The Expression of Constitutively Active Isotypes of Protein Kinase C to Investigate Preconditioning*

Jing Zhao, Oliver Renner, Lionel Wightman, Peter H. Sugden, Luisa Stewart, Andrew D. Miller, David S. Latchman, and Michael S. Marber

From the Department of Cardiology, United Medical and Dental Schools of Guy’s and St Thomas’ Hospitals, London, SE1 7EH the Division of Cardiac Medicine and the Department of Chemistry, Imperial College of Science, Technology, and Medicine, London, SW3 6LY and the Department of Molecular Pathology, University College London Medical School, London WIP 6DB, United Kingdom

The role of protein kinase C (PKC) in ischemic preconditioning remains controversial because of difficulties with both its measurement and pharmacological manipulation. We investigated preconditioning in isolated neonatal rat cardiocytes by expressing constitutively active isotypes of PKC. Observations at differing durations of simulated ischemia suggested β-galactosidase (β-gal) activity reflected viability within transfected myocytes. Preconditioning with 90 min of ischemia significantly increased β-gal activity and myocyte survival after 6 h of ischemia; an effect abolished by PKC inhibitors. After co-transfection with plasmids encoding β-gal and either constitutively active mutants of PKC-δ, PKC-α, wild type PKC-δ, or empty vector, cardiocytes were subjected to 6 h of ischemia. Only PKC-δ, rendered constitutively active by a limited deletion within the pseudosubstrate domain, consistently increased resistance to simulated ischemia (β-gal activity was 85.6 ± 11.9% versus 53.7 ± 6.5% (p ≤ 0.01) and dead myocytes 46.8 ± 3.4% versus 68.7 ± 2.8% (p ≤ 0.01)). Since transfection was apparent in only 5–12% of cells, the results suggested a protective bystander effect that was confirmed by co-culture of transfected myocytes with untransfected myocytes. In neonatal cardiocytes expression of active PKC-δ increases resistance to simulated ischemia. This observation may provide further insight into the mechanism and possible avenues for therapeutic exploitation of preconditioning.

Ischemic preconditioning describes the resistance to myocardial infarction that follows short, sublethal episodes of ischemia (1). The protection associated with ischemic preconditioning is profound but, unfortunately, short lived (2) and prone to tachyphylaxis (3). These features have cast doubts on the eventual clinical utility of this powerful protective phenomenon (4). For this and other reasons intense research activities and pharmacological manipulation of PKC activity to mimic or block preconditioning and measurement of activity or translocation of PKC has been used. Unfortunately, the most specific inhibitors, peptide fragments of pseudosubstrate domains, are difficult to use in vivo and require sarcolemmal permeabilization to use in vitro (14).

The studies of PKC in preconditioning thus far have used pharmacological manipulation of PKC activity to mimic or block preconditioning and measurement of activity or translocation of PKC has been used. Unfortunately, the most specific inhibitors, peptide fragments of pseudosubstrate domains, are difficult to use in vivo and require sarcolemmal permeabilization to use in vitro (14).

1 The abbreviations used are: PKC, protein kinase C; β-gal, β-galactosidase; DOPE, dioleoyl-ε-aminocapric acid; DC-Chol, 3β[14C]-N-octadecyl-α-dimethylaminoethanolcarboxamidomethylcholsterol; FCS, fetal calf serum; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; ANOVA, analysis of variance; FPLSD, Fischer protected least significant difference; pCAGGS, eukaryotic expression plasmid.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Cardiology, Guy’s and St. Thomas’ Medical and Dental Schools, St. Thomas’ Hospital, London SE1 7EH, UK. Tel.: 44 (0) 171 922-8191; Fax: 44 (0) 171 960-5659; E-mail: m.marber@rayne.umds.ac.uk.

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
culation to infer involvement, but seldom both in a single study (13). The conclusions of these studies are difficult to reconcile because some investigators (13, 14–16) report PKC activation is both a sufficient and necessary component of the ischemic preconditioning pathway, and others (10, 11, 13) suggest there is no involvement. The reason for this dichotomy is thought to lie in the complexities and uncertainties of pharmacological manipulation of the PKC pathway (6, 12, 13). This issue is of critical importance if preconditioning is to be mimicked and exploited by specific manipulation of its signaling pathway.

