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A METHOD FOR TREATING CARDIAC DYSFUNCTION AND PHARMACEUTICAL COMPOSITIONS USEFUL THEREFOR

- 5 The present invention relates generally to a method for, and pharmaceutical compositions useful in, the prophylaxis and/or treatment of cardiac dysfunction in a mammal by the administration of an effective amount of an agent capable of blocking or inhibiting the effect or release of inositol(1,4,5)*tris*phosphate in cardiac tissue.
- 10 Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.
- Throughout this specification, unless the context requires otherwise, the word
- 15 "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.
- 20 The phosphatidylinositol (PtdIns) turnover pathway is a complex signal transduction system that mediates a diverse range of neurotransmitter and hormone induced responses in a great variety of cells (1). The pathway usually involves the receptor mediated hydrolysis of a membrane phospholipid
- 25 phosphatidylinositol(4,5)*bis*phosphate (PtdIns(4,5)P₂) by a specific phospholipase C (PLC) causing the release of two well described second messengers *sn*-1,2-diacylglycerol (DAG) and inositol(1,4,5)*tris*phosphate, hereinafter denoted as Ins(1,4,5)P₃. DAG activates protein kinase C (PKC) and Ins(1,4,5)P₃ releases calcium from specific intracellular stores. The two arms of the pathway either separately or in concert, control a wide range of cellular responses including
- 30 contraction, secretion and mitogenesis (2). Ins(1,4,5)P₃ is metabolised rapidly within the cell by dephosphorylation via Ins(1,4)P₂ to Ins(4)P₁ and by phosphorylation to Ins(1,3,4,5)P₄, which is further metabolised to produce a wide range of inositol

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phosphate isomers (Figure 1). $\text{Ins}(1,3,4,5)\text{P}_4$ itself has been suggested to have a role in calcium sequestration (3) and roles for other inositol phosphates have been suggested (4).

- 5 The PtdIns pathway in the heart is activated by α_1 -adrenergic and muscarinic receptors, endothelin and stretch (5). Compared with non-muscle tissues, the heart is insensitive to $\text{Ins}(1,4,5)\text{P}_3$ in terms of calcium release (6), with the addition of high concentrations of $\text{Ins}(1,4,5)\text{P}_3$ causing a slow leakage of calcium rather than the rapid release seen in most cells (7). In addition, $\text{Ins}(1,4,5)\text{P}_3$ enhances calcium oscillations
10 which are associated with the development of arrhythmias (8, 9), but not calcium induced-calcium release, the mechanism involved in excitation-contraction coupling.

The present inventors have previously reported that PtdIns turnover pathway in the heart differs from that described in non-muscle cells. In heart, phosphorylation
15 products of $[\text{}^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ are barely detectable, indicating reduced $\text{Ins}(1,4,5)\text{P}_3$ kinase activity (10). More recent studies have shown that little $\text{Ins}(1,4,5)\text{P}_3$ is released and metabolised either by phosphorylation or dephosphorylation and that most of the accumulated inositol phosphates derive from $\text{Ins}(1,4)\text{P}_2$ rather than $\text{Ins}(1,4,5)\text{P}_3$ (11). These findings suggest that $\text{Ins}(1,4,5)\text{P}_3$ does not play a major
20 role in the healthy heart.

However, there is evidence that the PtdIns pathway and $\text{Ins}(1,4,5)\text{P}_3$ may be more important under pathological conditions such as myocardial ischaemia and reperfusion. α_1 -Adrenoceptor stimulation, which is associated with inositol
25 phosphate release, is capable of inducing arrhythmias both ventricular tachycardia (VT) and ventricular fibrillations (VF) during both ischaemia and reperfusion. Such responses are not observed under normoxic conditions. Ischaemia has been reported to increase α_1 -adrenoceptor density in a number of myocardial preparations (12) as well as increasing responsiveness to noradrenaline stimulation (13). Reperfusion of
30 ischaemic myocardium, is associated with large calcium accumulations, apparently associated with α_1 -adrenoceptor stimulation (14). These calcium accumulations have been implicated in reperfusion-induced injury. Furthermore, α_1 -adrenoceptor

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blockade, by a number of antagonists, has been shown to be anti-arrhythmic under both ischaemic and reperfusion condition (15).

In work leading to the present invention, detailed studies were carried out in isolated
5 perfused rat hearts to determine and characterise the effects of ischaemia and reperfusion on the PtdIns turnover pathway, and to investigate a possible functional role for the second messenger $\text{Ins}(1,4,5)\text{P}_3$ under these pathological conditions.

It was surprisingly discovered that reperfusion with oxygenated medium after global
10 ischaemia caused a rapid and quantitatively large rise in $\text{Ins}(1,4,5)\text{P}_3$ which could be correlated in time with the observed development of arrhythmias. Furthermore, it was observed that by blocking the formation of $\text{Ins}(1,4,5)\text{P}_3$ in the heart, using the aminoglycosides neomycin, gentamicin or streptomycin (being agents known to inhibit the formation of $\text{Ins}(1,4,5)\text{P}_3$) it was possible to completely abolish, or
15 significantly reduce arrhythmias occurring following reperfusion of the ischaemic heart.

Accordingly, one aspect of the present invention contemplates a method for the prophylaxis or treatment of cardiac dysfunction in a mammal said method comprising
20 administering to said mammal an effective amount of an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.

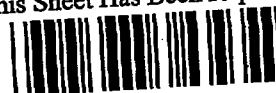
Another aspect of the present invention is directed to a pharmaceutical composition useful in the prophylaxis and/or treatment of cardiac dysfunction comprising an agent
25 capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents.

Yet another aspect of the present invention relates to an agent for use in blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.

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Still yet another aspect of the present invention is directed to the use of an agent which blocks formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue in the manufacture

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of a medicament for the treatment or prophylaxis of cardiac arrhythmia.

Optionally another anti-cardiac dysfunction agent such as an anti-arrhythmic drug is administered in combination with agents which block or inhibit the effect or release
5 of $\text{Ins}(1,4,5)\text{P}_3$. Such agents including, but not limited to, agents which induce thrombolysis for example, streptokinase and tissue plasminogen activator or other anti-arrhythmic agents for example, amiodorone, sotalol and lignocaine.

In a preferred embodiment, the mammal is a human, a livestock animal such as a
10 sheep, cow, horse, donkey or pig or a laboratory test animal such as a mouse, rat, rabbit, guinea pig, cat or dog. Most preferably, the mammal is a human.

The term "cardiac dysfunction" is used in its most general sense and relates to any condition which causes a reduction in effective pumping action of the heart such that
15 tissues are inadequately perfused. More particularly, it covers or may result from the following conditions: cardiac arrhythmia, cardiomyopathy, allograft rejection (cardiac), coronary angioplasty, cardio-pulmonary by-pass surgery and electrophysiological studies. The present invention is particularly directed to the prophylaxis and/or treatment of cardiac arrhythmia. Reference hereinafter to cardiac
20 arrhythmia should be taken as covering all conditions contemplated above.

The present invention is described in detail hereafter in terms of the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in rat cardiac tissue. However, it is understood that it extends to the effect of the $\text{Ins}(1,4,5)\text{P}_3$ inhibitor in all mammals and in particular humans
25 and livestock animals. Accordingly, any reference herein to the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in rats is meant to be applicable to the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in all mammals and in particular humans and livestock animals.

The term "cardiac arrhythmia" is used herein to denote conditions in a mammal
30 where there is an irregular heart action caused by physiological or pathological disturbances in discharge of cardiac impulses or their transmission through the conducting tissue of the heart, and includes tachycardia and fibrillations of particular

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cardiac sites, including but not limited to, atrial, essential, nodal, paroxysmal atrial, paroxysmal nodal, paroxysmal ventricular, polymorphic ventricular, reflex and sinus and ventricular fibrillation. Accordingly, the treatment of cardiac arrhythmia is taken to be returning or maintaining the regular action of the heart by the administration of
5 an effective amount of an agent which blocks or inhibits the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ for a time and under conditions sufficient to maintain, or return, the regular action of the heart. Cardiac arrhythmia may occur following a disease condition or result from trauma or therapy and the present invention is not limited to any one or more causes of cardiac arrhythmia. Typically, cardiac arrhythmia will
10 occur following myocardial infarction, as a consequence of reperfusion following cardio-pulmonary by-pass surgery, and in patients having unstable angina.

The present invention also extends to preventative therapy whereby an agent which blocks the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ is administered to prevent or reduce the
15 likelihood of cardiac arrhythmia developing.

The agents capable of blocking the effect or inhibiting the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ which are used in accordance with the present invention include, for example:

- 20 (i) agents which affect the biochemical release of $\text{Ins}(1,4,5)\text{P}_3$ from the phospholipid $\text{PtdIns}(4,5)\text{P}_2$ to cause reduction in $\text{Ins}(1,4,5)\text{P}_3$ formation. Such agents include the class of compounds known as the aminoglycosides, for example; gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivative thereof, and the class of compounds
25 known as polyamines, for example; spermine or derivative thereof as well as agents which bind the phospholipase C (PLC) enzyme to produce inhibition; and
- (ii) agents which block the effects of $\text{Ins}(1,4,5)\text{P}_3$, for example, agents which block the $\text{Ins}(1,4,5)\text{P}_3$ receptor, or antagonist molecules to $\text{Ins}(1,4,5)\text{P}_3$.

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Accordingly, in one preferred embodiment, the present invention provides a method for treating or preventing cardiac arrhythmias in a mammal which method comprises

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administering to said mammal an effective amount of an aminoglycoside for a time and under conditions sufficient to modulate cardiac arrhythmia.

Preferably the aminoglycoside is one of the group comprising gentamicin,
5 tobramycin, amikacin, netilmicin, kanamycin, streptomycin, neomycin or derivatives thereof. Even more preferably the aminoglycoside is neomycin or derivative thereof. In a most preferred embodiment the amino glycoside is gentamicin.

In a further preferred embodiment the invention provides a method for treating or
10 preventing cardiac arrhythmias in a mammal which method comprises administering to said mammal an effective amount of a polyamine molecule for a time and under condition sufficient to modulate cardiac arrhythmia.

Preferably, in this aspect of the invention the polyamine is spermine or its
15 derivatives. In a more preferred aspect the polyamine is spermine.

Furthermore, the route of administration of the polyamine or aminoglycoside or other agent blocking or inhibiting the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ is preferably by oral, parenteral or buccal administration but other routes may be equally applicable with
20 only minor modifications to the methods contemplated herein. The effective amount of the agent will depend on the mammal and the condition to be treated, however, the amount to be administered to the mammal will need to be non-toxic. In general, the effective amount of agent will be a daily dose in the range of 0.1 → 100 mg/kg, more preferably 1-100 mg/kg and even more preferably 5-60 mg/kg.

25

Pharmaceutically acceptable carriers or diluents for use in the composition of this invention are well known to persons skilled in the art, and by way of example are described in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

30

The method and pharmaceutical compositions described in accordance with the present invention will be useful *inter alia* in the treatment or prevention of cardiac

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arrhythmias occurring as a result of various surgical procedures, or underlying cardiac disease.

Agents capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ are generally first
5 tested *in vitro* in perfused heart tissue such as isolated perfused rat hearts. Potentially useful agents are then tested *in vivo* in a small animal model, such as rats, mice, guinea pigs or rabbits. Agents which are still of potential interest following this initial *in vivo* screen may then be tested in a larger animal model such as dogs, horses, pigs or mini-pigs. Clinical trials in human subjects may then follow using
10 agents which still show signs of useful activity. A suitable protocol for clinical trials of anti-arrhythmic agents is described by Heidbuchel *et al.* (36).

The present invention is further described by the following non-limiting Figures and Examples.

15

In the Figures:

Figure 1 is a diagrammatic representation of the phosphatidylinositol turnover pathway. The pathway predominating in heart is boxed.

20

Figure 2 shows HPLC profiles of TCA extracted inositol phosphates from isolated perfused rat hearts.

A: control heart; B: heart subjected to 30 minutes global myocardial ischaemia. Long arrows indicate elution position of nucleotide standards.

25

Figure 3 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in cpm/mg protein in isolated perfused rat hearts subjected to increasing periods of global myocardial ischaemia.

30 Figure 4 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in isolated perfused rat hearts subject to ischaemia and subsequent reperfusion following a 20 minute ischaemic period.

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Figure 5 is a graphical representation showing accumulation of individual ^3H -labelled inositol phosphates in isolated perfused rat hearts subject to noradrenaline stimulation (A) ischaemia and reperfusion (B) and ischaemia and reperfusion in the presence of reserpine (C).

Figure 6 is a graphical representation showing the effect of various neomycin concentrations on ^3H -labelled inositol phosphate accumulation within 2 minutes reperfusion following 20 minutes global ischaemia as a percentage of untreated 2 minutes reperfusion response.

Figure 7 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in cpm in neonatal myocytes. Note the low levels of $\text{Ins}(1,4,5)\text{P}_3$.

Figure 8 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates cpm in adrenal glomerulosa cells. Note the low levels of $\text{Ins}(1,4,5)\text{P}_3$.

20

EXAMPLE 1

INOSITOL PHOSPHATE EXPERIMENTS

The inositol phosphate response in heart

Earlier studies in the inventors' laboratory showed that the pathway of inositol phosphate release and metabolism in heart tissue was different from that in other cells in that products of dephosphorylation of the Ca^{2+} -releasing messenger $\text{Ins}(1,4,5)\text{P}_3$ were observed but the phosphorylation products were not (10). The metabolic pathways involved are shown in Figure 1. Subsequent studies provided an explanation for these findings by demonstrating that the primary release product of phosphatidylinositol-specific phospholipase C (PLC) in heart was $\text{Ins}(1,4)\text{P}_2$ rather than $\text{Ins}(1,4,5)\text{P}_3$ (11). This suggested that heart tissue avoided the generation of $\text{Ins}(1,4,5)\text{P}_3$ and provided the basis for the current studies. More recent studies have

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provided a mechanistic basis for this unusual pathway by showing that the "cardiac" pathway correlates with the presence of an atypical GTP-binding coupling protein called Gh (16). Gh couples to a different phospholipase C from PLC- β which couples receptors to typical GTP-binding proteins (17). To further test the association of Gh with the cardiac inositol phosphate pathway, effects of an inhibitor of PLC- β was investigated (18). This compound, U-73122, did not inhibit the accumulation of inositol phosphates in heart tissue under noradrenaline stimulation but was a fully effective inhibitor in isolated neonatal cardiomyocytes which lack Gh and have an inositol phosphate metabolic pathway indistinguishable from non-muscle cells. Thus, the phosphatidylinositol turnover pathway in heart tissue differs from that in other cell types in a number of important aspects.

The inventors investigated the possibility that some alteration in the pathway was responsible for the arrhythmic action of α_1 -adrenergic agonists under conditions of myocardial ischaemia and reperfusion.

A) METHODS

*Inositol phosphate response in isolated perfused rat hearts **

Adult male sprague dawley rats 250-300g were used. Where indicated, rats were treated with reserpine (5 mg/kg I.P. for 18hrs prior to killing) to deplete endogenous noradrenaline stores.. Rats were heparinized 1U/g I.P. 30 minutes prior to killing by decapitation. Hearts were rapidly excised and immediately chilled in ice-cold saline. Under ice-cold solution, hearts were cannulated via the ascending aorta as described by Langendorff and perfused retrogradely at a rate of 5 ml/min with HEPES-buffered Krebs solution equilibrated with 5%CO₂-95%O₂ at 37°C. Initial perfusion was in a non-recirculating manner to remove internal blood. Hearts were allowed to warm up gradually and, when free of internal blood, transferred to a 37°C water-jacketed organ bath containing carbogenated Krebs medium, and perfusion continued in a recirculating manner. Following a 15 minute equilibration period, hearts were labelled with [³H]-inositol, 2 μ Ci/ml for 2 h. Hearts were monitored throughout the labelling period for strong rhythmic beating. Any hearts which did not fulfil these requirements were discarded.

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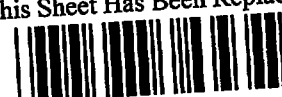
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Following labelling, the [^3H]-inositol-containing medium was removed, and replaced with Krebs medium containing propranolol (10^{-6} M) and lithium chloride (50 mM) to block β -adrenergic receptors and to inhibit InsP metabolism by InsP 1'-phosphatase respectively (20). Hearts were perfused with propranolol, LiCl solution for 10 minutes prior to ischaemia. Antagonists, when administered, were included in this 10 minute preincubation. Normothermic global ischaemia was induced by terminating perfusion for various times up to 30 minutes. Reperfusion was examined following a 20 minute ischaemic period by resuming perfusion at 5 ml/min. Reactions were terminated by freezing hearts in liquid N_2 following excision at the atrioventricular junction. Frozen ventricles were weighed and inositol phosphates extracted as described below.

Extraction of inositol phosphates

Inositol phosphates were extracted from ventricle samples in 3.5 ml ice-cold 5% trichloroacetic acid (TCA), 2.5 mM EDTA, 5 mM phytate solution, as described previously (21). Briefly, ventricles were homogenised with two 10 second passes of a "Polytron" homogenizer separated by 15 second periods on ice. Samples were homogenised further using a Potter-Elvehjem homogeniser followed by brief sonication. Homogenised samples were centrifuged (5000 rpm, 10min, 4°C) to remove TCA insoluble material. Supernatants were collected, and the remaining pellet re-extracted in 1.5 ml TCA, EDTA, phytate. Supernatants were pooled and freon, tri-n-octylamine (1:1 v/v 0.75 ml:1ml TCA solution) was added, mixed thoroughly, and phases separated by low speed centrifugation (2000 rpm, 10 min 4°C). The resultant aqueous phase, containing inositol phosphates was collected and treated with proteinase K (50 $\mu\text{g/ml}$, 2hr, 50°C). This process was necessary to preserve the chromatography column. Treated samples were passed through a 1 ml Dowex-50 column (4% cross-linked 4-400 mesh size) and eluted with 1 ml water (22). Urea (0.05M final) was added (23) and samples lyophilized prior to high performance liquid chromatography (HPLC) analysis. Recovery of inositol phosphates using this method was greater than 95% for each isomer, as estimated by addition of [^3H]-labelled standards to extractions of unlabelled hearts.

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Separation and quantitation of inositol phosphates

Inositol phosphates were separated by an anion-exchange HPLC system using a Whatman Partisil 10 μ SAX column in a Waters Radial Compression Unit, employing a complex ammonium phosphate gradient, pH 3.8 from 0-2M as described previously (24). Inositol phosphates were detected and quantitated using an on-line β -counter (model CR, Radiomatic Instruments). All peaks were identified by comparison with commercial standards. Nucleotide standards AMP, ADP, and ATP were run with samples to assess column performance.

10 *Noradrenaline determination in heart perfusate*

Aliquots of heart perfusate for noradrenaline estimation were collected from ischaemic tissue and at various intervals of reperfusion following 20 minutes MI. Samples were analysed by HPLC and quantitated by electrochemical detection as described previously. (25).

15

Ins(1,4,5)P₃ Mass Analysis

Mass of Ins(1,4,5)P₃ was measured using a competitive binding assay (Amersham TRK 1000) essentially as described previously (26). Ventricle samples were prepared as described above except for the omission of [³H]-label during the 2 hr perfusion of hearts prior to ischaemia, and for the use of 5 mM ATP instead of phytate in the extraction procedure. 5 mM phytate was found to cross-react in the Ins(1,4,5)P₃ assay.

Protein Determination

25 Protein concentration was determined in aliquots of TCA pellet following InsP extraction using a modified Lowry method with Bovine Serum Albumin as a standard.

Statistics

30 Values presented are mean $\pm$ SEM, for n=4 unless otherwise stated. Statistical analysis of results involved Students' unpaired "t-test" for all InsP data.

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B) RESULTS

Effects of global ischaemia on the accumulation of [3 H]-labelled inositol phosphates

TCA extracts of [3 H]inositol-labelled hearts contained compounds identified as
5 Ins(1/3)P₁, Ins(4)P₁, Ins(1,4)P₂ and Ins(1,4,5)P₃. As reported previously, [3 H]-
Ins(1,3,4,5)P₄ and its metabolic products were not evident (Figure 2a). Thirty minute
perfusion with oxygenated medium resulted in a time dependent increase in [3 H]-
label in inositol phosphates (Figure 3). In contrast, global ischaemia caused an altered
distribution of inositol phosphates (Figure 2b), while the total amount of label
10 remained unchanged. As shown in Figure 3, decreases in Ins(1,4)P₂ and
Ins(1,4,5)P₃, were observed together with concurrent increase in the degradation
products Ins(1)P₁ and Ins(4)P₁. Significant changes in [3 H]-labelled inositol
phosphates were observed after 5 min of ischaemia. The redistribution process
observed under ischaemic conditions was independent of the endogenous
15 noradrenaline released during ischaemia, as it was unaffected by both depletion of
noradrenaline stores by reserpinization, or by α_1 adrenoceptor blockade with
prazosin.

