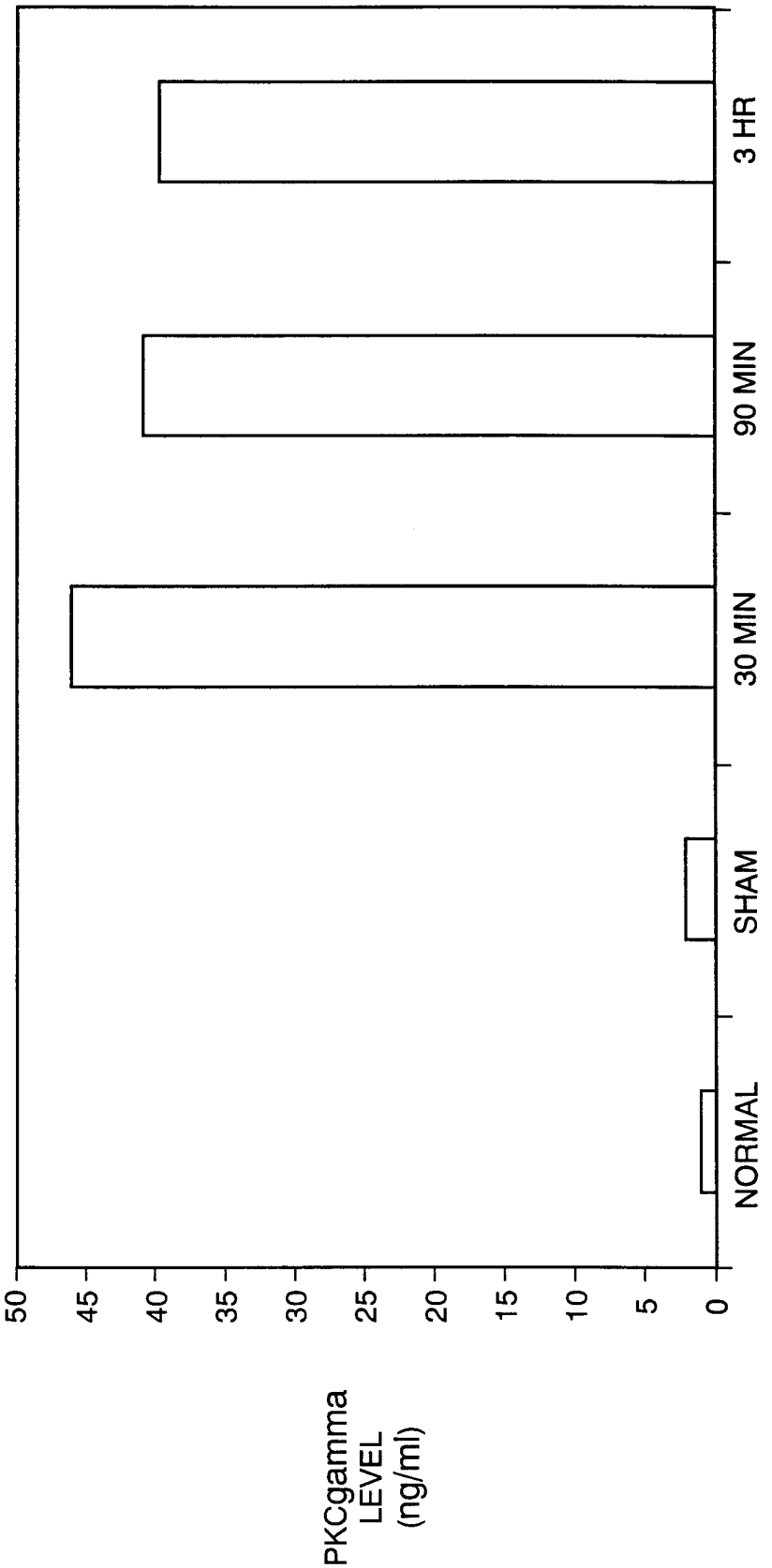
PKCgamma
LEVEL
(ng/ml)
0
5
10
15
20
25
30
35
40
45
50
NORMAL
SHAM
30 MIN
90 MIN
3 HR
TIME AFTER OCCLUSION