Recently PKC isoforms have become available that have been rendered constitutively active by limited amino-terminal deletions within the pseudosubstrate domain (17, 18). These alterations are thought to prevent, or decrease the probability of, interactions with the catalytic site and thus reduce the potential for steric interference of substrate phosphorylation (6). Cells have been transfected with expression plasmids encoding these mutationally active PKC isoforms to specifically interrogate the PKC pathway and determine the cross-talk between this and other possible parallel signal transduction pathways (17). In addition, downstream events are preserved because there is transcripational activation of an atrial naturetic factor promoter/reporter construct in cardiac myocytes expressing constitutively active PKC isoforms (18), an observation that suggests they, like more physiological stimuli, are capable of triggering a hypertrophic response (18). Our aim was to use these mutant constitutively active PKC isoforms to investigate ischemic preconditioning.

We have developed a system of simulated ischemia in cultured neonatal rat ventricular myocytes that shares many features of ischemic preconditioning in vivo and is similar to a previously characterized model (19). By using this system we demonstrate that β-gal can be used as a marker of viability in transfected myocytes exposed to simulated ischemia. By co-transfecting expression vectors for β-gal and mutant constitutively active PKC isoforms, we have examined resistance to simulated ischemia. We believe that this is the first report of the use of genetic manipulation to investigate preconditioning.

**EXPERIMENTAL PROCEDURES**

**Materials—** Dulbecco's modified Eagle's medium, medium 199 (M199), bovine serum albumin, horse serum, fetal calf serum (FCS), bovine testicular collagenase was from Worthington. Trypsin-0.05% phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and benzamidine were from Sigma-Aldrich (Gillingham, UK). [γ-32P]ATP was from Amersham Corp (Little Chalfont, UK). HEPES and the neutral phospholipid dioleoyl-<i>L</i>-phosphatidylethanolamine (DOPE) were from Sigma (Poole, UK). Cationic amphotericin B (Am B) was from Liposome (Paisley, UK). Collagenase was from Worthington. Trypan blue, pancreatin, and penicillin/streptomycin were from Life Technologies, Inc. (Paisley, UK). DE52 was from Waterman (Maidstone, UK). Monoclonal isooform-specific anti-PKC antibodies were from Affinity Research Products Ltd. (Exeter, UK). Peroxidase-conjugated rabbit anti-mouse immunoglobulins were from Dako (High Wycombe, UK).

**Isolation and Culture of Rat Ventricular Cardiomyocytes—** Neonatal rat ventricular cardiomyocytes were prepared from 1- to 2-day-old Sprague-Dawley rats as described previously (21). Brieﬂy, cells from neonatal rat ventricles were dispersed in a series of incubations at 37 °C in HEPES-buffered salt solution containing 0.6 mg/ml pancreatin and 0.5 mg/ml collagenase. The dispersed cells were plated for at least 30 min to minimize fibroblast contamination, and the unattached cells were re-plated on 6-well gelatin-coated plates at a density of 1–1.5 million cells/well. Fibroblast contamination was less than 5%. The cardiomyocytes were cultured at 37 °C in 5% CO2 in room air for 24 h. Dulbecco’s modiﬁed Eagle’s medium:M199, supplemented with 10% horse serum, 5% FCS, and 100 units/ml penicillin/streptomycin for the first 24 h. Thereafter cells were maintained in an identical medium with a reduced serum concentration of 1% FCS. Under these conditions, in excess of 80% of cells beat spontaneously for the duration of the experiment. Experiments were performed after 1–3 days in culture.

**Expression Vectors for Transfection of Neonatal Cardiomyocytes—** The high efﬁciency eukaryotic expression plasmid, pCAGGS, was used for all transfections (22). This plasmid contains the cytomegalovirus immediate early enhancer and chicken β-actin promoter with first intron upstream of a multiple cloning site. It has been shown previously that this heterologous promoter is transcriptionally active in cardiac muscle differentiation (23).

The plasmid pCAGGS-β-gal was constructed from pBCH110 (Promega, St Albans, UK) by excising the β-gal gene between HindIII and BamHI sites and inserting into the vector pCAGGS at the BamHI site. The β-gal gene lacked sequences conferring nuclear localization. PKC constructs were subcloned into pCAGGS from plasmids provided by Dr. Peter Parker, Imperial Cancer Research Fund Laboratories, London, UK. Three PKC isoforms were studied: 1) wild type PKC-δ; 2) PKC-δ with a limited deletion of the inhibitory pseudosubstrate subdomain (residues 151–160); and 3) PKC-α with a limited deletion of the inhibitory pseudosubstrate subdomain (residues 22–28). These mutant PKC isoforms have previously been shown to code for constitutively active functional proteins (17, 18).