In contrast to normoxic perfusion, total accumulation of inositol phosphates in
20 myocardium remained unchanged throughout the 30 minute ischaemic period studied,
such that the decreases in [3 H]-label of Ins(1,4,5)P₃ and Ins(1,4)P₂ could be
accounted for by increases in the metabolic products Ins(1/3)P₁ and Ins(4)P₁. These
data indicate that global ischaemia causes an inhibition of PtdIns-PLC activity, such
that no inositol phosphate release occurs, even in the presence of locally high
25 concentrations of noradrenaline.

Control of inositol phosphate release under ischaemic conditions

More recent modelling studies have shown that the altered metabolism of inositol
phosphates during ischaemia is not related either to the reduced pH or the high
30 ambient extracellular K⁺ concentration, under these conditions. Rather, our studies
indicated that the change in inositol phosphate profiles could be explained solely by
the decrease in ATP content. Moderate anoxia reduced ATP content from 8.3 ± 1



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$\mu\text{moles/g}$ dry weight to 4.5 ± 0.4 (mean $\pm$ SEM, $n=3$, $p<.01$) and this resulted in a reduction in content of $\text{Ins}(1,4)\text{P}_2$. Larger reductions in ATP (to 0.718 ± 0.14), as occurs in global ischaemia, resulted in reduced $\text{Ins}(1,4,5)\text{P}_3$ as well as $\text{Ins}(1,4)\text{P}_2$. Both profiles, moderate and severe ischaemia, could be modelled using inhibitors of

5 ATP generation.

Under conditions of moderate ischaemia, release of $\text{Ins}(1,4,5)\text{P}_3$ occurs in response to noradrenaline, the ambient levels of which are high under these conditions. Reperfusion following moderate ischaemia further enhanced this response (see

10 below). Thus, whether $\text{Ins}(1,4,5)\text{P}_3$ release occurs under ischaemic conditions depends on the ATP content of the tissue and thus on the severity of the ischaemia. However, enhanced release is always observed with reperfusion.

15 *Effects of post-ischaemic reperfusion on the accumulation of ^3H -labelled inositol phosphates*

Reperfusion with oxygenated medium following 20 minutes global myocardial ischaemia resulted in a rapid increase in accumulation of $[^3\text{H}]$ -labelled inositol phosphates. This activation was biphasic, with a rapid and transient accumulation occurring between 1 and 2 minutes reperfusion, followed by a slow, quantitatively

20 smaller, secondary accumulation which continued past 20 minutes (Figure 4).

- The rapid accumulation of inositol phosphates caused by 2 minutes post-ischaemic reperfusion was dependent on the release of endogenous noradrenaline, as it was prevented by prior reserpine treatment (Figure 5c). The release of $[^3\text{H}]$ -labelled

25 $\text{Ins}(1,4,5)_3$ could be reinstated in reserpinized animals by perfusion with 10^{-4} M noradrenaline. Prazosin ($10 \mu\text{M}$) treatment inhibited the 2 minute reperfusion inositol phosphate response, demonstrating mediation via α_1 -adrenoceptors. Thus, the immediate reperfusion $\text{Ins}(1,4,5)\text{P}_3$ response requires neuronally released noradrenaline and is mediated by α_1 -adrenoceptors.

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Even though the reperfusion $\text{Ins}(1,4,5)\text{P}_3$ response was caused by noradrenaline stimulation of α_1 -adrenoceptors, it differed from the noradrenaline response under normoxic conditions in a number of ways. First, the release of inositol phosphates was quantitatively greater. Second, the profile of released inositol phosphates was different in that the primary release product under reperfusion conditions was $\text{Ins}(1,4,5)\text{P}_3$ whereas $\text{Ins}(1,4)\text{P}_2$ predominates under normoxic conditions (11). Third, inositol phosphate release under reperfusion conditions was inhibited by a number of compounds which did not inhibit release under normoxic conditions, as discussed in detail below (Table 1).

The 2 minute reperfusion response required the reintroduction of oxygen, as reperfusion with O_2 free (N_2 saturated) medium, did not activate inositol phosphate release. This finding is of great interest as recent reports indicate that oxidants increase the sensitivity of $\text{Ins}(1,4,5)\text{P}_3$ receptors (27). Thus, by two different mechanisms, increased release and increased sensitivity, $\text{Ins}(1,4,5)\text{P}_3$ can be more effective and more damaging under conditions of ischaemia and reperfusion.

Noradrenaline concentration under conditions of ischaemia and reperfusion

Endogenous noradrenaline is known to accumulate in myocardial extracellular space under ischaemic conditions (28). In the current studies the noradrenaline concentration was measured in the tissue and surrounding perfusate, to verify such accumulation in the inventors' model. Control values ($9.87 \pm 1.54 \times 10^{-10} \text{ M}$) were sampled following 10 minutes recirculating perfusion with Krebs' medium containing propranolol and LiCl prior to initiation of ischaemia. 20 minutes global ischaemia caused an accumulation of noradrenaline within the myocardial extracellular space ($8.19 \pm 2.03 \times 10^{-7} \text{ M}$). This noradrenaline was released rapidly with reperfusion and levels remained elevated at 2 minute reperfusion ($1.26 \pm 0.28 \times 10^{-8} \text{ M}$), although diluted around 40 fold when released into the 10 ml recirculating perfusate. Perfusate noradrenaline levels dropped significantly following 20 minutes reperfusion ($1.06 \pm 0.5 \times 10^{-9} \text{ M}$) to a level not different from those of controls ($P > 0.3$). Noradrenaline concentrations in the perfusate after 2 minutes reperfusion averaged

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1.54 $\pm$ 0.5 $\times 10^{-8}$ M. Such concentrations are not effective in stimulating inositol phosphate release from non-ischaemic myocardium. This demonstrates an increased sensitivity or availability of ventricular α_1 -adrenoceptors during 2 minutes post-ischaemic reperfusion. Previous studies in the inventors' laboratory have shown that

5 nerve stimulation of non-ischaemic myocardium produces little increase in Inositol phosphate accumulation. Thus, cardiac α_1 -adrenoceptors responds to neuronally released noradrenaline only under conditions of ischaemia and reperfusion.

Effect of aminoglycosides on the reperfusion- induced [3 H]-labelled Inositol phosphate response

10

The aminoglycoside antibiotic neomycin has been well characterised as an inhibitor of Ins(1,4,5) P_3 release (29)(30). The effects of neomycin on the 2 minute reperfusion-induced [3 H]-labelled Inositol phosphate response were investigated. Neomycin (0.05 mM), added 10 minutes prior to ischaemia and maintained

15 throughout the procedure, did not significantly inhibit the accumulation of [3 H]-labelled inositol phosphates. Higher concentrations, 0.5 mM and 5 mM, produced a 45% and 55% average inhibition of [3 H]-labelled Inositol phosphate accumulation respectively, compared with 2 minutes post-ischaemic reperfusion in the absence of neomycin (Figure 6). These results are different from findings in healthy heart tissue

20 where 5 mM neomycin caused a 20% reduction in accumulation of [3 H]Ins(1,4,5) P_3 but was without effect on the accumulations of Inositol phosphate₁ and Inositol phosphate₂. Lower concentrations were ineffective. These data indicate an increase in sensitivity of myocardial tissue to neomycin of more than 10 fold during post-ischaemic reperfusion.

25

Subsequent experiments were performed to establish whether this inhibitory effect of neomycin was a class effect of aminoglycosides and if so whether other members might have higher potency. Streptomycin and gentamicin were chosen for these studies with a view to using available plasma assays for *in vivo* studies. As is shown

30 in Table 1, both gentamicin and streptomycin were more potent inhibitors of reperfusion-induced Ins(1,4,5) P_3 release than neomycin. Thus, inhibition of reperfusion-induced Ins(1,4,5) P_3 release is a class effect of the aminoglycosides.

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As shown in Table 1, the polyamine, spermine, also was tested and shown to be inhibitory.

More recent studies have investigated the possible contribution of thrombin to the
5 reperfusion arrhythmias. The studies described above used medium-perfused hearts. However, *in vivo* myocardial ischaemia and reperfusion are associated with local high concentrations of thrombin associated with the thrombus formation responsible for the occlusion. Recent studies from other laboratories have pointed to a direct pro-arrhythmic effect of the thrombus *in vivo*. Studies in the inventors' laboratory have
10 shown that thrombin directly stimulates release of $\text{Ins}(1,4,5)\text{P}_3$ in heart tissue and that this release is inhibited by gentamicin at similar concentrations to those required for inhibition of the noradrenaline response under reperfusion conditions.

In summary, reperfusion of hearts following global ischaemia causes release of
15 $\text{Ins}(1,4,5)\text{P}_3$ and this can either be effected either by neuronally released noradrenaline or by thrombin. Both of these agents would be expected to be present in the heart under ischaemic conditions *in vivo*.

EXAMPLE 2

20 MYOCARDIAL FUNCTION *IN VITRO*

A) METHODS

- *Reperfusion-induced arrhythmias in in situ perfused rat hearts*

A model of *in situ* perfused rat hearts subjected to 20 minutes coronary artery occlusion to induce ischaemia, followed by reperfusion was used to investigate
25 reperfusion-induced arrhythmias.

Rats (250-300g) were anaesthetised (pentobarbitone 60mg/kg, I.P.) and heparinised (200U/rat I.V.) The thorax was then opened and a metal cannula (ID 1 mm) inserted and tied into the ascending aorta, to commence Langendorff perfusion *in situ* (31).
30 The interval from opening the chest to commencing heart perfusion was between 50 and 70 seconds. The perfusate was Krebs buffer, with coronary flow controlled by a Ismatac peristaltic pump at a rate of 5 ml/g heart weight/min for the non-ischaemic

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perfusion. At this flow rate the coronary perfusion pressure was between 32-38 mmHg, measured by pressure transducer (Gould Statham model p23) connected to the sidearm of the perfusion line. Following aortic cannulation, pulmonary vessels were tied off. In some preparations, ventricular pressure was measured by inserting a
5 Millar micro-tip transducer catheter into the left ventricular cavity via the apex. All parameters- coronary perfusion pressure, ventricular pressure, and epicardial ECG, were constantly recorded on a Grass model 7 polygraph. Two hearts were perfused and simultaneously studied.

- 10 A silk suture (4.0) was positioned loosely around the left coronary artery 2-3 mm from its origin. Two silk releasing rings were placed around the suture for subsequent release of occlusion. Successful occlusion of the artery, inducing regional ischaemia, was indicated by increases in coronary perfusion pressure of at least 30%. Perfusion pressure was adjusted to non-ischaemic perfusion levels during the
15 ischaemic period.

Drugs were added to the perfusion via a Harvard Model 22 pump. The drug flow was less than 1% of perfusion flow to ensure constant perfusate parameters. Unless mentioned otherwise, the drugs were present 10 minutes before, and during the entire
20 ischaemic and reperfusion periods.

Heart preparations were allowed to stabilise for at least 20 minutes prior to commencing studies. Following 10 minute drug incubation, the left coronary artery was ligated to induce local ischaemia, for a period of 20 minutes. At the end of this
25 ischaemic period, the ligature was released and hearts perfused at pre-ischaemic flow for a period of 5 minutes. Ventricular arrhythmias, occurring during ischaemia or during the first 2 minutes of reperfusion were defined according to the Lambeth Conventions (32).

30 *Statistics*

Incidence of arrhythmias was analysed using a χ^2 test, with $n \geq 8$ for each group.

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B) RESULTS

Effects of aminoglycosides on reperfusion- induced arrhythmias

The initial reperfusion-induced release of the second messenger $\text{Ins}(1,4,5)\text{P}_3$ occurs at a time point associated with reperfusion-induced arrhythmias. Based on the considerations outlined above, this suggested a possible role for $\text{Ins}(1,4,5)\text{P}_3$ in the development of arrhythmias. Therefore, reperfusion-induced arrhythmias were quantitated, and the effects of compounds which inhibit the $\text{Ins}(1,4,5)\text{P}_3$ response under these conditions were investigated.

10

An ischaemic model of coronary occlusion and subsequent reperfusion in *in situ* perfused rat hearts was used, and provided a high percentage of ventricular arrhythmias under both ischaemic and perfused conditions. Twenty minutes regional ischaemia by coronary artery occlusion resulted in 83% (10/12) incidence of VT. As shown in Table 2, reperfusion following this ischaemic period resulted in VT and degenerative VF in 10 out of the 12 rats studied. In all cases, a large number of premature ventricular beats were observed. None of the hearts which became arrhythmic with VT and subsequent VF recovered during the 5 minute reperfusion period studied.

20

The effects of neomycin on these reperfusion-induced arrhythmias are shown in table 2. Neomycin at 0.05 mM had no significant effect on the induced arrhythmias with 6 out of 8 rats studied showing both severe VT and VF throughout reperfusion. In contrast, and in parallel with [^3H]-labelled Inositol phosphate studies, higher concentrations of neomycin (0.5 mM) prevented the occurrence of reperfusion-induced arrhythmias with none of the 10 rats studied exhibiting VT or VF. Similar effects were observed with 5 mM neomycin. The inhibition of reperfusion-induced arrhythmias by 0.5 mM neomycin was independent of any haemodynamic effects as heart rate and ventricular pressure, while dropping upon initial introduction of the drug, recovered to levels not significantly differing from control during the 10 minute incubation period prior to occlusion.

30

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As is shown in Table 1, streptomycin, gentamicin and spermine also inhibited reperfusion arrhythmias in parallel with their effects on reperfusion-induced release of $\text{Ins}(1,4,5)\text{P}_3$. Gentamicin was the most potent agent tested followed by streptomycin. At the higher concentrations gentamicin and streptomycin also produced a partial
5 inhibition of arrhythmias occurring during the ischaemic phase. These data further suggest the possibility of a contribution by $\text{Ins}(1,4,5)\text{P}_3$ to these arrhythmias in addition to its apparently essential role in reperfusion arrhythmias.

Thus, inhibition of hydrolysis of phosphatidylinositol(4,5)bisphosphate
10 ($\text{PtdIns}(4,5)\text{P}_2$) under reperfusion conditions prevents the development of reperfusion arrhythmias.

Effect of staurosporine on reperfusion-induced arrhythmias

In addition to $\text{Ins}(1,4,5)\text{P}_3$, the second messenger DAG is also produced upon
15 hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. DAG activates various isoforms of protein kinase C which in turn controls a number of cellular processes. Thus, it was possible that the anti-arrhythmic effects of the aminoglycosides was be due to inhibition of DAG production and subsequent PKC activation. Therefore, the effects of a potent protein kinase C inhibitor staurosporine (33) on reperfusion-induced arrhythmias was
20 investigated. Staurosporine (10^{-7} M, a concentration known to inhibit PKC in the myocardium under reperfusion conditions (34)) did not significantly alter the percentage incidence of reperfusion-induced arrhythmias from untreated controls as shown in Table 1.

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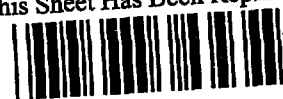


TABLE 1:

	Ins(1,4,5)P ₃		Reperfusion-induced arrhythmias			
			VT		VF	
5		mass	p	%	p	%
	control	200 ± 18		83		83
	0.05 mM neomycin	184 ± 7	ns	75	ns	70
	0.5 mM neomycin	121 ± 11	<.025	0	<.005	0
	5 mM neomycin	104 ± 12	<.025	0	<.005	0
10	0.5 mM spermine	182 ± 4	ns	63	ns	36
	5 mM spermine	160 ± 9	<.05	30	<.025	20
	PTX (25 µg/kg)	128 ± 28	<.05	40	ns	10
	0.015 mM genta	145 ± 2	<.05	58	<.025	25
	0.15 mM genta	136 ± 22	<.05	27	<.025	27
15	1.5 mM genta	102 ± 17	<.01	17	<.025	8
	0.015 mM strep	201 ± 16	ns	45	ns	27
	0.15 mM strep	167 ± 5	<.05	25	<.025	6
	1.5 mM strep	94 ± 11	<.01	45	<.025	27
	.1 µM stauro	nd		83	ns	69
20						

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Table 1: Effects of a number of agents on the release of $\text{Ins}(1,4,5)\text{P}_3$ and reperfusion-induced arrhythmias. Values shown are $\text{Ins}(1,4,5)\text{P}_3$ content in pmol/mg protein, mean $\pm$ SEM of data from 4 different hearts 2 minutes after commencing reperfusion following 20 min global ischaemia, and % incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) in the experimental group ($n \geq 8$). Pertussis toxin (PTX) was given by I.P. injection 48 h prior to experimentation. "Genta" is gentamicin, "strep" is streptomycin and "stauro" is staurosporine. Statistical analysis of $\text{Ins}(1,4,5)\text{P}_3$ mass data was performed using Student's unpaired "t" test and analysis of arrhythmia data using a χ^2 test. p values are relative to control.

Effect of inhibition of phospholipase C β

To date, few direct inhibitors of phosphatidylinositol-specific phospholipase C have been available. However, one compound, a steroid derivative known as U-73122, has been shown to inhibit PLC- β isoforms. As explained above, this agent did not inhibit release of $\text{Ins}(1,4,5)\text{P}_3$ in intact heart preparations and was without anti-arrhythmic activity. These data are summarised in Table 2.

Taken together, the data derived from a number of different compounds show that inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ causes an inhibition of reperfusion arrhythmias.

Arrhythmic effect of thrombin under reperfusion conditions

The finding that thrombin directly stimulated release of $\text{Ins}(1,4,5)\text{P}_3$ in heart indicates the possibility that thrombin might cause arrhythmias directly *via* this mechanism. Experiments were performed using hearts from reserpinized rats so that arrhythmic events initiated by noradrenaline were eliminated. Reperfusion arrhythmias were not observed in hearts from reserpinized animals. However, when thrombin (2 U/ml) was added to the perfusate a high incidence of reperfusion arrhythmias was observed. Furthermore, these arrhythmias were inhibited by gentamicin, indicating a relationship to release of $\text{Ins}(1,4,5)\text{P}_3$ (Table 2).

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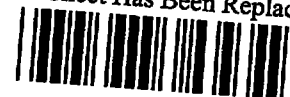


TABLE 2:

	VT%	VF%	n	p
reserpine	44	0	9	
5 thrombin (2 U/ml)	89	67	9	<.05
thrombin + gentamicin	60	0	5	<0.05*
control	100	63	46	
U-73122 (10^{-6} M)	88	61	12	ns

- 10 Table 2: Arrhythmic effect of thrombin and its inhibition by gentamicin. * relative to thrombin. Other p values are relative to control.

In summary, agents which stimulate release of $\text{Ins}(1,4,5)\text{P}_3$ under reperfusion conditions are pro-arrhythmic and agents which inhibit release are anti-arrhythmic.

15

EXAMPLE 3

IN VIVO STUDIES

A) METHODS

- 20 Gentamicin was chosen for studies of anti-arrhythmic action of aminoglycoside *in vivo* because it is the most potent agent *in vitro* and because an assay method for measuring plasma levels is currently available. Rats were not heparinised so that effects of thrombin will be contributory to observed arrhythmias.

In vivo model for coronary artery occlusion and reperfusion

- 25 Rats were anaesthetised with pentobarbitone (60 mg/Kg IP) and artificially ventilated with room air. The chests were opened via the fourth intercostal space, and the heart exposed. A 4.0 suture was placed around the left coronary artery and then passed through a guided polyethylene cannula. The thorax was closed leaving the threaded cannula outside the chest. The right carotid artery was cannulated for recording of



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arterial blood pressure. After stabilisation for 20-30 minutes, the left coronary artery was ligated by pulling the ends of the ligature through the tube and securing them by clamping the tube together with the threads. The ischaemic period was 10 minutes followed by reperfusion. Arterial blood pressure and a lead II ECG was recorded 3 minutes before and during ischaemia, and for the first 5 minutes of reperfusion. In the *in vivo* rat model, the highest incidence of reperfusion arrhythmias occurs after a 10 minute period of coronary artery occlusion. The size of the ischaemic area was determined at the end of the experiment by the dye method. There was no difference in ischaemic area between hearts from control animals and those receiving gentamicin. Gentamicin was infused at a dose of 2.5-10 mg/Kg/min prior to initiation of ischaemia and maintained throughout the protocol. This caused a moderate reduction in blood pressure and heart rate.