FIG. 6

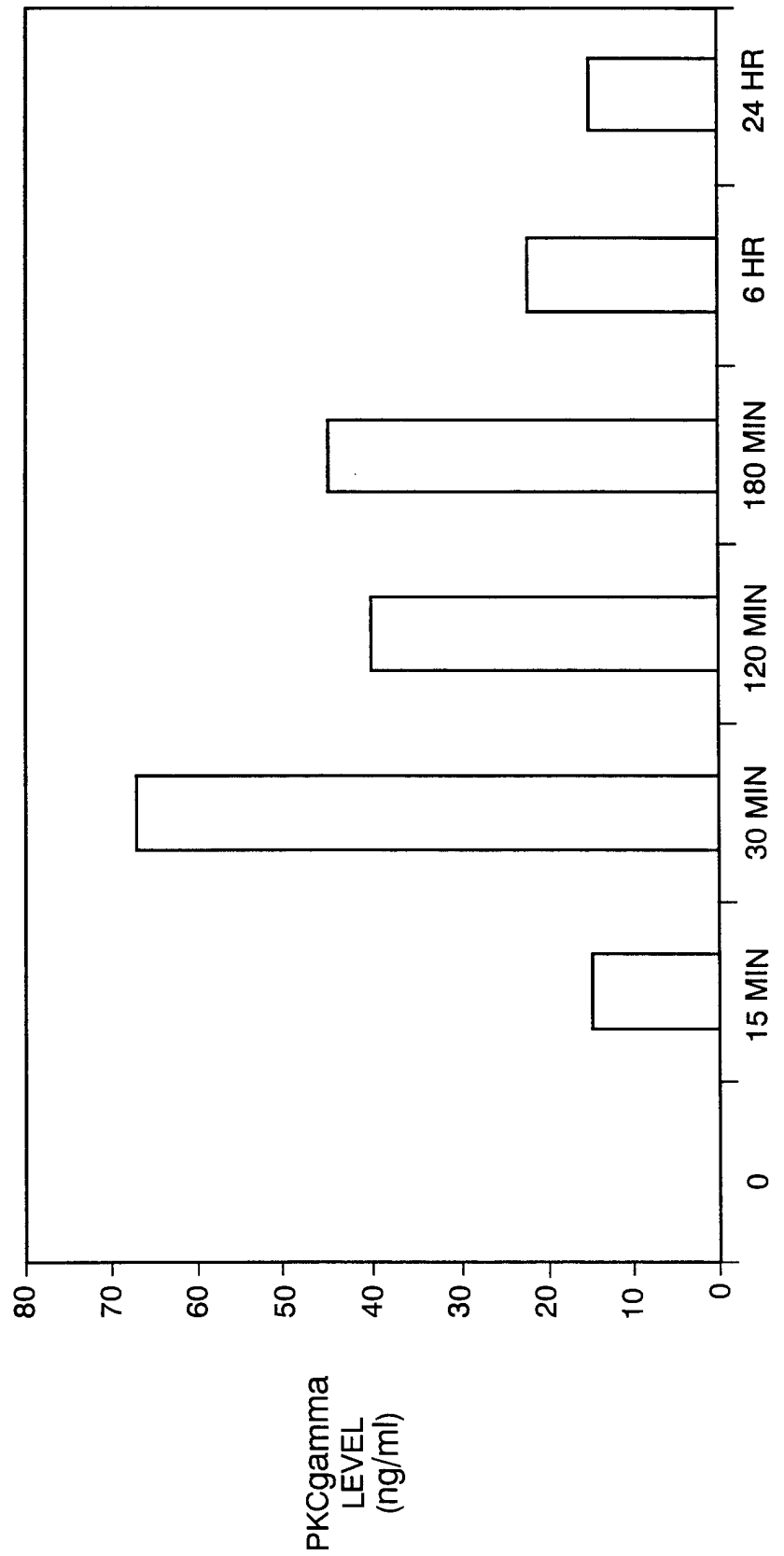
PKCgamma
LEVEL
(ng/ml)
0
10
20
30
40
50
60
70
80
0
15 MIN
30 MIN
120 MIN
180 MIN
6 HR
24 HR
TIME AFTER OCCLUSION

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24337

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 33/53
US CL : 436/526

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)
U.S. : 435/ 7.1,7.94,975 ; 436/518,523,526,533,538,546,63; 530,380

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: caplus, embase, medline, lca, caba

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HARRIS et al. Anti-CD3-Induced Changes in Protein Kinase C Isozymes Expression in Human CD4+ and CD8+ T Lymphocytes.Harris et al Journal of Clinical immunology. 1995, Vol. 15, No. 5, pp 232-241, page 237 second col., page 233 first col. methods.	1, 2, 4-6,27, 28
Y	KRUPINSKI et al. Protein Kinase C expression and activity in the human brain after ischemic stroke. Acta Neurobiol. Exp. 1998, Vol. 98, pages 13-21, fugure 3, page 19 discussion.	6-24
Y	US 5,223,398 A (KORTRIGHT et al.) 29 June 1993, col. 5, lines 5-30.	3,25,26
A	WIELOCH et al. Intracellular Signal Transduction in the Posrischemic Brain. Advances in Neurology Cellular and Molecular Mechanisms of Ischemic Brain Damage. Vol. 71, pages 372-374.	1,7,17,20
A	YOSHIDA et al. Translocation of protein Kinase C-alpha, gamma and epsilon isoforms in ischemic rat heart. Biochemica et Biophysica Acts. 1996, Vol. 1317, pages 36-44.	1-28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family 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 application or patent 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

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Date of the actual completion of the international search	Date of mailing of the international search report
12 October 2000 (12.10.2000)	14 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Long V Le Telephone No. 703-308-1235

Form PCT/ISA/210 (second sheet) (July 1998)

专利名称(译)	用于检测中枢神经系统损伤的分析		
公开(公告)号	EP1210599A4	公开(公告)日	2004-12-08
申请号	EP2000959900	申请日	2000-08-25
[标]申请(专利权)人(译)	威盛成像		
申请(专利权)人(译)	VIATECH IMAGING，LLC		
当前申请(专利权)人(译)	VIATECH IMAGING，LLC		
[标]发明人	CORNELL BELL ANN MADDEN KATHLEEN S RIBLET LESLIE A		
发明人	CORNELL-BELL, ANN MADDEN, KATHLEEN, S. RIBLET, LESLIE, A.		
IPC分类号	G01N33/573 G01N33/53 G01N33/545 G01N33/553 G01N33/577 G01N33/58 G01N33/68		
CPC分类号	G01N33/6893 G01N2800/324 Y10S435/975		
优先权	09/384356 1999-08-27 US		
其他公开文献	EP1210599A1		
外部链接	Espacenet		

摘要(译)

公开了一种用于快速诊断以缺血事件为特征的疾病的方法，所述缺血事件例如中风，短暂性脑缺血发作，头部创伤，心肌梗塞或导致颅血流中断的其他损伤。该方法涉及检测外周血中蛋白激酶C（PKCg）的γ同种型的存在，其表示对中枢神经系统组织的损伤和至少血脑屏障的瞬时破坏。该测定可以例如由紧急医疗人员在允许在中枢神经系统发生永久性损伤之前治疗患者的时间范围内进行。