Plasmids were purified by alkaline lysis of the bacterial host (DH1Alpha) followed by polyethylene glycol precipitation.

**Transfection of Neonatal Cardiomyocytes—** Cardiocytes at 60–70% confluency were transfected by incubation with complexes of DNA with DC-Chol/DOPE cationic liposomes. These liposomes were prepared in the following way. DC-Chol (12 μmol) and a chloroform solution of DOPE (6 μml) were combined in dichloromethane (10 ml) under nitrogen and then diluted with sterile 20 mM HEPES buffer, pH 7.8. The two-phase system under nitrogen was sonicated for 3 min at ambient temperature. Organic solvent was then removed under reduced pressure, and the resulting liposomes were sonicated for a further 3 min prior to use. The liposome-DNA complexes were prepared by mixing DNA (0.1% w/v) with liposome (0.1% w/v) in H2O at a ratio of 1:3 (w/w). The liposome-DNA complexes were allowed to stand at room temperature for 15–30 min before use. One ml of serum-free maintenance medium with 20–40 μl of DNA/liposome mixture was added per well of a 6-well plate. Cells were then incubated at 37 °C in room air supplemented with 5% CO2 for 1 h. Thereafter maintenance medium containing 1% FCS was gently overlaid, and the cells were returned to the incubator. Cells extract for gene activity were assayed 45–72 h after transfection. By using pCAGGS-β-gal as a reporter, transfection efficiency was consistently between 5 and 12%, staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl b-galactopyranoside.

**Ischemia Model—** The cells were washed three times with PBS before addition of 1 ml of ischemia buffer (118 mM NaCl, 24 mM NaHCO3, 1 mM Na2HPO4·H2O, 2.5 mM CaCl2·2H2O, 1.2 mM MgCl2, 0.5 mM sodium pyruvate, 10 mM HEPES, 20 mM sodium lactate, and 16 mM glucose) and pre-gassed with 5% CO2, 95% argon. On addition of ischemia buffer spontaneous contraction within the monolayer ceased. The cells were then transferred to a purpose-built ischemia chamber and incubated at 37 °C in 5% CO2, 95% argon for up to 12 h. The O2 content of the atmosphere inside the chamber was <1% for the duration of the experiment as measured by an on-line oxygen meter (Grifﬁn and George, Falmouth, UK).

**Co-culture Model—** Neonatal cardiomyocytes were prepared and cultured as described above either on standard 6-well plates or within a cell culture insert (Falcon, Oxford, UK). The inserts, designed to fit within the wells of a 6-well plate, were perforated with 1.0-μm pores allowing free communication of media between insert and well. The cells within the insert and the well were transfected and cultured apart and only combined immediately before simulated ischemia. To ensure that both cell monolayers were bathed in buffer, volumes had to be increased to 2 ml during simulated ischemia.

**Evaluation of Cell Viability—** After the cells had been subjected to simulated ischemia, remaining ischemia buffer was gently aspirated and saved for LDH determination, and cells were detached by washing in PBS with trypsin. Following centrifugation a small aliquot of cells was incubated in PBS with 0.4% trypsin blue for a few seconds before placing in a hemocytometer for counting under phase contrast. Dead cells, permeable to trypsin blue, were counted as a percentage of the total number of cells in at least 25 grids of the hemocytometer so that at least 200 cells were counted in total. Examination of the initial ischemia buffer revealed no cells.

**Measurement of LDH Activity—** On opening the ischemia chamber (re-oxygenation) 200-μl samples of the ischemia buffer were gently collected for the determination of LDH. The following day a spectrophotometric LDH enzyme assay was performed with a Sigma assay kit (TOX-7).

**Western Blotting Analysis—** After transfection the cells were washed three times with PBS and harvested in 1 ml of hot electrophore-
sis sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, and 2% β-mercaptoethanol) and then boiled for an additional 5 min. The cell extracts were then centrifuged for 5 min to remove insoluble material. The protein concentration of the diluted supernatant was determined by the Pierce method (Pierce) before adding 0.003% bromophenol blue.