As is shown in Table 3, gentamicin inhibited the development of reperfusion arrhythmias *in vivo*. The arrhythmias generated under these conditions would be expected to be initiated by noradrenaline and by thrombin and possibly also by other agents. It is important to note that gentamicin was effective also against the arrhythmias occurring under ischaemic conditions. As indicated above, release of $\text{Ins}(1,4,5)\text{P}_3$ also can occur under these conditions, depending on the severity of the ischaemia.

TABLE 3:

	VT%	VF%	n	p
control	100	68	13	
gentamicin	30	0	10	<0.01

Table 3: Effect of gentamicin on reperfusion arrhythmias *in vivo*.

In summary, gentamicin is a very effective agent in inhibiting reperfusion arrhythmias *in vivo*.

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EXAMPLE 4
CARDIAC SELECTIVE PLC INHIBITOR

5 Studies from the inventors show that inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ under conditions of post-ischaemic reperfusion inhibits the development of reperfusion arrhythmias. This could be achieved either by directly binding to and inhibiting the PLC enzyme which hydrolyses $\text{PtdIns}(4,5)\text{P}_2$ to $\text{Ins}(1,4,5)\text{P}_3$ or by binding $\text{PtdIns}(4,5)\text{P}_2$ and thereby inhibiting PLC activity less directly. Given that the PtdIns turnover pathway occurs in all cell types, it is important to incorporate some selectivity into inhibitory agents if they are to be effective clinical tools. The PLC which is coupled to α_1 -adrenoceptors in heart provides one possible target for a direct enzyme inhibitor because it is a different enzyme from any of the currently described PLC classes (17). However, agents like the aminoglycosides which bind $\text{PtdIns}(4,5)\text{P}_2$ can also be appropriate. The inventors have shown that the potency of these compounds increases by more than an order of magnitude under ischaemic conditions. Furthermore, even though the aminoglycosides act by binding $\text{PtdIns}(4,5)\text{P}_2$, the relative potencies of the aminoglycosides in causing an inhibition of PLC activity differ for the different PLC isoforms and neomycin rather than gentamicin is generally most potent (Table 4). $\text{PtdIns}(4,5)\text{P}_2$ is an activator rather than a substrate for phosphatidylcholine-specific phospholipase D. For this enzyme, aminoglycosides have been shown to inhibit the activation by $\text{PtdIns}(4,5)\text{P}_2$ but the rank order of potencies was different from observed in studies of PLC. Thus, even though the aminoglycosides act by binding $\text{PtdIns}(4,5)\text{P}_2$, this binding clearly affects different enzymes which interact with $\text{PtdIns}(4,5)\text{P}_2$ differently.

Thus, the development of a suitably selective agent will be possible by either of these mechanisms.

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TABLE 4:

	PC-PLD	PI-PLC	PI-PLC	PI-PLC
	brain	kidney cy	kidney me	heart rep
neomycin	65	30	200	400
5 streptomycin	125	380	2850	150
gentamicin	ND	130	500	15

Table 4: Relative potencies of neomycin, streptomycin and gentamicin in inhibiting PLC hydrolysing $\text{PtdIns}(4,5)\text{P}_2$ or in inhibiting the stimulatory effect of $\text{PtdIns}(4,5)\text{P}_2$ on PLC activity. Values shown are average EC_{50} values $\times 10^{-6}$ M. Kidney cy and kidney me refer to cytosolic and membrane enzymes, respectively. Heart rep refers to hearts under reperfusion conditions. Brain and kidney data are from the literature (35).

EXAMPLE 5

ANTI-ARRHYTHMIC AGENTS

Currently available anti-arrhythmic agents are only poorly effective and their use is often limited by significant side effects. Furthermore, these agents target primarily the arrhythmias occurring during ischaemia and are essentially ineffective against the arrhythmias which occur following reperfusion (36). Gentamicin *in vitro* and *in vivo* is very effective in inhibiting reperfusion arrhythmias. These findings set the stage for the development of an entirely new class of anti-arrhythmic agent based on the inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$. Furthermore, it is likely that arrhythmias developed *in vivo* are mechanistically more complex than those observed in *in vitro* model systems and that $\text{Ins}(1,4,5)\text{P}_3$ may have a wider role than classical "reperfusion" arrhythmias, as suggested both by our modelling studies and by the *in vivo* experiments. Conditions such as unstable angina likely involve repeated

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transient occlusions of the coronary artery and thus repeated episodes of ischaemia and reperfusion. Thus, agents which act by inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ may have wider clinical implications than classical "reperfusion" arrhythmias.

- 5 Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and
- 10 all combinations of any two or more of said steps or features.

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CLAIMS:

1. A method for the prophylaxis or treatment of cardiac dysfunction in a mammal said method comprising administering to said mammal an effective amount of an agent capable of blocking formation or release of inositol (1,4,5) triphosphate ($\text{Ins}(1,4,5)\text{P}_3$) in cardiac tissue.
2. A method according to claim 1 wherein the cardiac dysfunction is a cardiac arrhythmia.
3. A method according to claim 2 wherein the cardiac arrhythmia comprises tachycardia and fibrillations of cardiac sites.
4. A method according to claim 1 or 2 or 3 wherein the agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
5. A method according to claim 4 wherein the agent is an aminoglycoside.
6. A method according to claim 5 wherein the aminoglycoside is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.
7. A method according to claim 4 wherein the agent is a polyamine.
8. A method according to claim 7 wherein the polyamine is spermine or a derivative thereof.
9. A method according to claim 1 or 2 or 3 wherein the agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
10. A method according to claim 9 wherein the agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

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11. A method according to claim 9 wherein the agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
12. A method according to claim 1 or 2 or 3 wherein the agent is administered by oral, parenteral or buccal administration.
13. A method according to claim 1 or 2 or 3 wherein the effective amount is from about 0.1 to about 100 mg per kilogram of body weight of the mammal.
14. A method according to claim 13 wherein the effective amount is from about 5 to about 60 mg per kilogram of body weight of the mammal.
15. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an anti-arrhythmia drug.
16. A method according to claim 15 wherein the anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.
17. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an agent capable of effecting thrombolysis.
18. A method according to claim 17 wherein the agent is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
19. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an agent which increases cardiac systolic and/or diastolic function.
20. A method according to claim 19 wherein said agent is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.
21. A method according to claim 1 or 2 or 3 wherein the mammal is a human.

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22. An agent for use in blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.
23. An agent according to claim 22 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
24. An agent according to claim 23 wherein said agent is an aminoglycoside.
25. An agent according to claim 24 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilimicin, kanamycin, streptomycin and neomycin or derivatives thereof.
26. An agent according to claim 23 wherein said agent is a polyamine.
27. An agent according to claim 26 wherein said agent is spermine or a derivative thereof.
28. An agent according to claim 22 wherein said agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
29. An agent according to claim 28 wherein said agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.
30. An agent according to claim 28 wherein said agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
31. An agent according to claim 22 or 23 or 28 wherein said agent further comprises another anti-arrhythmia drug.
32. An agent according to claim 31 wherein said other anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.

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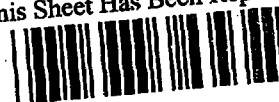




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33. An agent according to claim 22 or 23 or 28 wherein said agent further comprises a component capable of effecting thrombolysis.
34. An agent according to claim 33 wherein the component is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
35. An agent according to claim 22 or 23 or 29 wherein said agent further comprises a component which increases cardiac systolic and/or diastolic function.
36. An agent according to claim 35 wherein said component is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.
37. A pharmaceutical composition useful in the prophylaxis and/or treatment of cardiac dysfunction comprising an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents.
38. A pharmaceutical composition according to claim 37 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
39. A pharmaceutical composition according to claim 38 wherein said agent is an aminoglycoside.
40. A pharmaceutical composition according to claim 39 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilimicin, kanamycin, streptomycin and neomycin or derivatives thereof.
41. A pharmaceutical composition according to claim 37 wherein said agent is a polyamine.
42. A pharmaceutical composition according to claim 41 wherein said agent is spermine or a derivative thereof.

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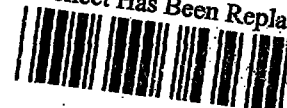




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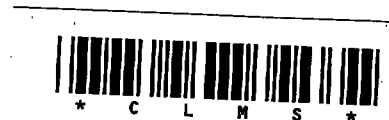
43. A pharmaceutical composition according to claim 37 wherein said agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
44. A pharmaceutical composition according to claim 43 wherein said agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.
45. A pharmaceutical composition according to claim 43 wherein said agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
46. A pharmaceutical composition according to claim 37 or 38 or 43 wherein said agent further comprises another anti-arrhythmia drug.
47. A pharmaceutical composition according to claim 46 wherein said other anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.
48. A pharmaceutical composition according to claim 38 or 39 or 43 wherein said composition further comprises an agent capable of effecting thrombolysis.
49. A pharmaceutical composition according to claim 48 wherein the agent is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
50. A pharmaceutical composition according to claim 37 or 38 or 43 wherein said composition further comprises an agent which increases cardiac systolic and/or diastolic function.
51. A pharmaceutical composition according to claim 50 wherein said agent is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.

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52. Use of an agent which blocks formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue in the manufacture of a medicament for the treatment or prophylaxis of cardiac arrhythmia.
53. A use according to claim 52 wherein the agent is an aminoglycoside.
54. A use according to claim 52 wherein the agent is a polyamine.



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ABSTRACT

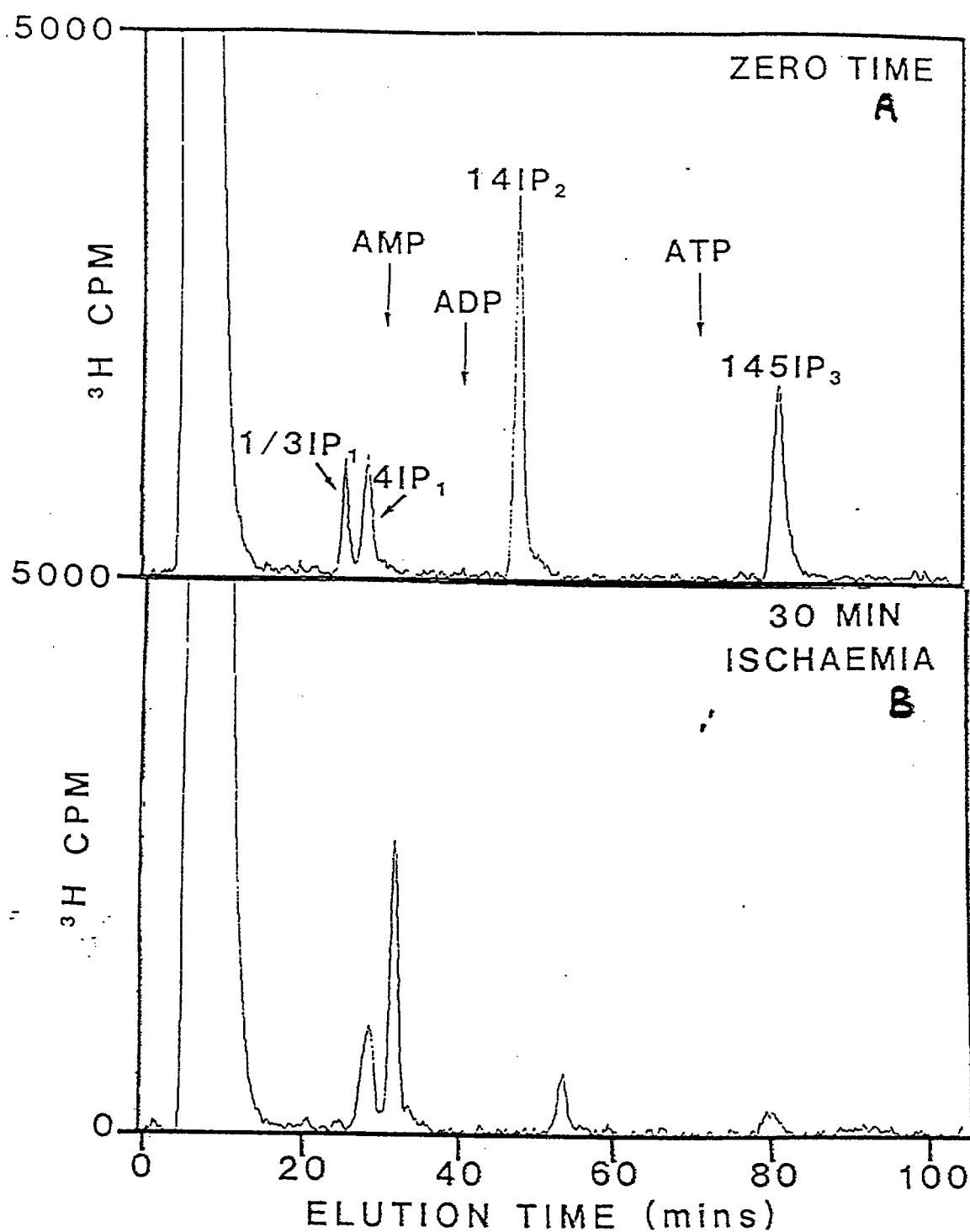
The present invention relates generally to a method for, and pharmaceutical compositions useful in, the prophylaxis and/or treatment of cardiac dysfunction in a mammal by the administration of an effective amount of an agent capable of blocking or inhibiting the effect or release of inositol(1,4,5)*tris*phosphate in cardiac tissue.

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FIGURE 2

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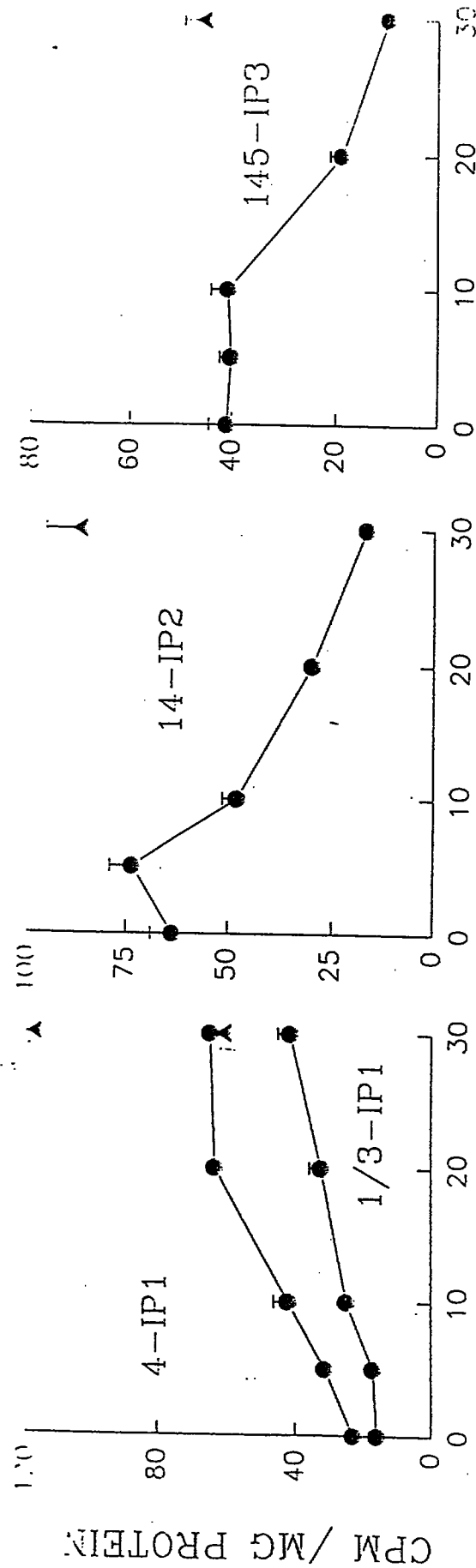


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FIGURE 3

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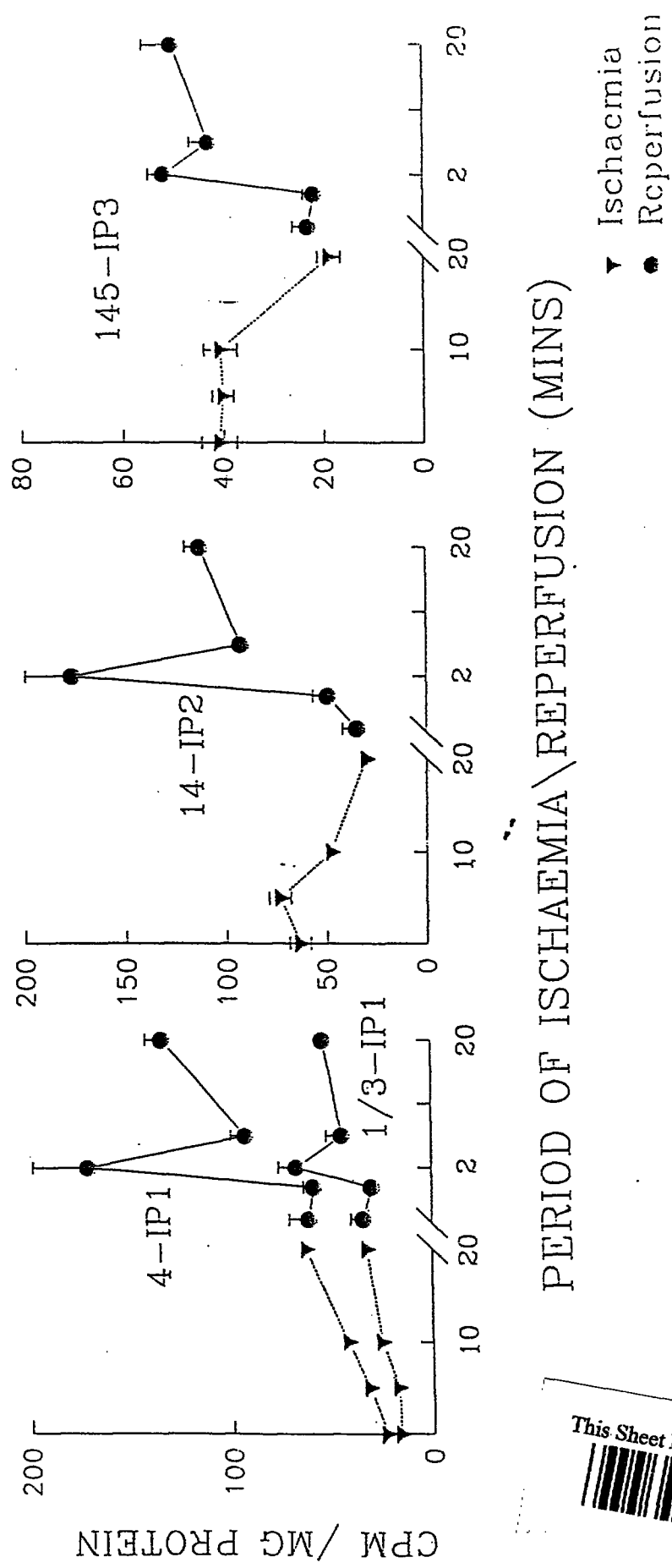
PERIOD OF ISCHAEMIA (MINS)

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▲ 30min control

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FIGURE 4

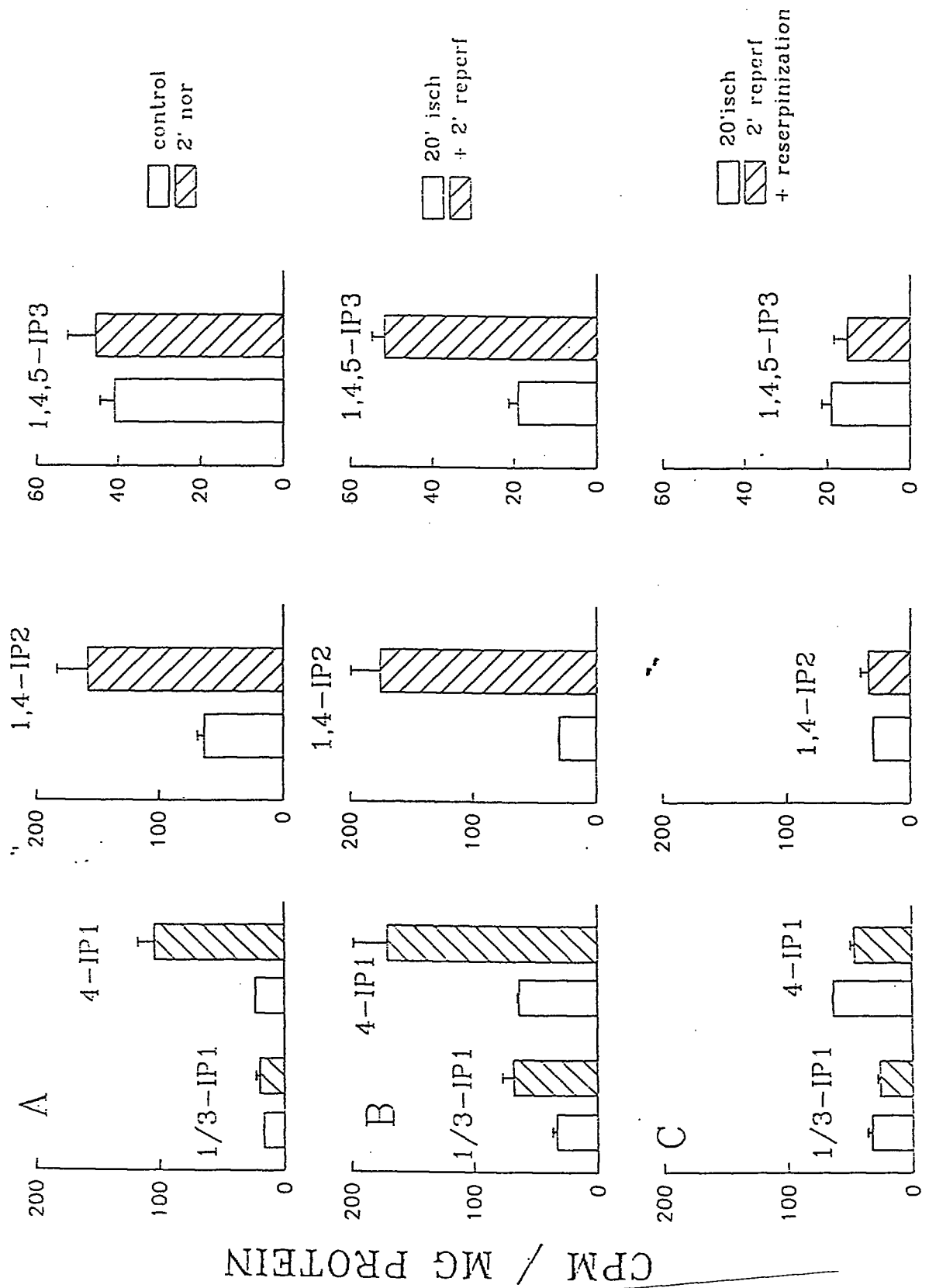
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FIGURE 5

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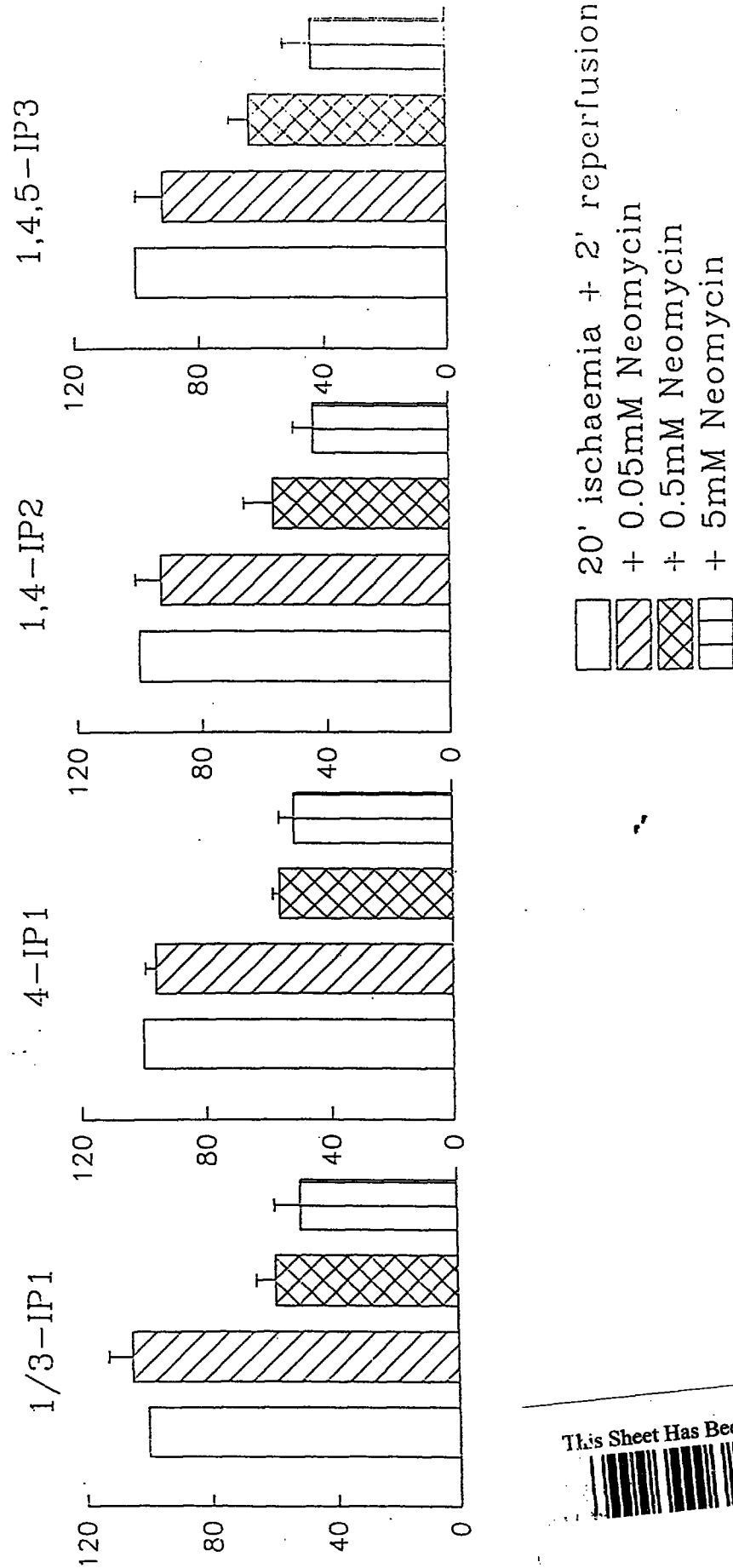


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FIGURE 6

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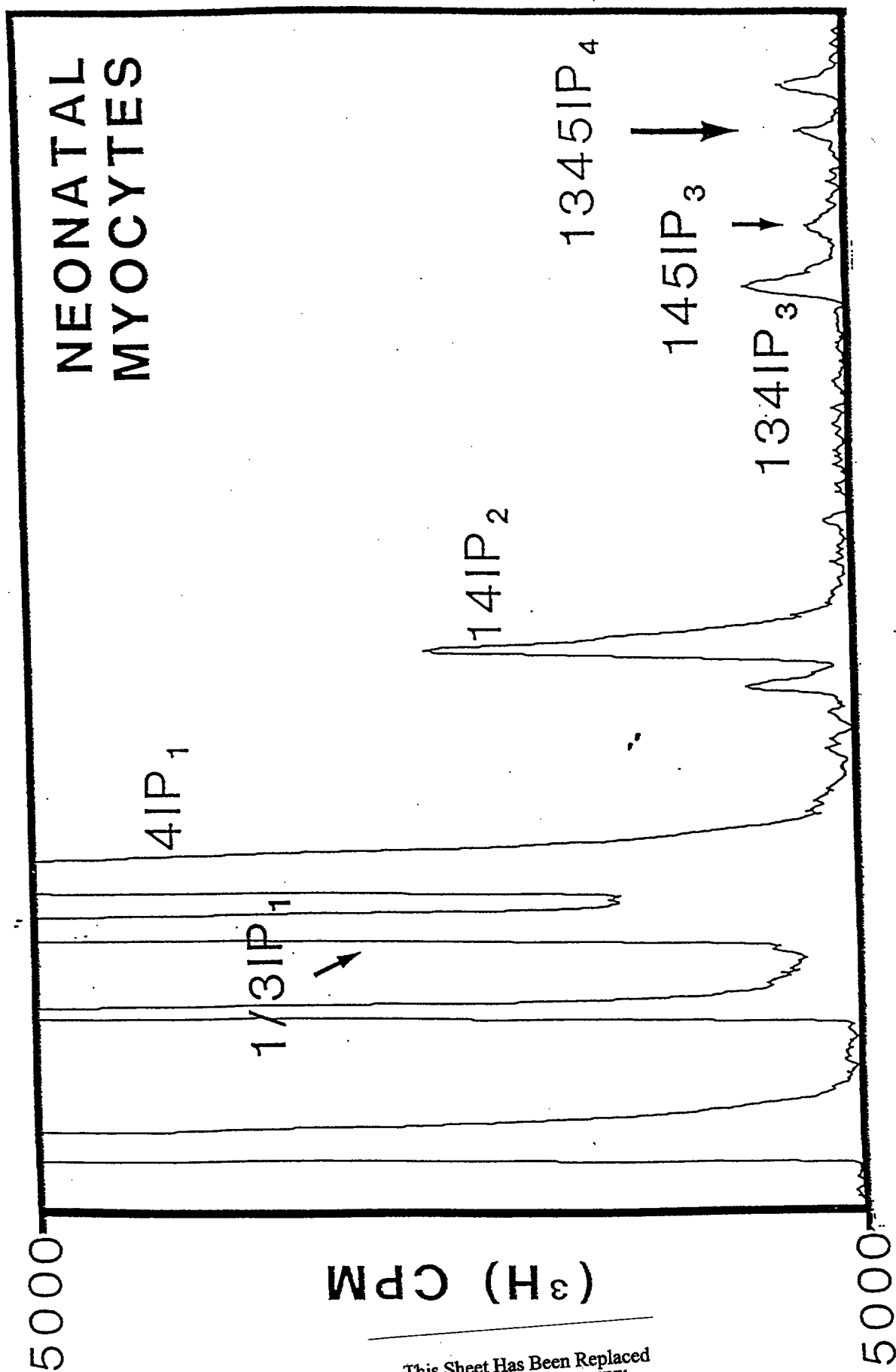


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FIGURE 7

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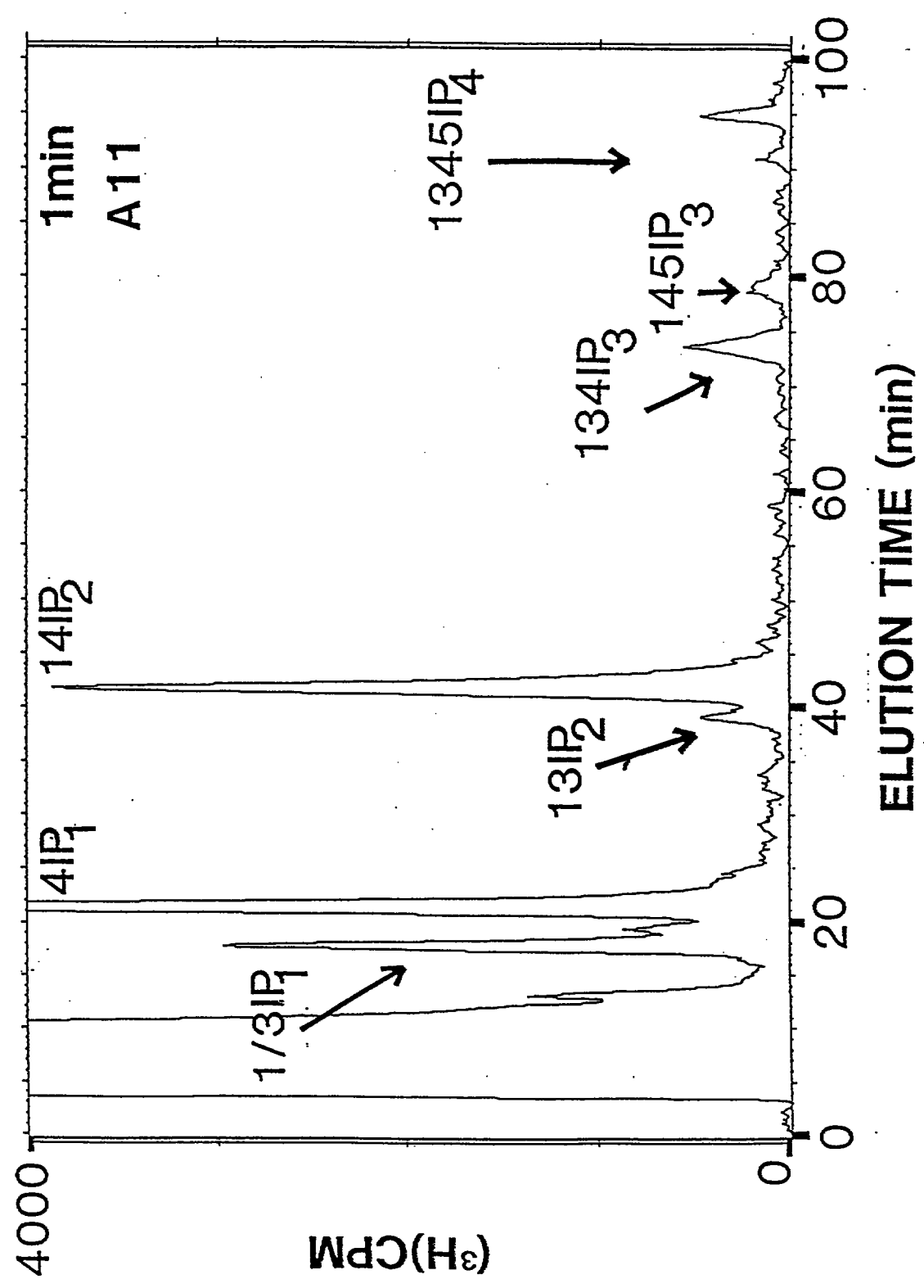
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FIGURE 8

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ADRENAL GLOMERULOSA



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REPLACEMENT SHEET

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A METHOD FOR TREATING CARDIAC DYSFUNCTION AND PHARMACEUTICAL COMPOSITIONS USEFUL THEREFOR

The present invention relates generally to a method for, and pharmaceutical compositions useful in, the prophylaxis and/or treatment of cardiac dysfunction in a mammal by the administration of an effective amount of an agent capable of blocking or inhibiting the effect or release of inositol(1,4,5)trisphosphate in cardiac tissue.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The phosphatidylinositol (PtdIns) turnover pathway is a complex signal transduction system that mediates a diverse range of neurotransmitter and hormone induced responses in a great variety of cells (1). The pathway usually involves the receptor mediated hydrolysis of a membrane phospholipid phosphatidylinositol(4,5)bispophosphate (PtdIns(4,5)P₂) by a specific phospholipase C (PLC) causing the release of two well described second messengers *sn*-1,2-diacylglycerol (DAG) and inositol(1,4,5)trisphosphate, hereinafter denoted as Ins(1,4,5)P₃. DAG activates protein kinase C (PKC) and Ins(1,4,5)P₃ releases calcium from specific intracellular stores. The two arms of the pathway either separately or in concert, control a wide range of cellular responses including contraction, secretion and mitogenesis (2). Ins(1,4,5)P₃ is metabolised rapidly within the cell by dephosphorylation via Ins(1,4)P₂ to Ins(4)P₁ and by phosphorylation to Ins(1,3,4,5)P₄, which is further metabolised to produce a wide range of inositol

phosphate isomers (Figure 1). $\text{Ins}(1,3,4,5)\text{P}_4$ itself has been suggested to have a role in calcium sequestration (3) and roles for other inositol phosphates have been suggested (4).

The PtdIns pathway in the heart is activated by α_1 -adrenergic and muscarinic receptors, endothelin and stretch (5). Compared with non-muscle tissues, the heart is insensitive to $\text{Ins}(1,4,5)\text{P}_3$ in terms of calcium release (6), with the addition of high concentrations of $\text{Ins}(1,4,5)\text{P}_3$ causing a slow leakage of calcium rather than the rapid release seen in most cells (7). In addition, $\text{Ins}(1,4,5)\text{P}_3$ enhances calcium oscillations which are associated with the development of arrhythmias (8, 9), but not calcium induced-calcium release, the mechanism involved in excitation-contraction coupling.

The present inventors have previously reported that PtdIns turnover pathway in the heart differs from that described in non-muscle cells. In heart, phosphorylation products of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ are barely detectable, indicating reduced $\text{Ins}(1,4,5)\text{P}_3$ kinase activity (10). More recent studies have shown that little $\text{Ins}(1,4,5)\text{P}_3$ is released and metabolised either by phosphorylation or dephosphorylation and that most of the accumulated inositol phosphates derive from $\text{Ins}(1,4)\text{P}_2$ rather than $\text{Ins}(1,4,5)\text{P}_3$ (11). These findings suggest that $\text{Ins}(1,4,5)\text{P}_3$ does not play a major role in the healthy heart.

However, there is evidence that the PtdIns pathway and $\text{Ins}(1,4,5)\text{P}_3$ may be more important under pathological conditions such as myocardial ischaemia and reperfusion. α_1 -Adrenoceptor stimulation, which is associated with inositol phosphate release, is capable of inducing arrhythmias both ventricular tachycardia (VT) and ventricular fibrillations (VF) during both ischaemia and reperfusion. Such responses are not observed under normoxic conditions. Ischaemia has been reported to increase α_1 -adrenoceptor density in a number of myocardial preparations (12) as well as increasing responsiveness to noradrenaline stimulation (13). Reperfusion of ischaemic myocardium, is associated with large calcium accumulations, apparently associated with α_1 -adrenoceptor stimulation (14). These calcium accumulations have been implicated in reperfusion-induced injury. Furthermore, α_1 -adrenoceptor

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blockade, by a number of antagonists, has been shown to be anti-arrhythmic under both ischaemic and reperfusion condition (15).

In work leading to the present invention, detailed studies were carried out in isolated perfused rat hearts to determine and characterise the effects of ischaemia and reperfusion on the PtdIns turnover pathway, and to investigate a possible functional role for the second messenger $\text{Ins}(1,4,5)\text{P}_3$ under these pathological conditions.

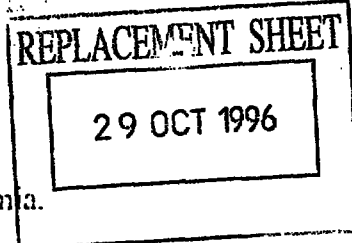
It was surprisingly discovered that reperfusion with oxygenated medium after global ischaemia caused a rapid and quantitatively large rise in $\text{Ins}(1,4,5)\text{P}_3$ which could be correlated in time with the observed development of arrhythmias. Furthermore, it was observed that by blocking the formation of $\text{Ins}(1,4,5)\text{P}_3$ in the heart, using the aminoglycosides neomycin, gentamicin or streptomycin (being agents known to inhibit the formation of $\text{Ins}(1,4,5)\text{P}_3$) it was possible to completely abolish, or significantly reduce arrhythmias occurring following reperfusion of the ischaemic heart.

Accordingly, one aspect of the present invention contemplates a method for the prophylaxis or treatment of cardiac dysfunction in a mammal said method comprising administering to said mammal an effective amount of an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.

Another aspect of the present invention is directed to a pharmaceutical composition useful in the prophylaxis and/or treatment of cardiac dysfunction comprising an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents.

Yet another aspect of the present invention relates to an agent for use in blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.

Still yet another aspect of the present invention is directed to the use of an agent which blocks formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue in the manufacture



of a medicament for the treatment or prophylaxis of cardiac arrhythmia.

Optionally another anti-cardiac dysfunction agent such as an anti-arrhythmic drug is administered in combination with agents which block or inhibit the effect or release of $\text{Ins}(1,4,5)\text{P}_3$. Such agents including, but not limited to, agents which induce thrombolysis for example, streptokinase and tissue plasminogen activator or other anti-arrhythmic agents for example, amiodorone, sotalol and lignocaine.

In a preferred embodiment, the mammal is a human, a livestock animal such as a sheep, cow, horse, donkey or pig or a laboratory test animal such as a mouse, rat, rabbit, guinea pig, cat or dog. Most preferably, the mammal is a human.