Measurement of β-Galactosidase Activity—β-Galactosidase activity was determined in the cell lysates (lysis buffer, 100 mM KH₂PO₄, 0.2% Triton X-100, 1 mM diithiothreitol, pH 7.8) using a Galacto-Light™ assay kit (BL 330G, Insight Biotechnology Ltd, London, UK). A β-gal standard was used to show that light output within the experimental range was directly proportional to β-gal concentration. β-Gal activity, expressed per mg of protein, was measured in cells harvested post-simulated ischemia (see evaluation of cell viability) and expressed as a percentage of activity in wells transfected with the same expression plasmid but lacking insert (empty vector), harvested in the same manner, and subjected to simultaneous simulated ischemia.

Partial Purification of PKC—The cells were washed with PBS and lysed in homogenization buffer (0.05 M Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.3% Triton X-100, 10 mM diithiothreitol, 10 mM benzamidine, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cells were then sonicated and centrifuged. The supernatant was collected and incubated with 0.5 ml of DEAE-cellulose (DE52) for 60 min at 4 °C with occasional gentle agitation. DE52 had been pre-equilibrated with 200 mM Tris, pH 7.5, and washed in buffer A at 4 °C (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 1 mM diithiothreitol). After incubation, the lysate/DE52 slurry was loaded on a column. The column was then washed with 3 ml of buffer A and PKC eluted with 0.3 M NaCl.

Results

The Simulation of Ischemia—To mimic the changes in extra-cellular ions occurring during ischemia in vivo, we used a bicarbonate-based buffer at pH 6.2 with 20 mM lactate, 16 mM K⁺, and no metabolic substrate (O₂ < 1%), at a volume of 1 ml, just sufficient to wet the cellular monolayer. By using this technique we exposed neonatal cardiocytes to simulated ischemia for increasing duration. The rate and extent of ischemic injury were determined by an assessment of trypan blue permeability and LDH release. Fig. 1, panel A, shows that the percentage of dead cells (as assessed by trypan blue) increased in a time-dependent and reproducible manner. Similar results were obtained for LDH release (see Fig. 1, panel B).

Our aim was to use this model to transfact a subpopulation of cells within the monolayer with genes encoding isotypes of PKC to determine sensitivity to simulated ischemia. However this would require a method of assessing viability in the subpopulation of cells taking up plasmid, expressing recombinant protein, and remaining attached post-ischemia. β-Gal is a cytoplasmic enzyme with a molecular mass of 43 kDa, less than that of other endogenous enzymes such as creatine kinase (81 kDa) and LDH (70 kDa) which are commonly used markers of cell viability. We therefore wished to examine if β-gal derived from a transfected plasmid could be used as an index of viability in the subpopulation of transfected cells. Fig. 1, panel C, shows that when β-gal activity, after different durations of simulated ischemia, is expressed as a percentage of activity prior to ischemia, there is a time-dependent attrition which is inversely related to trypan blue exclusion and supernatant LDH activity. Thus, after 8 h of simulated ischemia β-gal activity was reduced to 38.3 ± 4.6% of the control activity (p < 0.01 versus control).

Preconditioning with Simulated Ischemia and the Role of PKC—Preconditioning with ischemia in vivo or simulated ischemia in vitro has been shown to delay necrosis/cell death in response to subsequent ischemia. We wished to investigate if
similar protection occurred in our model. Cardiocytes were subjected to varying durations of simulated ischemia before returning cultures to maintenance medium in a standard incubator (room air supplemented with 5% CO₂) for 30 min. After this recovery phase cultures were exposed once again to ischemia buffer in an atmosphere of 95% argon, 5% CO₂ for periods of 6 h. These preliminary experiments demonstrated that 90 min of simulated ischemia was not a sufficiently severe stress to cause cell death but would consistently reduce injury after 6 h of simulated ischemia.

To determine whether this pretreatment represented a delay in cell death, we repeated the time course, as described above, with and without preconditioning (see Fig. 2). 90 min of simulated ischemia consistently and significantly delayed cell death as measured by trypan blue exclusion. Moreover, when measured after 6 h of simulated ischemia in cells transfected with pCAGGS-β-gal, the residual β-gal activity in preconditioned cells was significantly greater than, and LDH leakage was significantly less than, that in non-preconditioned cells (see Table IA).

In addition we wished to examine the role of PKC activation in this particular model of preconditioning. By using two different PKC inhibitors chelerythrine and staurosporine at previously reported concentrations (25), during the 90 min of preconditioning ischemia, we were consistently able to block the protection seen in our model (see Table IB). Paradoxically the presence of the PKC inhibitor during conditioning ischemia seemed to sensitize the cardiocytes, even though the PKC inhibitors did not cause cell death in the presence of prolonged simulated ischemia in non-preconditioned cells (Table II).

Expression and Activity of PKC Following Transfection—A high efficiency eukaryotic expression plasmid was used to introduce and drive recombinant PKC expression in myocyte cultures. Transfection was achieved with cationic liposome-DNA complexes as described above. With this technique, 48–72 h post-transfection, we noticed appreciable increases in PKC immunoreactivity within protein samples derived from the whole monolayer despite transfection efficiencies of only 5–12% as assessed by staining with a chromogenic substrate (see Fig. 3). In untransfected cells PKC-δ (72 kDa) and PKC-α (84 kDa) immunoreactivities were weak. Transfection with PKC-δ deletion or PKC-δ wild type resulted in a similar accumulation of protein (see Fig. 3).

To ascertain if the recombinant PKC visible on immunoblots was functional, we measured maximally activable PKC in the transfected cells. Table III shows that PKC activities were significantly enhanced in cells transfected with the PKC-δ or PKC-α compared with cells transfected with empty vector alone. These differences in total PKC activity were noticeable

### Table I

| Cell death (%) | LDH release % | β-Galactosidase activity % |
|----------------|---------------|---------------------------|
| A              |               |                           |
| Control        | 70.3 ± 2.1b   | 100                       |
| Preconditioning| 45.1 ± 2.5ab  | 84.6 ± 2.8b              |
| B              |               |                           |
| Control        | 65.6 ± 5.4    | 100                       |
| Preconditioning| 30.0 ± 3.8b   | 93.2 ± 2.5b              |
| Preconditioning + 10 µM chelerythrine | 64.3 ± 7.7 | 118.6 ± 8.7 |
| Preconditioning + 1 µM staurosporine | 64.3 ± 0.6 | 109.7 ± 9.1 |

**p < 0.01 versus control.*** *p < 0.05 versus control.

### Table II

| Cell death (%) | LDH release % | β-Galactosidase activity % |
|----------------|---------------|---------------------------|
| Control        | 80.0 ± 1.5    | 100                       |
| Control + 10 µM chelerythrine | 78.0 ± 2.4 | 82.5 ± 3.1 |
| Control + 1 µM staurosporine | 77.9 ± 3.1 | 117.4 ± 9.0 |

**p < 0.05 versus control.*** *p < 0.01 versus control.

### Table III

| PKC activity (%) | -δ deletion (n = 7) | -δ wild type (n = 7) | -α deletion (n = 5) |
|------------------|---------------------|---------------------|---------------------|
| PKC activity (%) | 153.80 ± 15.52b    | 128.74 ± 8.53b      | 115.60 ± 6.18b      |

**p < 0.01 versus PKC activity of pCAGGS lacking insert.*** *p < 0.05. PKC activity of pCAGGS lacking insert.
Cardiocytes, cardiocytes transfected with pCAGGS alone or encoding PKC-\(d\) (type PKC-\(d\) with a 7-amino acid deletion, pCAGGS within the multiple cloning site) together with pCAGGS lacking insert. Percentage cell death is defined as the percentage of cells permeable to trypan blue expressed as a percentage of the total number of myocytes. Values are mean ± S.E.

Table IV

| Procedure                          | Trypan blue | LDH release | \(\beta\)-Gal activity |
|-----------------------------------|------------|-------------|-----------------------|
| Non-transfected                   | 80.3 ± 2.0 | 120.6 ± 10.6| 3.5 ± 0.5             |
| (\(n = 12\))                      |            | (\(n = 12\))| (\(n = 12\))          |
| cAGGS                             | 80.1 ± 1.1 | 100         | 100                   |
| (\(n = 28\))                      |            | (\(n = 28\))| (\(n = 28\))          |
| PKC-\(d\) deletion                | 65.7 ± 1.0*| 76.8 ± 4.2* | 165.4 ± 14.4*         |
| (\(n = 28\))                      |            | (\(n = 28\))| (\(n = 28\))          |
| PKC-\(d\) deletion + 1 \(\mu\)M | 83.3 ± 1.2 | 136.4 ± 6.9*| 120.1 ± 8.3           |
| staurosporine                     | (\(n = 28\))| (\(n = 28\))| (\(n = 28\))          |

\(^a\) \(p < 0.05\) versus empty vector. Comparisons by one-way ANOVA. Post hoc comparisons by FPLSD method.