The term "cardiac dysfunction" is used in its most general sense and relates to any condition which causes a reduction in effective pumping action of the heart such that tissues are inadequately perfused. More particularly, it covers or may result from the following conditions: cardiac arrhythmia, cardiomyopathy, allograft rejection (cardiac), coronary angioplasty, cardio-pulmonary by-pass surgery and electrophysiological studies. The present invention is particularly directed to the prophylaxis and/or treatment of cardiac arrhythmia. Reference hereinafter to cardiac arrhythmia should be taken as covering all conditions contemplated above.

The present invention is described in detail hereafter in terms of the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in rat cardiac tissue. However, it is understood that it extends to the effect of the $\text{Ins}(1,4,5)\text{P}_3$ inhibitor in all mammals and in particular humans and livestock animals. Accordingly, any reference herein to the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in rats is meant to be applicable to the effect of $\text{Ins}(1,4,5)\text{P}_3$ inhibition in all mammals and in particular humans and livestock animals.

The term "cardiac arrhythmia" is used herein to denote conditions in a mammal where there is an irregular heart action caused by physiological or pathological disturbances in discharge of cardiac impulses or their transmission through the conducting tissue of the heart, and includes tachycardia and fibrillations of particular

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cardiac sites, including but not limited to, atrial, essential, nodal, paroxysmal atrial, paroxysmal nodal, paroxysmal ventricular, polymorphic ventricular, reflex and sinus and ventricular fibrillation. Accordingly, the treatment of cardiac arrhythmia is taken to be returning or maintaining the regular action of the heart by the administration of an effective amount of an agent which blocks or inhibits the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ for a time and under conditions sufficient to maintain, or return, the regular action of the heart. Cardiac arrhythmia may occur following a disease condition or result from trauma or therapy and the present invention is not limited to any one or more causes of cardiac arrhythmia. Typically, cardiac arrhythmia will occur following myocardial infarction, as a consequence of reperfusion following cardio-pulmonary by-pass surgery, and in patients having unstable angina.

The present invention also extends to preventative therapy whereby an agent which blocks the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ is administered to prevent or reduce the likelihood of cardiac arrhythmia developing.

The agents capable of blocking the effect or inhibiting the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ which are used in accordance with the present invention include, for example:

- (i) agents which affect the biochemical release of $\text{Ins}(1,4,5)\text{P}_3$ from the phospholipid $\text{PtdIns}(4,5)\text{P}_2$ to cause reduction in $\text{Ins}(1,4,5)\text{P}_3$ formation. Such agents include the class of compounds known as the aminoglycosides, for example; gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivative thereof, and the class of compounds known as polyamines, for example; spermine or derivative thereof as well as agents which bind the phospholipase C (PLC) enzyme to produce inhibition; and
- (ii) agents which block the effects of $\text{Ins}(1,4,5)\text{P}_3$, for example, agents which block the $\text{Ins}(1,4,5)\text{P}_3$ receptor, or antagonist molecules to $\text{Ins}(1,4,5)\text{P}_3$.

Accordingly, in one preferred embodiment, the present invention provides a method for treating or preventing cardiac arrhythmias in a mammal which method comprises

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administering to said mammal an effective amount of an aminoglycoside for a time and under conditions sufficient to modulate cardiac arrhythmia.

Preferably the aminoglycoside is one of the group comprising gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin, neomycin or derivatives thereof. Even more preferably the aminoglycoside is neomycin or derivative thereof. In a most preferred embodiment the amino glycoside is gentamicin.

In a further preferred embodiment the invention provides a method for treating or preventing cardiac arrhythmias in a mammal which method comprises administering to said mammal an effective amount of a polyamine molecule for a time and under condition sufficient to modulate cardiac arrhythmia.

Preferably, in this aspect of the invention the polyamine is spermine or its derivatives. In a more preferred aspect the polyamine is spermine.

Furthermore, the route of administration of the polyamine or aminoglycoside or other agent blocking or inhibiting the effect or release of $\text{Ins}(1,4,5)\text{P}_3$ is preferably by oral, parenteral or buccal administration but other routes may be equally applicable with only minor modifications to the methods contemplated herein. The effective amount of the agent will depend on the mammal and the condition to be treated, however, the amount to be administered to the mammal will need to be non-toxic. In general, the effective amount of agent will be a daily dose in the range of $0.1 \rightarrow 100 \text{ mg/kg}$, more preferably $1-100 \text{ mg/kg}$ and even more preferably $5-60 \text{ mg/kg}$.

Pharmaceutically acceptable carriers or diluents for use in the composition of this invention are well known to persons skilled in the art, and by way of example are described in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

The method and pharmaceutical compositions described in accordance with the present invention will be useful *inter alia* in the treatment or prevention of cardiac

arrhythmias occurring as a result of various surgical procedures, or underlying cardiac disease.

Agents capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ are generally first tested *in vitro* in perfused heart tissue such as isolated perfused rat hearts. Potentially useful agents are then tested *in vivo* in a small animal model, such as rats, mice, guinea pigs or rabbits. Agents which are still of potential interest following this initial *in vivo* screen may then be tested in a larger animal model such as dogs, horses, pigs or mini-pigs. Clinical trials in human subjects may then follow using agents which still show signs of useful activity. A suitable protocol for clinical trials of anti-arrhythmic agents is described by Heidbuchel *et al.* (36).

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a diagrammatic representation of the phosphatidylinositol turnover pathway. The pathway predominating in heart is boxed.

Figure 2 shows HPLC profiles of TCA extracted inositol phosphates from isolated perfused rat hearts.

A: control heart; B: heart subjected to 30 minutes global myocardial ischaemia. Long arrows indicate elution position of nucleotide standards.

Figure 3 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in cpm/mg protein in isolated perfused rat hearts subjected to increasing periods of global myocardial ischaemia.

Figure 4 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in isolated perfused rat hearts subject to ischaemia and subsequent reperfusion following a 20 minute ischaemic period.

Figure 5 is a graphical representation showing accumulation of individual ^3H -labelled inositol phosphates in isolated perfused rat hearts subject to noradrenaline stimulation (A) ischaemia and reperfusion (B) and ischaemia and reperfusion in the presence of reserpine (C).

Figure 6 is a graphical representation showing the effect of various neomycin concentrations on ^3H -labelled inositol phosphate accumulation within 2 minutes reperfusion following 20 minutes global ischaemia as a percentage of untreated 2 minutes reperfusion response.

Figure 7 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates in cpm in neonatal myocytes. Note the low levels of $\text{Ins}(1,4,5)\text{P}_3$.

Figure 8 is a graphical representation showing the accumulation of ^3H -labelled inositol phosphates cpm in adrenal glomerulosa cells. Note the low levels of $\text{Ins}(1,4,5)\text{P}_3$.

EXAMPLE 1 INOSITOL PHOSPHATE EXPERIMENTS

The inositol phosphate response in heart

Earlier studies in the inventors' laboratory showed that the pathway of inositol phosphate release and metabolism in heart tissue was different from that in other cells in that products of dephosphorylation of the Ca^{2+} -releasing messenger $\text{Ins}(1,4,5)\text{P}_3$ were observed but the phosphorylation products were not (10). The metabolic pathways involved are shown in Figure 1. Subsequent studies provided an explanation for these findings by demonstrating that the primary release product of phosphatidylinositol-specific phospholipase C (PLC) in heart was $\text{Ins}(1,4)\text{P}_2$ rather than $\text{Ins}(1,4,5)\text{P}_3$ (11). This suggested that heart tissue avoided the generation of $\text{Ins}(1,4,5)\text{P}_3$ and provided the basis for the current studies. More recent studies have

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provided a mechanistic basis for this unusual pathway by showing that the "cardiac" pathway correlates with the presence of an atypical GTP-binding coupling protein called Gh (16). Gh couples to a different phospholipase C from PLC- β which couples receptors to typical GTP-binding proteins (17). To further test the association of Gh with the cardiac inositol phosphate pathway, effects of an inhibitor of PLC- β was investigated (18). This compound, U-73122, did not inhibit the accumulation of inositol phosphates in heart tissue under noradrenaline stimulation but was a fully effective inhibitor in isolated neonatal cardiomyocytes which lack Gh and have an inositol phosphate metabolic pathway indistinguishable from non-muscle cells. Thus, the phosphatidylinositol turnover pathway in heart tissue differs from that in other cell types in a number of important aspects.

The inventors investigated the possibility that some alteration in the pathway was responsible for the arrhythmic action of α_1 -adrenergic agonists under conditions of myocardial ischaemia and reperfusion.

A) METHODS

Inositol phosphate response in isolated perfused rat hearts

Adult male sprague dawley rats 250-300g were used. Where indicated, rats were treated with reserpine (5 mg/kg I.P. for 18hrs prior to killing) to deplete endogenous noradrenaline stores.. Rats were heparinized 1U/g I.P. 30 minutes prior to killing by decapitation. Hearts were rapidly excised and immediately chilled in ice-cold saline. Under ice-cold solution, hearts were cannulated via the ascending aorta as described by Langendorff and perfused retrogradely at a rate of 5 ml/min with HEPES-buffered Krebs solution equilibrated with 5%CO₂-95%O₂ at 37°C. Initial perfusion was in a non-recirculating manner to remove internal blood. Hearts were allowed to warm up gradually and, when free of internal blood, transferred to a 37°C water-jacketed organ bath containing carbogenated Krebs medium, and perfusion continued in a recirculating manner. Following a 15 minute equilibration period, hearts were labelled with [³H]-inositol, 2 μ Ci/ml for 2 h. Hearts were monitored throughout the labelling period for strong rhythmic beating. Any hearts which did not fulfil these requirements were discarded.

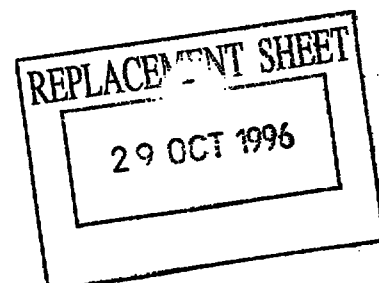
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Following labelling, the [^3H]-inositol-containing medium was removed, and replaced with Krebs medium containing propranolol (10^{-6} M) and lithium chloride (50 mM) to block β -adrenergic receptors and to inhibit InsP metabolism by InsP 1'-phosphatase respectively (20). Hearts were perfused with propranolol, LiCl solution for 10 minutes prior to ischaemia. Antagonists, when administered, were included in this 10 minute preincubation. Normothermic global ischaemia was induced by terminating perfusion for various times up to 30 minutes. Reperfusion was examined following a 20 minute ischaemic period by resuming perfusion at 5 ml/min. Reactions were terminated by freezing hearts in liquid N_2 following excision at the atrioventricular junction. Frozen ventricles were weighed and inositol phosphates extracted as described below.

Extraction of inositol phosphates

Inositol phosphates were extracted from ventricle samples in 3.5 ml ice-cold 5% trichloroacetic acid (TCA), 2.5 mM EDTA, 5 mM phytate solution, as described previously (21). Briefly, ventricles were homogenised with two 10 second passes of a "Polytron" homogenizer separated by 15 second periods on ice. Samples were homogenised further using a Potter-Elvehjem homogeniser followed by brief sonication. Homogenised samples were centrifuged (5000 rpm, 10min, 4°C) to remove TCA insoluble material. Supernatants were collected, and the remaining pellet re-extracted in 1.5 ml TCA, EDTA, phytate. Supernatants were pooled and freon, tri-n-octylamine (1:1 v/v 0.75 ml:1ml TCA solution) was added, mixed thoroughly, and phases separated by low speed centrifugation (2000 rpm, 10 min 4°C). The resultant aqueous phase, containing inositol phosphates was collected and treated with proteinase K (50 $\mu\text{g}/\text{ml}$, 2hr, 50°C). This process was necessary to preserve the chromatography column. Treated samples were passed through a 1 ml Dowex-50 column (4% cross-linked 4-400 mesh size) and eluted with 1 ml water (22). Urea (0.05M final) was added (23) and samples lyophilized prior to high performance liquid chromatography (HPLC) analysis. Recovery of inositol phosphates using this method was greater than 95% for each isomer, as estimated by addition of [^3H]-labelled standards to extractions of unlabelled hearts.



Separation and quantitation of inositol phosphates

Inositol phosphates were separated by an anion-exchange HPLC system using a Whatman Partisil 10 μ SAX column in a Waters Radial Compression Unit, employing a complex ammonium phosphate gradient, pH 3.8 from 0-2M as described previously (24). Inositol phosphates were detected and quantitated using an on-line β -counter (model CR, Radiomatic Instruments). All peaks were identified by comparison with commercial standards. Nucleotide standards AMP, ADP, and ATP were run with samples to assess column performance.

Noradrenaline determination in heart perfusate

Aliquots of heart perfusate for noradrenaline estimation were collected from ischaemic tissue and at various intervals of reperfusion following 20 minutes MI. Samples were analysed by HPLC and quantitated by electrochemical detection as described previously. (25).

Ins(1,4,5)P₃ Mass Analysis

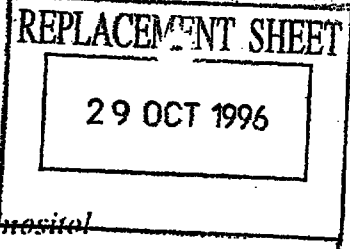
Mass of Ins(1,4,5)P₃ was measured using a competitive binding assay (Amersham TRK 1000) essentially as described previously (26). Ventricle samples were prepared as described above except for the omission of [³H]-label during the 2 hr perfusion of hearts prior to ischaemia, and for the use of 5 mM ATP instead of phytate in the extraction procedure. 5 mM phytate was found to cross-react in the Ins(1,4,5)P₃ assay.

Protein Determination

Protein concentration was determined in aliquots of TCA pellet following InsP extraction using a modified Lowry method with Bovine Serum Albumin as a standard.

Statistics

Values presented are mean $\pm$ SEM, for n=4 unless otherwise stated. Statistical analysis of results involved Students' unpaired "t-test" for all InsP data.



B) RESULTS

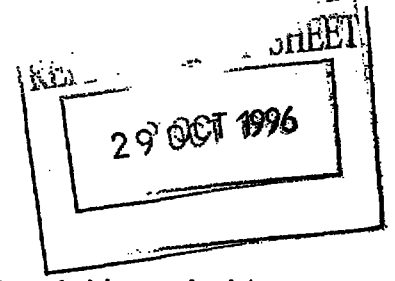
Effects of global ischaemia on the accumulation of [^3H]-labelled inositol phosphates

TCA extracts of [^3H]inositol-labelled hearts contained compounds identified as $\text{Ins}(1/3)\text{P}_1$, $\text{Ins}(4)\text{P}_1$, $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$. As reported previously, [^3H]- $\text{Ins}(1,3,4,5)\text{P}_4$ and its metabolic products were not evident (Figure 2a). Thirty minute perfusion with oxygenated medium resulted in a time dependent increase in [^3H]-label in inositol phosphates (Figure 3). In contrast, global ischaemia caused an altered distribution of inositol phosphates (Figure 2b), while the total amount of label remained unchanged. As shown in Figure 3, decreases in $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$, were observed together with concurrent increase in the degradation products $\text{Ins}(1)\text{P}_1$ and $\text{Ins}(4)\text{P}_1$. Significant changes in [^3H]-labelled inositol phosphates were observed after 5 min of ischaemia. The redistribution process observed under ischaemic conditions was independent of the endogenous noradrenaline released during ischaemia, as it was unaffected by both depletion of noradrenaline stores by reserpinization, or by α_1 adrenoceptor blockade with prazosin.

In contrast to normoxic perfusion, total accumulation of inositol phosphates in myocardium remained unchanged throughout the 30 minute ischaemic period studied, such that the decreases in [^3H]-label of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,4)\text{P}_2$ could be accounted for by increases in the metabolic products $\text{Ins}(1/3)\text{P}_1$ and $\text{Ins}(4)\text{P}_1$. These data indicate that global ischaemia causes an inhibition of PtdIns-PLC activity, such that no inositol phosphate release occurs, even in the presence of locally high concentrations of noradrenaline.

Control of inositol phosphate release under ischaemic conditions

More recent modelling studies have shown that the altered metabolism of inositol phosphates during ischaemia is not related either to the reduced pH or the high ambient extracellular K^+ concentration, under these conditions. Rather, our studies indicated that the change in inositol phosphate profiles could be explained solely by the decrease in ATP content. Moderate anoxia reduced ATP content from 8.3 ± 1



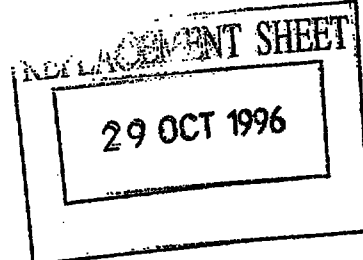
$\mu\text{moles/g}$ dry weight to 4.5 ± 0.4 (mean $\pm$ SEM, $n=3$, $p<.01$) and this resulted in a reduction in content of $\text{Ins}(1,4)\text{P}_2$. Larger reductions in ATP (to 0.718 ± 0.14), as occurs in global ischaemia, resulted in reduced $\text{Ins}(1,4,5)\text{P}_3$ as well as $\text{Ins}(1,4)\text{P}_2$. Both profiles, moderate and severe ischaemia, could be modelled using inhibitors of ATP generation.

Under conditions of moderate ischaemia, release of $\text{Ins}(1,4,5)\text{P}_3$ occurs in response to noradrenaline, the ambient levels of which are high under these conditions. Reperfusion following moderate ischaemia further enhanced this response (see below). Thus, whether $\text{Ins}(1,4,5)\text{P}_3$ release occurs under ischaemic conditions depends on the ATP content of the tissue and thus on the severity of the ischaemia. However, enhanced release is always observed with reperfusion.

Effects of post-ischaemic reperfusion on the accumulation of ^3H -labelled inositol phosphates

Reperfusion with oxygenated medium following 20 minutes global myocardial ischaemia resulted in a rapid increase in accumulation of $[\text{}^3\text{H}]$ -labelled inositol phosphates. This activation was biphasic, with a rapid and transient accumulation occurring between 1 and 2 minutes reperfusion, followed by a slow, quantitatively smaller, secondary accumulation which continued past 20 minutes (Figure 4).

The rapid accumulation of inositol phosphates caused by 2 minutes post-ischaemic reperfusion was dependent on the release of endogenous noradrenaline, as it was prevented by prior reserpine treatment (Figure 5c). The release of $[\text{}^3\text{H}]$ -labelled $\text{Ins}(1,4,5)\text{P}_3$ could be reinstated in reserpinized animals by perfusion with 10^{-4} M noradrenaline. Prazosin ($10 \mu\text{M}$) treatment inhibited the 2 minute reperfusion inositol phosphate response, demonstrating mediation via α_1 -adrenoceptors. Thus, the immediate reperfusion $\text{Ins}(1,4,5)\text{P}_3$ response requires neuronally released noradrenaline and is mediated by α_1 -adrenoceptors.



Even though the reperfusion $\text{Ins}(1,4,5)\text{P}_3$ response was caused by noradrenaline stimulation of α_1 -adrenoceptors, it differed from the noradrenaline response under normoxic conditions in a number of ways. First, the release of inositol phosphates was quantitatively greater. Second, the profile of released inositol phosphates was different in that the primary release product under reperfusion conditions was $\text{Ins}(1,4,5)\text{P}_3$ whereas $\text{Ins}(1,4)\text{P}_2$ predominates under normoxic conditions (11). Third, inositol phosphate release under reperfusion conditions was inhibited by a number of compounds which did not inhibit release under normoxic conditions, as discussed in detail below (Table 1).

The 2 minute reperfusion response required the reintroduction of oxygen, as reperfusion with O_2 free (N_2 saturated) medium, did not activate inositol phosphate release. This finding is of great interest as recent reports indicate that oxidants increase the sensitivity of $\text{Ins}(1,4,5)\text{P}_3$ receptors (27). Thus, by two different mechanisms, increased release and increased sensitivity, $\text{Ins}(1,4,5)\text{P}_3$ can be more effective and more damaging under conditions of ischaemia and reperfusion.

Noradrenaline concentration under conditions of ischaemia and reperfusion

Endogenous noradrenaline is known to accumulate in myocardial extracellular space under ischaemic conditions (28). In the current studies the noradrenaline concentration was measured in the tissue and surrounding perfusate, to verify such accumulation in the inventors' model. Control values ($9.87 \pm 1.54 \times 10^{-10} \text{ M}$) were sampled following 10 minutes recirculating perfusion with Krebs' medium containing propranolol and LiCl prior to initiation of ischaemia. 20 minutes global ischaemia caused an accumulation of noradrenaline within the myocardial extracellular space ($8.19 \pm 2.03 \times 10^{-7} \text{ M}$). This noradrenaline was released rapidly with reperfusion and levels remained elevated at 2 minute reperfusion ($1.26 \pm 0.28 \times 10^{-8} \text{ M}$), although diluted around 40 fold when released into the 10 ml recirculating perfusate. Perfusate noradrenaline levels dropped significantly following 20 minutes reperfusion ($1.06 \pm 0.5 \times 10^{-9} \text{ M}$) to a level not different from those of controls ($P > 0.3$). Noradrenaline concentrations in the perfusate after 2 minutes reperfusion averaged

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$1.54 \pm 0.5 \times 10^{-8}$ M. Such concentrations are not effective in stimulating inositol phosphate release from non-ischaemic myocardium. This demonstrates an increased sensitivity or availability of ventricular α_1 -adrenoceptors during 2 minutes post-ischaemic reperfusion. Previous studies in the inventors' laboratory have shown that nerve stimulation of non-ischaemic myocardium produces little increase in Inositol phosphate accumulation. Thus, cardiac α_1 -adrenoceptors responds to neuronally released noradrenaline only under conditions of ischaemia and reperfusion.

Effect of aminoglycosides on the reperfusion-induced [3 H]-labelled Inositol phosphate response

The aminoglycoside antibiotic neomycin has been well characterised as an inhibitor of Ins(1,4,5) P_3 release (29)(30). The effects of neomycin on the 2 minute reperfusion-induced [3 H]-labelled Inositol phosphate response were investigated. Neomycin (0.05 mM), added 10 minutes prior to ischaemia and maintained throughout the procedure, did not significantly inhibit the accumulation of [3 H]-labelled inositol phosphates. Higher concentrations, 0.5 mM and 5 mM, produced a 45% and 55% average inhibition of [3 H]-labelled Inositol phosphate accumulation respectively, compared with 2 minutes post-ischaemic reperfusion in the absence of neomycin (Figure 6). These results are different from findings in healthy heart tissue where 5 mM neomycin caused a 20% reduction in accumulation of [3 H]Ins(1,4,5) P_3 but was without effect on the accumulations of Inositol phosphate₁ and Inositol phosphate₂. Lower concentrations were ineffective. These data indicate an increase in sensitivity of myocardial tissue to neomycin of more than 10 fold during post-ischaemic reperfusion.

Subsequent experiments were performed to establish whether this inhibitory effect of neomycin was a class effect of aminoglycosides and if so whether other members might have higher potency. Streptomycin and gentamicin were chosen for these studies with a view to using available plasma assays for *in vivo* studies. As is shown in Table 1, both gentamicin and streptomycin were more potent inhibitors of reperfusion-induced Ins(1,4,5) P_3 release than neomycin. Thus, inhibition of reperfusion-induced Ins(1,4,5) P_3 release is a class effect of the aminoglycosides.

As shown in Table 1, the polyamine, spermine, also was tested and shown to be inhibitory.

More recent studies have investigated the possible contribution of thrombin to the reperfusion arrhythmias. The studies described above used medium-perfused hearts. However, *in vivo* myocardial ischaemia and reperfusion are associated with local high concentrations of thrombin associated with the thrombus formation responsible for the occlusion. Recent studies from other laboratories have pointed to a direct pro-arrhythmic effect of the thrombus *in vivo*. Studies in the inventors' laboratory have shown that thrombin directly stimulates release of $\text{Ins}(1,4,5)\text{P}_3$ in heart tissue and that this release is inhibited by gentamicin at similar concentrations to those required for inhibition of the noradrenaline response under reperfusion conditions.

In summary, reperfusion of hearts following global ischaemia causes release of $\text{Ins}(1,4,5)\text{P}_3$ and this can either be effected either by neuronally released noradrenaline or by thrombin. Both of these agents would be expected to be present in the heart under ischaemic conditions *in vivo*.

EXAMPLE 2

MYOCARDIAL FUNCTION *IN VITRO*

A) METHODS

Reperfusion-induced arrhythmias in in situ perfused rat hearts

A model of *in situ* perfused rat hearts subjected to 20 minutes coronary artery occlusion to induce ischaemia, followed by reperfusion was used to investigate reperfusion-induced arrhythmias.

Rats (250-300g) were anaesthetised (pentobarbitone 60mg/kg, I.P.) and heparinised (200U/rat I.V.) The thorax was then opened and a metal cannula (ID 1 mm) inserted and tied into the ascending aorta, to commence Langendorff perfusion *in situ* (31). The interval from opening the chest to commencing heart perfusion was between 50 and 70 seconds. The perfusate was Krebs buffer, with coronary flow controlled by a Ismatac peristaltic pump at a rate of 5 ml/g heart weight/min for the non-ischaemic

perfusion. At this flow rate the coronary perfusion pressure was between 32-38 mmHg, measured by pressure transducer (Gould Statham model p23) connected to the sidearm of the perfusion line. Following aortic cannulation, pulmonary vessels were tied off. In some preparations, ventricular pressure was measured by inserting a Millar micro-tip transducer catheter into the left ventricular cavity via the apex. All parameters- coronary perfusion pressure, ventricular pressure, and epicardial ECG, were constantly recorded on a Grass model 7 polygraph. Two hearts were perfused and simultaneously studied.

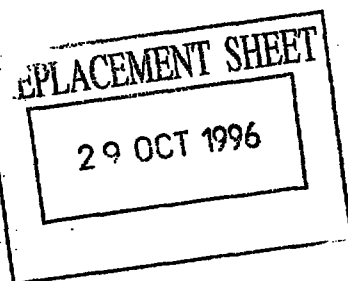
A silk suture (4.0) was positioned loosely around the left coronary artery 2-3 mm from its origin. Two silk releasing rings were placed around the suture for subsequent release of occlusion. Successful occlusion of the artery, inducing regional ischaemia, was indicated by increases in coronary perfusion pressure of at least 30%. Perfusion pressure was adjusted to non-ischaemic perfusion levels during the ischaemic period.

Drugs were added to the perfusion via a Harvard Model 22 pump. The drug flow was less than 1% of perfusion flow to ensure constant perfusate parameters. Unless mentioned otherwise, the drugs were present 10 minutes before, and during the entire ischaemic and reperfusion periods.

Heart preparations were allowed to stabilise for at least 20 minutes prior to commencing studies. Following 10 minute drug incubation, the left coronary artery was ligated to induce local ischaemia, for a period of 20 minutes. At the end of this ischaemic period, the ligature was released and hearts perfused at pre-ischaemic flow for a period of 5 minutes. Ventricular arrhythmias, occurring during ischaemia or during the first 2 minutes of reperfusion were defined according to the Lambeth Conventions (32).

Statistics

Incidence of arrhythmias was analysed using a χ^2 test, with $n \geq 8$ for each group.



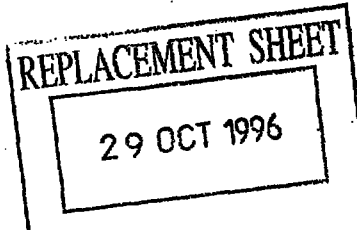
B) RESULTS

Effects of aminoglycosides on reperfusion-induced arrhythmias

The initial reperfusion-induced release of the second messenger $\text{Ins}(1,4,5)\text{P}_3$ occurs at a time point associated with reperfusion-induced arrhythmias. Based on the considerations outlined above, this suggested a possible role for $\text{Ins}(1,4,5)\text{P}_3$ in the development of arrhythmias. Therefore, reperfusion-induced arrhythmias were quantitated, and the effects of compounds which inhibit the $\text{Ins}(1,4,5)\text{P}_3$ response under these conditions were investigated.

An ischaemic model of coronary occlusion and subsequent reperfusion in *in situ* perfused rat hearts was used, and provided a high percentage of ventricular arrhythmias under both ischaemic and reperfused conditions. Twenty minutes regional ischaemia by coronary artery occlusion resulted in 83% (10/12) incidence of VT. As shown in Table 2, reperfusion following this ischaemic period resulted in VT and degenerative VF in 10 out of the 12 rats studied. In all cases, a large number of premature ventricular beats were observed. None of the hearts which became arrhythmic with VT and subsequent VF recovered during the 5 minute reperfusion period studied.

The effects of neomycin on these reperfusion-induced arrhythmias are shown in table 2. Neomycin at 0.05 mM had no significant effect on the induced arrhythmias with 6 out of 8 rats studied showing both severe VT and VF throughout reperfusion. In contrast, and in parallel with [^3H]-labelled Inositol phosphate studies, higher concentrations of neomycin (0.5 mM) prevented the occurrence of reperfusion-induced arrhythmias with none of the 10 rats studied exhibiting VT or VF. Similar effects were observed with 5 mM neomycin. The inhibition of reperfusion-induced arrhythmias by 0.5 mM neomycin was independent of any haemodynamic effects as heart rate and ventricular pressure, while dropping upon initial introduction of the drug, recovered to levels not significantly differing from control during the 10 minute incubation period prior to occlusion.



As is shown in Table 1, streptomycin, gentamicin and spermine also inhibited reperfusion arrhythmias in parallel with their effects on reperfusion-induced release of $\text{Ins}(1,4,5)\text{P}_3$. Gentamicin was the most potent agent tested followed by streptomycin. At the higher concentrations gentamicin and streptomycin also produced a partial inhibition of arrhythmias occurring during the ischaemic phase. These data further suggest the possibility of a contribution by $\text{Ins}(1,4,5)\text{P}_3$ to these arrhythmias in addition to its apparently essential role in reperfusion arrhythmias.

Thus, inhibition of hydrolysis of phosphatidylinositol(4,5)bisphosphate ($\text{PtdIns}(4,5)\text{P}_2$) under reperfusion conditions prevents the development of reperfusion arrhythmias.

Effect of staurosporine on reperfusion-induced arrhythmias

In addition to $\text{Ins}(1,4,5)\text{P}_3$, the second messenger DAG is also produced upon hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$. DAG activates various isoforms of protein kinase C which in turn controls a number of cellular processes. Thus, it was possible that the anti-arrhythmic effects of the aminoglycosides was be due to inhibition of DAG production and subsequent PKC activation. Therefore, the effects of a potent protein kinase C inhibitor staurosporine (33) on reperfusion-induced arrhythmias was investigated. Staurosporine (10^{-7} M, a concentration known to inhibit PKC in the myocardium under reperfusion conditions (34)) did not significantly alter the percentage incidence of reperfusion-induced arrhythmias from untreated controls as shown in Table 1.

TABLE 1:

	Ins(1,4,5)P ₃		Reperfusion-induced arrhythmias			
	mass	p	VT		VF	
			%	p	%	p
control	200 ± 18		83		83	
0.05 mM neomycin	184 ± 7	ns	75	ns	70	ns
0.5 mM neomycin	121 ± 11	<.025	0	<.005	0	<.005
5 mM neomycin	104 ± 12	<.025	0	<.005	0	<.005
0.5 mM spermine	182 ± 4	ns	63	ns	36	<.025
5 mM spermine	160 ± 9	<.05	30	<.025	20	<.005
PTX (25 µg/kg)	128 ± 28	<.05	40	ns	10	<.001
0.015 mM genta	145 ± 2	<.05	58	<.025	25	<.025
0.15 mM genta	136 ± 22	<.05	27	<.025	27	<.025
1.5 mM genta	102 ± 17	<.01	17	<.025	8	<.025
0.015 mM strep	201 ± 16	ns	45	ns	27	<.025
0.15 mM strep	167 ± 5	<.05	25	<.025	6	<.025
1.5 mM strep	94 ± 11	<.01	45	<.025	27	<.025
.1µM stauro	nd		83	ns	69	ns

Table 1: Effects of a number of agents on the release of $\text{Ins}(1,4,5)\text{P}_3$ and reperfusion-induced arrhythmias. Values shown are $\text{Ins}(1,4,5)\text{P}_3$ content in pmol/mg protein, mean $\pm$ SEM of data from 4 different hearts 2 minutes after commencing reperfusion following 20 min global ischaemia, and % incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) in the experimental group ($n \geq 8$). Pertussis toxin (PTX) was given by I.P. injection 48 h prior to experimentation. "Genta" is gentamicin, "strep" is streptomycin and "stauro" is staurosporine. Statistical analysis of $\text{Ins}(1,4,5)\text{P}_3$ mass data was performed using Student's unpaired "t" test and analysis of arrhythmia data using a χ^2 test. p values are relative to control.

Effect of inhibition of phospholipase C β

To date, few direct inhibitors of phosphatidylinositol-specific phospholipase C have been available. However, one compound, a steroid derivative known as U-73122, has been shown to inhibit PLC- β isoforms. As explained above, this agent did not inhibit release of $\text{Ins}(1,4,5)\text{P}_3$ in intact heart preparations and was without anti-arrhythmic activity. These data are summarised in Table 2.

Taken together, the data derived from a number of different compounds show that inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ causes an inhibition of reperfusion arrhythmias.

Arrhythmic effect of thrombin under reperfusion conditions

The finding that thrombin directly stimulated release of $\text{Ins}(1,4,5)\text{P}_3$ in heart indicates the possibility that thrombin might cause arrhythmias directly *via* this mechanism. Experiments were performed using hearts from reserpinized rats so that arrhythmic events initiated by noradrenaline were eliminated. Reperfusion arrhythmias were not observed in hearts from reserpinized animals. However, when thrombin (2 U/ml) was added to the perfusate a high incidence of reperfusion arrhythmias was observed. Furthermore, these arrhythmias were inhibited by gentamicin, indicating a relationship to release of $\text{Ins}(1,4,5)\text{P}_3$ (Table 2).

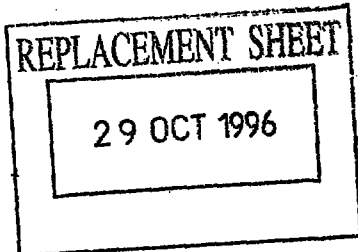


TABLE 2:

	VT%	VF%	n	p
reserpine	44	0	9	
thrombin (2 U/ml)	89	67	9	<.05
thrombin + gentamicin	60	0	5	<0.05*
control	100	63	46	
U-73122 (10^{-6} M)	88	61	12	ns

Table 2: Arrhythmic effect of thrombin and its inhibition by gentamicin. * relative to thrombin. Other p values are relative to control.

In summary, agents which stimulate release of $\text{Ins}(1,4,5)\text{P}_3$ under reperfusion conditions are pro-arrhythmic and agents which inhibit release are anti-arrhythmic.

EXAMPLE 3

IN VIVO STUDIES

A) METHODS

Gentamicin was chosen for studies of anti-arrhythmic action of aminoglycoside *in vivo* because it is the most potent agent *in vitro* and because an assay method for measuring plasma levels is currently available. Rats were not heparinised so that effects of thrombin will be contributory to observed arrhythmias.

In vivo model for coronary artery occlusion and reperfusion

Rats were anaesthetised with pentobarbitone (60 mg/Kg IP) and artificially ventilated with room air. The chests were opened via the forth intercostal space, and the heart exposed. A 4.0 suture was placed around the left coronary artery and then passed through a guided polyethylene cannula. The thorax was closed leaving the threaded cannula outside the chest. The right carotid artery was cannulated for recording of

arterial blood pressure. After stabilisation for 20-30 minutes, the left coronary artery was ligated by pulling the ends of the ligature through the tube and securing them by clamping the tube together with the threads. The ischaemic period was 10 minutes followed by reperfusion. Arterial blood pressure and a lead II ECG was recorded 3 minutes before and during ischaemia, and for the first 5 minutes of reperfusion. In the *in vivo* rat model, the highest incidence of reperfusion arrhythmias occurs after a 10 minute period of coronary artery occlusion. The size of the ischaemic area was determined at the end of the experiment by the dye method. There was no difference in ischaemic area between hearts from control animals and those receiving gentamicin. Gentamicin was infused at a dose of 2.5-10 mg/Kg/min prior to initiation of ischaemia and maintained throughout the protocol. This caused a moderate reduction in blood pressure and heart rate.

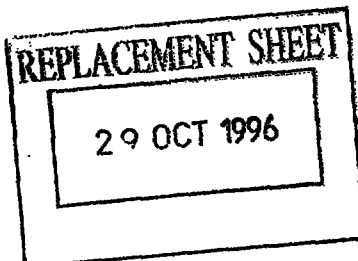
As is shown in Table 3, gentamicin inhibited the development of reperfusion arrhythmias *in vivo*. The arrhythmias generated under these conditions would be expected to be initiated by noradrenaline and by thrombin and possibly also by other agents. It is important to note that gentamicin was effective also against the arrhythmias occurring under ischaemic conditions. As indicated above, release of $\text{Ins}(1,4,5)\text{P}_3$ also can occur under these conditions, depending on the severity of the ischaemia.

TABLE 3:

	VT%	VF%	n	p
control	100	68	13	
gentamicin	30	0	10	<0.01

Table 3: Effect of gentamicin on reperfusion arrhythmias *in vivo*.

In summary, gentamicin is a very effective agent in inhibiting reperfusion arrhythmias *in vivo*.



EXAMPLE 4
CARDIAC SELECTIVE PLC INHIBITOR

Studies from the inventors show that inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ under conditions of post-ischaemic reperfusion inhibits the development of reperfusion arrhythmias. This could be achieved either by directly binding to and inhibiting the PLC enzyme which hydrolyses $\text{PtdIns}(4,5)\text{P}_2$ to $\text{Ins}(1,4,5)\text{P}_3$ or by binding $\text{PtdIns}(4,5)\text{P}_2$ and thereby inhibiting PLC activity less directly. Given that the PtdIns turnover pathway occurs in all cell types, it is important to incorporate some selectivity into inhibitory agents if they are to be effective clinical tools. The PLC which is coupled to α_1 -adrenoceptors in heart provides one possible target for a direct enzyme inhibitor because it is a different enzyme from any of the currently described PLC classes (17). However, agents like the aminoglycosides which bind $\text{PtdIns}(4,5)\text{P}_2$ can also be appropriate. The inventors have shown that the potency of these compounds increases by more than an order of magnitude under ischaemic conditions. Furthermore, even though the aminoglycosides act by binding $\text{PtdIns}(4,5)\text{P}_2$, the relative potencies of the aminoglycosides in causing an inhibition of PLC activity differ for the different PLC isoforms and neomycin rather than gentamicin is generally most potent (Table 4). $\text{PtdIns}(4,5)\text{P}_2$ is an activator rather than a substrate for phosphatidylcholine-specific phospholipase D. For this enzyme, aminoglycosides have been shown to inhibit the activation by $\text{PtdIns}(4,5)\text{P}_2$ but the rank order of potencies was different from observed in studies of PLC. Thus, even though the aminoglycosides act by binding $\text{PtdIns}(4,5)\text{P}_2$, this binding clearly affects different enzymes which interact with $\text{PtdIns}(4,5)\text{P}_2$ differently.

Thus, the development of a suitably selective agent will be possible by either of these mechanisms.

TABLE 4:

	PC-PLD	PI-PLC	PI-PLC	PI-PLC
	brain	kidney cy	kidney me	heart rep
neomycin	65	30	200	400
streptomycin	125	380	2850	150
gentamicin	ND	130	500	15

Table 4: Relative potencies of neomycin, streptomycin and gentamicin in inhibiting PLC hydrolysing $\text{PtdIns}(4,5)\text{P}_2$ or in inhibiting the stimulatory effect of $\text{PtdIns}(4,5)\text{P}_2$ on PLC activity. Values shown are average EC_{50} values $\times 10^{-6}$ M. Kidney cy and kidney me refer to cytosolic and membrane enzymes, respectively. Heart rep refers to hearts under reperfusion conditions. Brain and kidney data are from the literature (35).

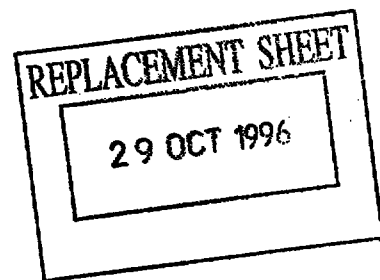
EXAMPLE 5

ANTI-ARRHYTHMIC AGENTS

Currently available anti-arrhythmic agents are only poorly effective and their use is often limited by significant side effects. Furthermore, these agents target primarily the arrhythmias occurring during ischaemia and are essentially ineffective against the arrhythmias which occur following reperfusion (36). Gentamicin *in vitro* and *in vivo* is very effective in inhibiting reperfusion arrhythmias. These findings set the stage for the development of an entirely new class of anti-arrhythmic agent based on the inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$. Furthermore, it is likely that arrhythmias developed *in vivo* are mechanistically more complex than those observed in *in vitro* model systems and that $\text{Ins}(1,4,5)\text{P}_3$ may have a wider role than classical "reperfusion" arrhythmias, as suggested both by our modelling studies and by the *in vivo* experiments. Conditions such as unstable angina likely involve repeated

transient occlusions of the coronary artery and thus repeated episodes of ischaemia and reperfusion. Thus, agents which act by inhibition of release of $\text{Ins}(1,4,5)\text{P}_3$ may have wider clinical implications than classical "reperfusion" arrhythmias.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.



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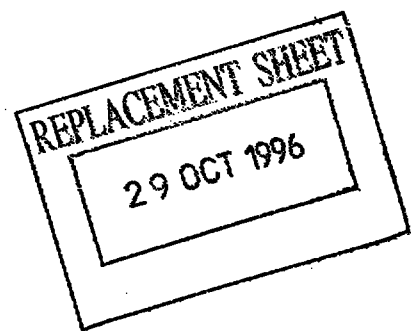
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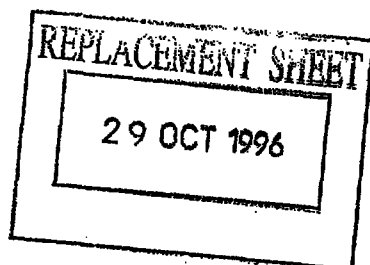
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CLAIMS:

1. A method for the prophylaxis or treatment of cardiac dysfunction in a mammal said method comprising administering to said mammal an effective amount of an agent capable of blocking formation or release of inositol (1,4,5) triphosphate ($\text{Ins}(1,4,5)\text{P}_3$) in cardiac tissue.
2. A method according to claim 1 wherein the cardiac dysfunction is a cardiac arrhythmia.
3. A method according to claim 2 wherein the cardiac arrhythmia comprises tachycardia and fibrillations of cardiac sites.
4. A method according to claim 1 or 2 or 3 wherein the agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
5. A method according to claim 4 wherein the agent is an aminoglycoside.
6. A method according to claim 5 wherein the aminoglycoside is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.
7. A method according to claim 4 wherein the agent is a polyamine.
8. A method according to claim 7 wherein the polyamine is spermine or a derivative thereof.
9. A method according to claim 1 or 2 or 3 wherein the agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
10. A method according to claim 9 wherein the agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

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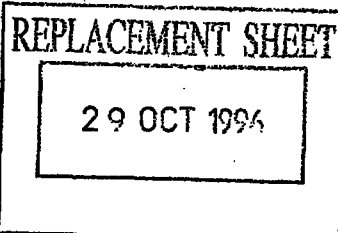
11. A method according to claim 9 wherein the agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
12. A method according to claim 1 or 2 or 3 wherein the agent is administered by oral, parenteral or buccal administration.
13. A method according to claim 1 or 2 or 3 wherein the effective amount is from about 0.1 to about 100 mg per kilogram of body weight of the mammal.
14. A method according to claim 13 wherein the effective amount is from about 5 to about 60 mg per kilogram of body weight of the mammal.
15. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an anti-arrhythmia drug.
16. A method according to claim 15 wherein the anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.
17. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an agent capable of effecting thrombolysis.
18. A method according to claim 17 wherein the agent is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
19. A method according to claim 1 or 2 or 3 further comprising the sequential or simultaneous administration of an agent which increases cardiac systolic and/or diastolic function.
20. A method according to claim 19 wherein said agent is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.
21. A method according to claim 1 or 2 or 3 wherein the mammal is a human.



22. An agent for use in blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue.
23. An agent according to claim 22 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
24. An agent according to claim 23 wherein said agent is an aminoglycoside.
25. An agent according to claim 24 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.
26. An agent according to claim 23 wherein said agent is a polyamine.
27. An agent according to claim 26 wherein said agent is spermine or a derivative thereof.
28. An agent according to claim 22 wherein said agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
29. An agent according to claim 28 wherein said agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ -receptor.
30. An agent according to claim 28 wherein said agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
31. An agent according to claim 22 or 23 or 28 wherein said agent further comprises another anti-arrhythmia drug.
32. An agent according to claim 31 wherein said other anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.

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33. An agent according to claim 22 or 23 or 28 wherein said agent further comprises a component capable of effecting thrombolysis.
34. An agent according to claim 33 wherein the component is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
35. An agent according to claim 22 or 23 or 29 wherein said agent further comprises a component which increases cardiac systolic and/or diastolic function.
36. An agent according to claim 35 wherein said component is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.
37. A pharmaceutical composition useful in the prophylaxis and/or treatment of cardiac dysfunction comprising an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents.
38. A pharmaceutical composition according to claim 37 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
39. A pharmaceutical composition according to claim 38 wherein said agent is an aminoglycoside.
40. A pharmaceutical composition according to claim 39 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.
41. A pharmaceutical composition according to claim 37 wherein said agent is a polyamine.
42. A pharmaceutical composition according to claim 41 wherein said agent is spermine or a derivative thereof.

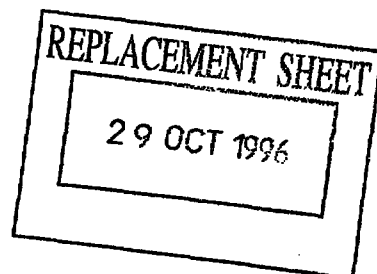
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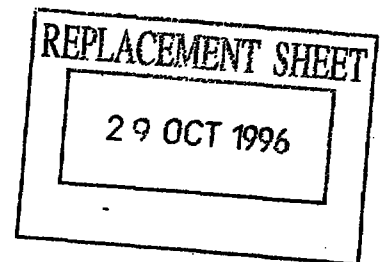
43. A pharmaceutical composition according to claim 37 wherein said agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.
44. A pharmaceutical composition according to claim 43 wherein said agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.
45. A pharmaceutical composition according to claim 43 wherein said agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.
46. A pharmaceutical composition according to claim 37 or 38 or 43 wherein said agent further comprises another anti-arrhythmia drug.
47. A pharmaceutical composition according to claim 46 wherein said other anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.
48. A pharmaceutical composition according to claim 38 or 39 or 43 wherein said composition further comprises an agent capable of effecting thrombolysis.
49. A pharmaceutical composition according to claim 48 wherein the agent is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
50. A pharmaceutical composition according to claim 37 or 38 or 43 wherein said composition further comprises an agent which increases cardiac systolic and/or diastolic function.
51. A pharmaceutical composition according to claim 50 wherein said agent is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.





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52. Use of an agent which blocks formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue in the manufacture of a medicament for the treatment or prophylaxis of cardiac arrhythmia.
53. A use according to claim 52 wherein the agent is an aminoglycoside.
54. A use according to claim 52 wherein the agent is a polyamine.



**A METHOD FOR TREATING CARDIAC DYSFUNCTION AND
PHARMACEUTICAL COMPOSITIONS USEFUL THEREFOR**

ABSTRACT

The present invention relates generally to a method for, and pharmaceutical compositions useful in, the prophylaxis and/or treatment of cardiac dysfunction in a mammal by the administration of an effective amount of an agent capable of blocking or inhibiting the effect or release of inositol(1,4,5)*tris*phosphate in cardiac tissue.

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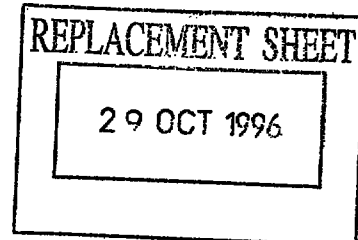


FIGURE 1

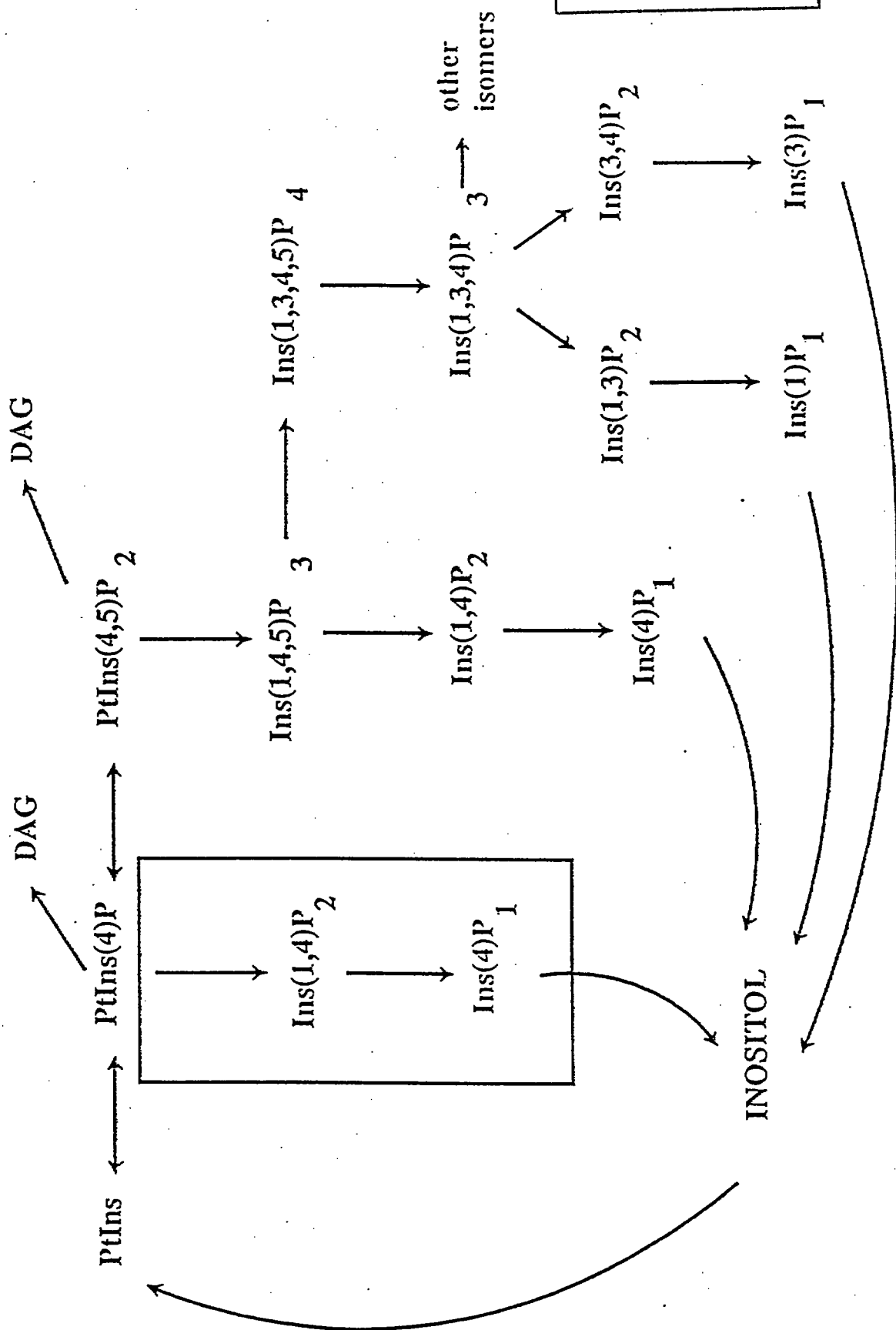


FIGURE 2

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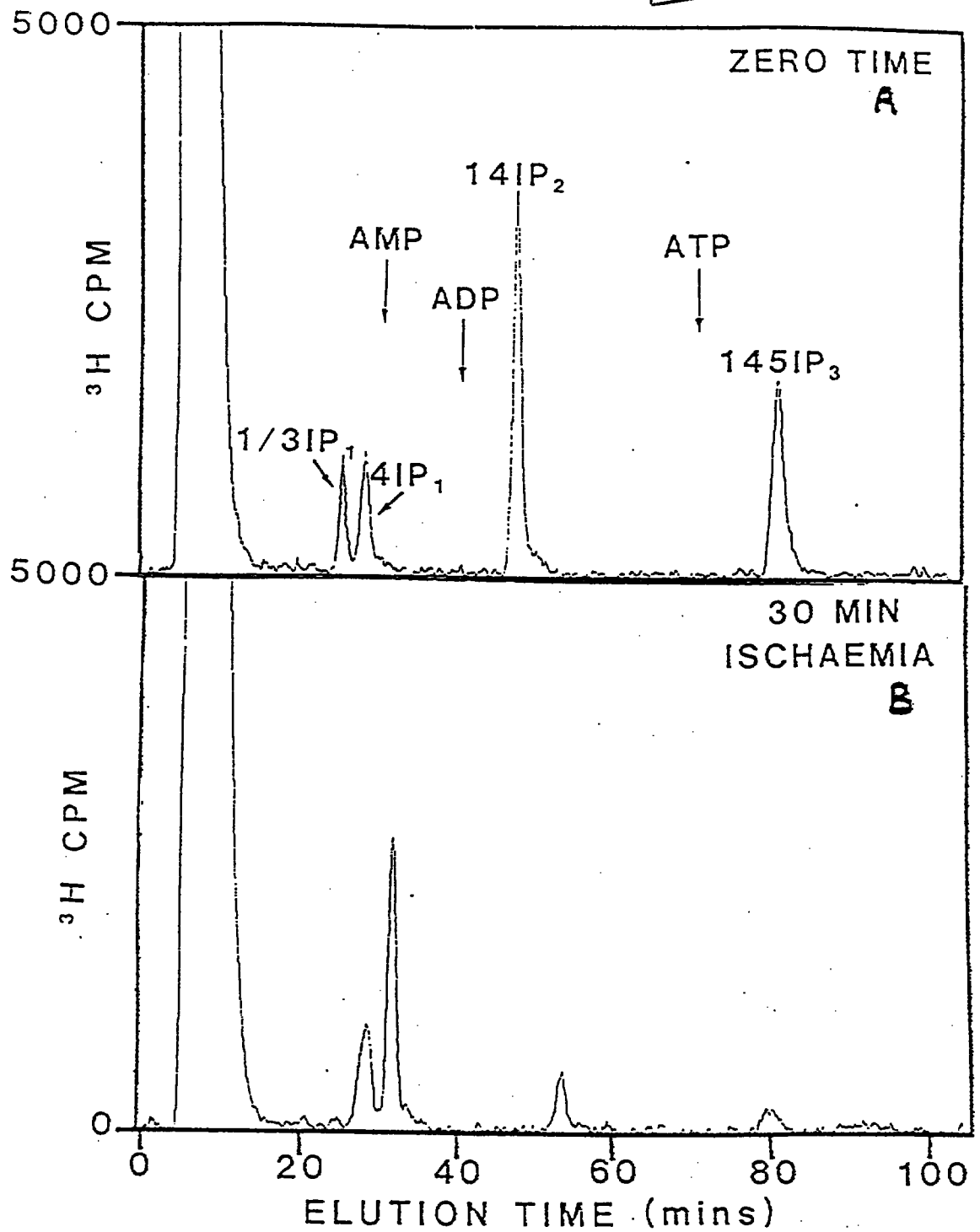
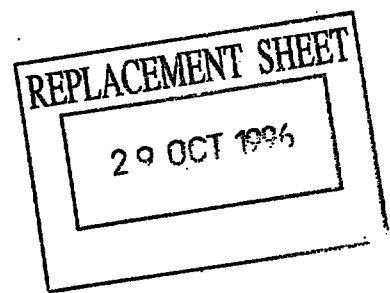
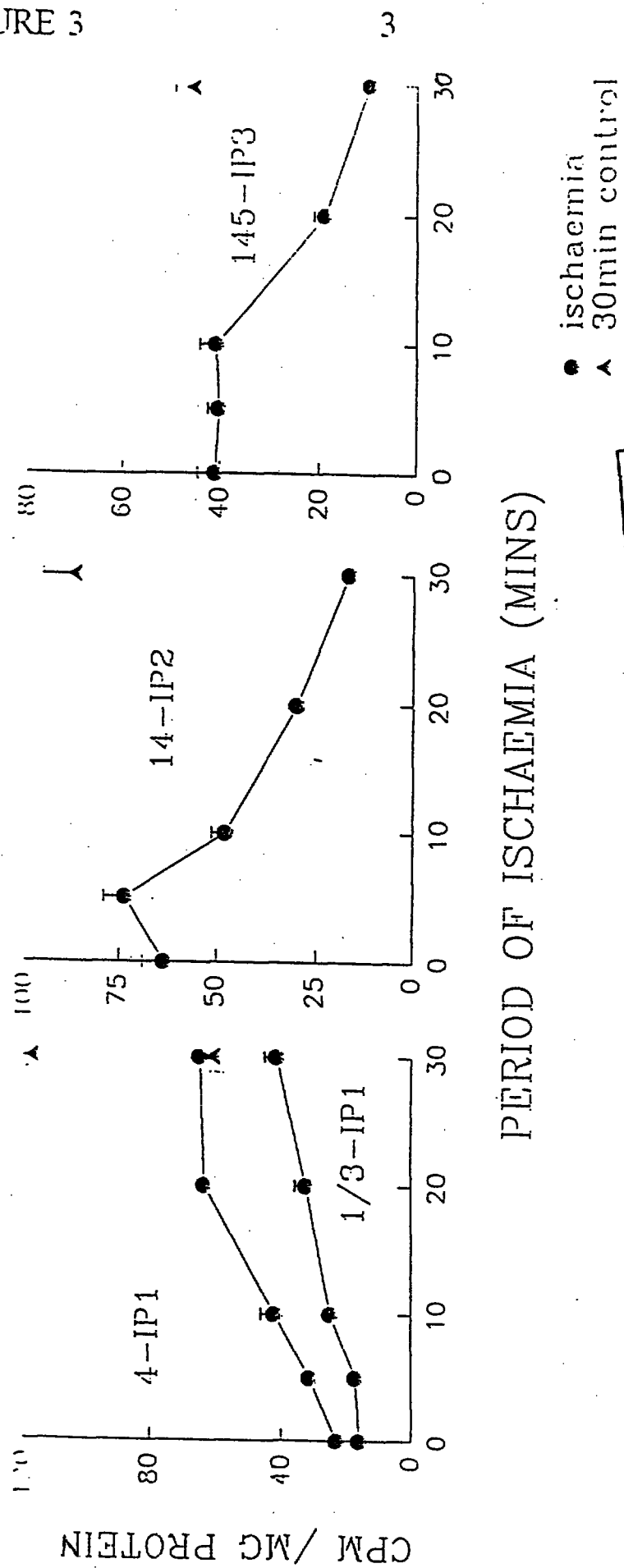


FIGURE 3



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FIGURE 4

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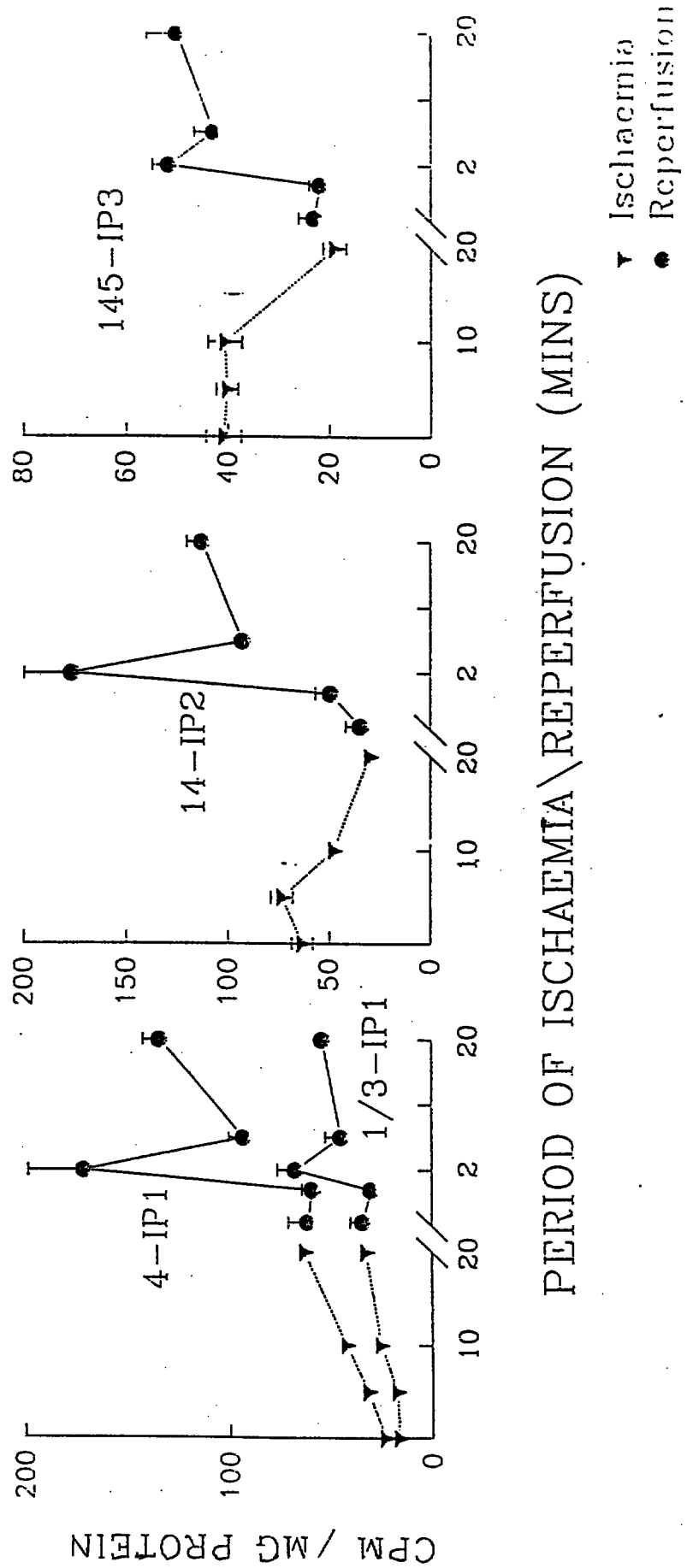


FIGURE 5

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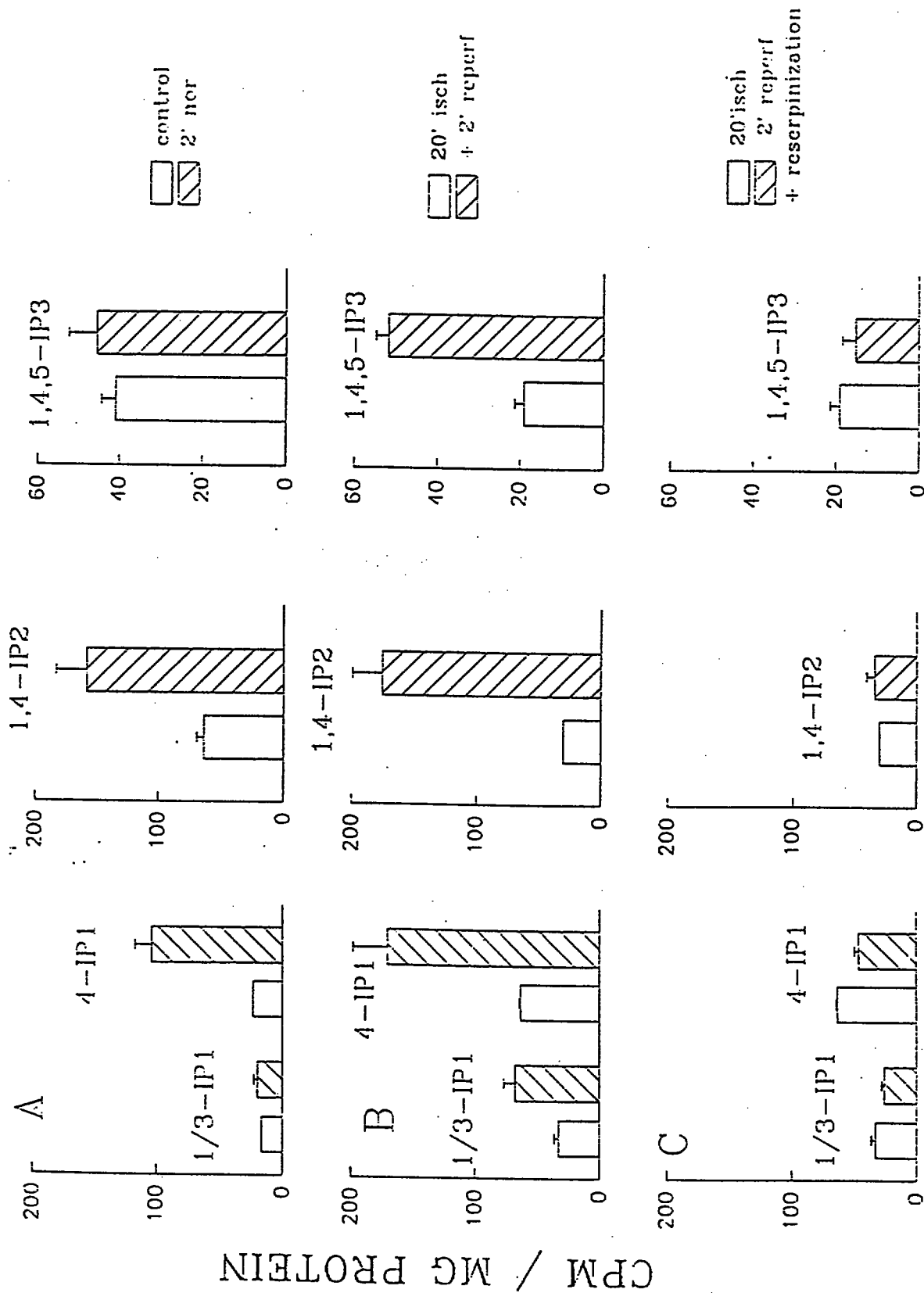
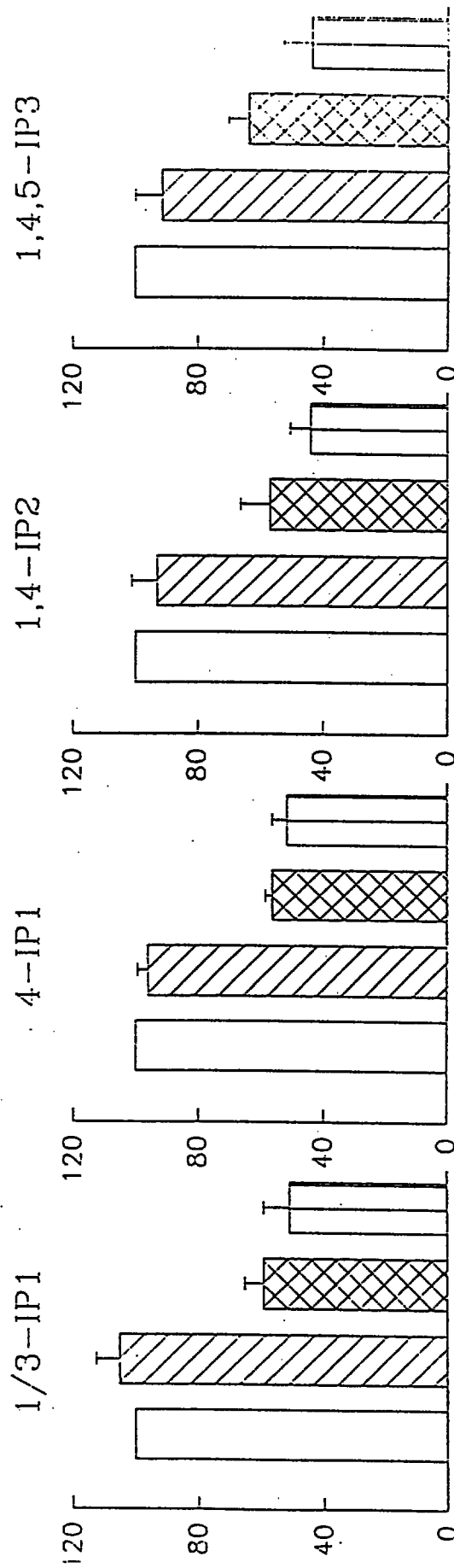


FIGURE 6

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20' ischaemia + 2' reperfusion
+ 0.05mM Neomycin
+ 0.5mM Neomycin
+ 5mM Neomycin

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FIGURE 7

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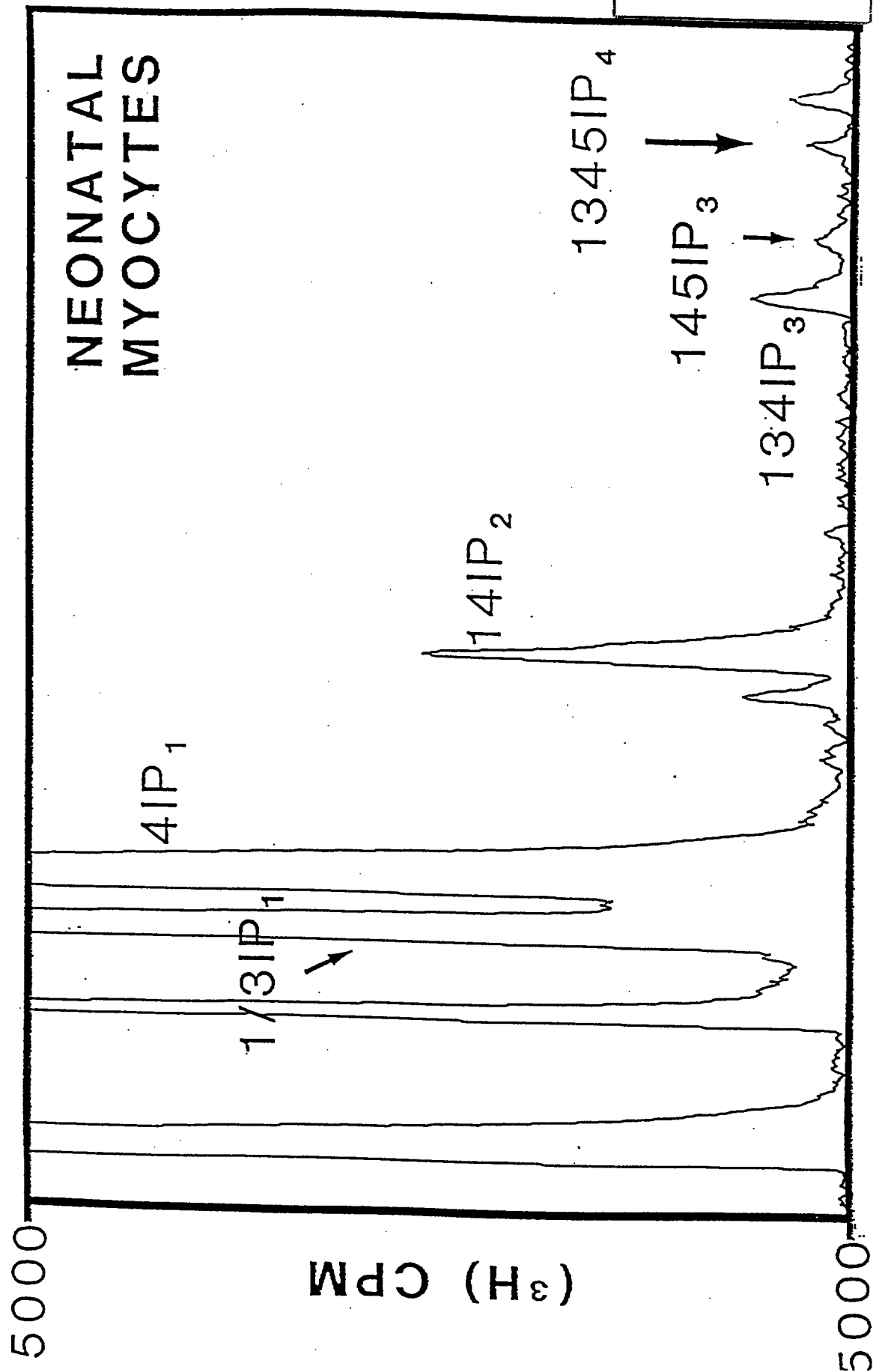
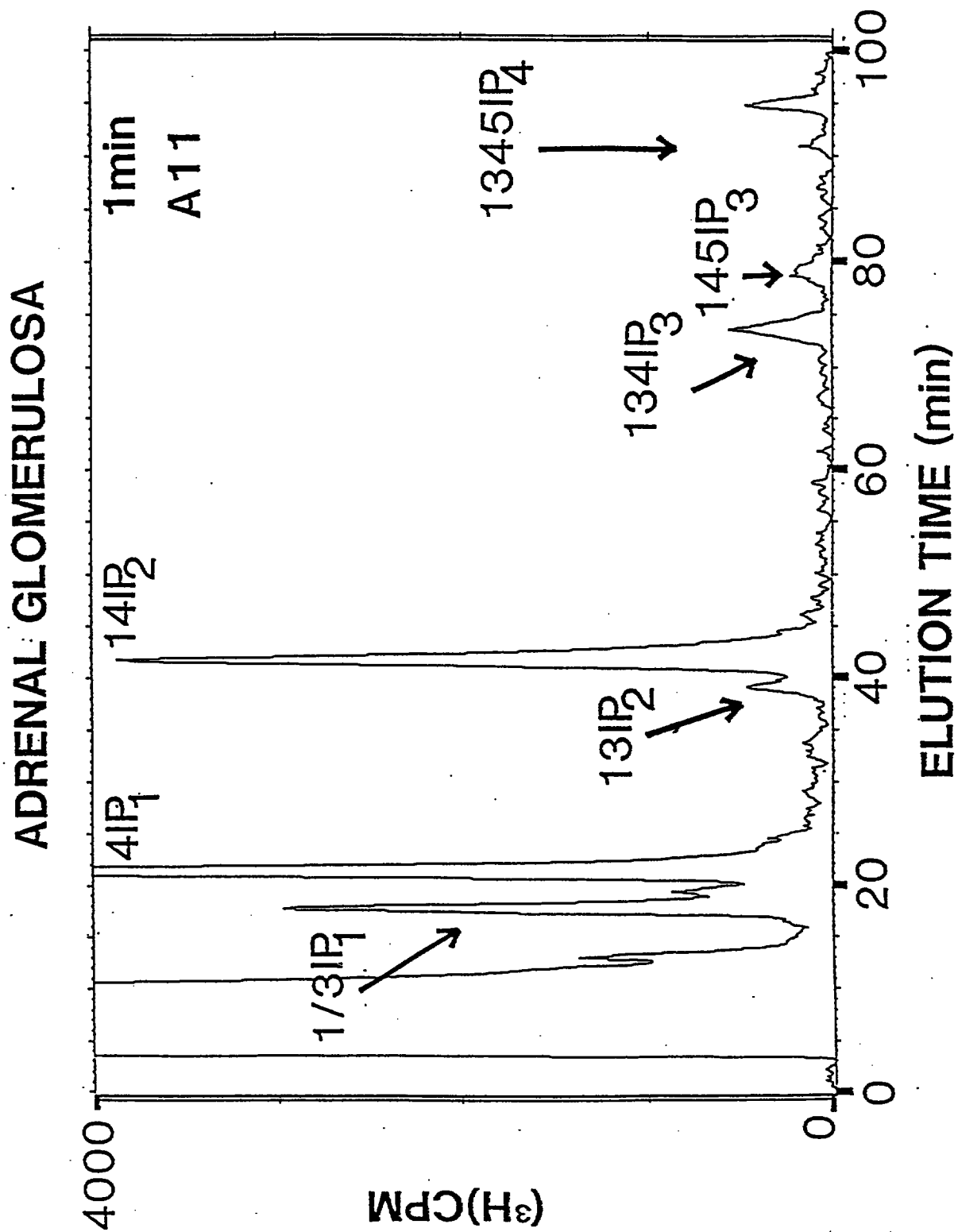


FIGURE 8

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SINGAPORE PATENT APPLICATION NO. 9605370-7

Baker Medical Research Institute

FIRST STATEMENT OF PROPOSED AMENDMENTS

1. At page 34, amend claim 22 to read "An agent when used in blocking formation or release of Ins(1,4,5)P₃ in cardiac tissue according to the method of claim 1."
2. At page 35, amend claim 37 to read "A pharmaceutical composition when used in the prophylaxis and/or treatment of cardiac dysfunction according to the method of claim 1 comprising an agent capable of blocking formation or release of Ins(1,4,5)P₃ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents."
3. At page 37, add new claim 55 as follows: "A method according to any one of claims 1 to 21 or an agent according to any one of claims 22 to 36 or a pharmaceutical composition according to any one of claims 37 to 51 or a use according to any one of claims 52 to 54 substantially as hereinbefore described with reference to the Figures and/or Examples."

12 December, 1997

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☐ Description : paged to paged
☒ Claim(s) : paged ^{21, 35, 37} to paged
☐ Drawing(s) : paged to paged
☐ Abstract : paged to paged

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22. An agent when used in blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue according to the method of claim 1.

23. An agent according to claim 22 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.

24. An agent according to claim 23 wherein said agent is an aminoglycoside.

25. An agent according to claim 24 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.

26. An agent according to claim 23 wherein said agent is a polyamine.

27. An agent according to claim 26 wherein said agent is spermine or a derivative thereof.

28. An agent according to claim 22 wherein said agent blocks the effects of $\text{Ins}(1,4,5)\text{P}_3$.

29. An agent according to claim 28 wherein said agent blocks the $\text{Ins}(1,4,5)\text{P}_3$ receptor.

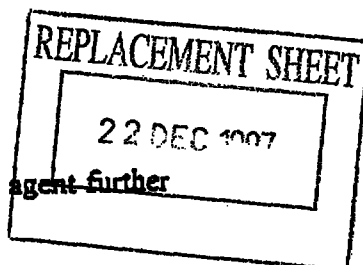
30. An agent according to claim 28 wherein said agent is an antagonist of $\text{Ins}(1,4,5)\text{P}_3$.

31. An agent according to claim 22 or 23 or 28 wherein said agent further comprises another anti-arrhythmia drug.

32. An agent according to claim 31 wherein said other anti-arrhythmia drug is selected from the list consisting of amiodorone, sotalol and lignocaine.

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33. An agent according to claim 22 or 23 or 28 wherein said agent further comprises a component capable of effecting thrombolysis.
34. An agent according to claim 33 wherein the component is selected from the list consisting of streptokinase, urokinase and tissue plasminogen activator.
35. An agent according to claim 22 or 23 or 29 wherein said agent further comprises a component which increases cardiac systolic and/or diastolic function.
36. An agent according to claim 35 wherein said component is selected from the list consisting of dioxin, dobutamine, nitrates, captopril, nifedipine and frusemide.
37. A pharmaceutical composition when used in the prophylaxis and/or treatment of cardiac dysfunction according to the method of claim 1 comprising an agent capable of blocking formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue together with one or more pharmaceutically acceptable carriers and/or diluents.
38. A pharmaceutical composition according to claim 37 wherein said agent inhibits or prevents release of $\text{Ins}(1,4,5)\text{P}_3$ from phospholipid $\text{PtdIns}(4,5)\text{P}_2$.
39. A pharmaceutical composition according to claim 38 wherein said agent is an aminoglycoside.
40. A pharmaceutical composition according to claim 39 wherein said agent is selected from the list consisting of gentamicin, tobramycin, amikacin, netilmicin, kanamycin, streptomycin and neomycin or derivatives thereof.
41. A pharmaceutical composition according to claim 37 wherein said agent is a polyamine.
42. A pharmaceutical composition according to claim 41 wherein said agent is spermine or a derivative thereof.

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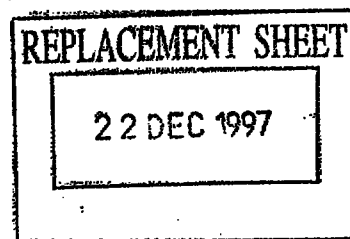
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52. Use of an agent which blocks formation or release of $\text{Ins}(1,4,5)\text{P}_3$ in cardiac tissue in the manufacture of a medicament for the treatment or prophylaxis of cardiac arrhythmia.

53. A use according to claim 52 wherein the agent is an aminoglycoside.

54. A use according to claim 52 wherein the agent is a polyamine.

55. A method according to any one of claims 1 to 21 or an agent according to any one of claims 22 to 36 or a pharmaceutical composition according to any one of claims 37 to 51 or a use according to any one of claims 52 to 54 substantially as hereinbefore described with reference to the Figures and/or Examples.



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