\(^b\) \(p < 0.01\) versus empty vector. Comparisons by one-way ANOVA. Post hoc comparisons by FPLSD method.

It is apparent from Fig. 4 that overexpression of the PKC-\(d\) deletion results in significant protection of the subpopulation of transfected cells as assessed by \(\beta\)-gal activity (panel A). Surprisingly, however, protection is not confined to the transfected cells since there is an increase in viability of the whole monolayer as measured by the global indices of trypan blue exclusion and LDH release. This degree of protection cannot be explained by an enhanced resistance to ischemia within just the 5–12% of cells successfully transfected.

The protection seen in non-transfected cells implies a bystander effect. Our cultures of neonatal cardiocytes beat synchronously probably indicating intact gap junctions. The bystander effect could thus be secondary to cell to cell communication through an extracellular or low molecular weight intercellular signal.

**Investigation of the Bystander Effect**—To investigate further the possibility of a bystander effect, we co-cultured cardiocytes transfected with different plasmids. The cardiocyte monolayer within the wells of a standard 6-well plate were transfected with pCAGGS encoding \(\beta\)-gal alone. The cardiocyte monolayers within an insertable well were co-transfected with pCAGGS encoding \(\beta\)-gal and pCAGGS encoding wild type PKC-\(d\).

Myocyte cultures were exposed to 6 h of simulated ischemia 48–72 h after transfection. At this time point there is appreciable recombinant PKC expression and activity (see Fig. 3 and Table I). It is apparent from Fig. 4 that overexpression of the PKC-\(d\) deletion results in significant protection of the subpopulation of transfected cells as assessed by \(\beta\)-gal activity (panel A). Surprisingly, however, protection is not confined to the transfected cells since there is an increase in viability of the whole monolayer as measured by the global indices of trypan blue exclusion and LDH release. This degree of protection cannot be explained by an enhanced resistance to ischemia within just the 5–12% of cells successfully transfected.

The protection seen in non-transfected cells implies a bystander effect. Our cultures of neonatal cardiocytes beat synchronously probably indicating intact gap junctions. The bystander effect could thus be secondary to cell to cell communication through an extracellular or low molecular weight intercellular signal.

**Investigation of the Bystander Effect**—To investigate further the possibility of a bystander effect, we co-cultured cardiocytes transfected with different plasmids. The cardiocyte monolayer within the wells of a standard 6-well plate were transfected with pCAGGS encoding \(\beta\)-gal alone. The cardiocyte monolayers within an insertable well were co-transfected with pCAGGS encoding \(\beta\)-gal and pCAGGS encoding either the PKC-\(d\) deletion, PKC-\(d\) wild type, or lacking any insert. These separately transfected monolayers were only combined in a single tissue culture well immediately before adding ischemia buffer and entering the ischemia chamber. After 6 h of simulated ischemia, cell viability in each monolayer was assessed separately. Fig. 5 shows viability of the monolayer transfected only with pCAGGS-\(\beta\)-gal. Despite the larger volume of ischemia buffer increasing dilution, the plasmid used to transfecƟ the upper monolayer influenced the viability of the lower monolayer. The results of these experiments suggest that during ischemia a diffusible factor is released from cells expressing the PKC-\(d\) deletion mutant that is capable of acting upon and protecting

**Experimental Procedures** are given in Table IV. The rationale behind the co-transfection approach was that viability in the transfected subpopulation of cells could be measured using \(\beta\)-gal activity as demonstrated above. This relies on the widely accepted finding that cells taking up one plasmid will also take up the others available (26). This assumption forms the basis of a variety of investigative techniques involving plasmid co-transfection (27). These experiments were performed in a blinded fashion the operator only being aware of the assignment of pCAGGS-\(\beta\)-gal and pCAGGS encoding wild type PKC-\(d\).

Myocyte cultures were exposed to 6 h of simulated ischemia 48–72 h after transfection. At this time point there is appreciable recombinant PKC expression and activity (see Fig. 3 and Table I). It is apparent from Fig. 4 that overexpression of the PKC-\(d\) deletion results in significant protection of the subpopulation of transfected cells as assessed by \(\beta\)-gal activity (panel A). Surprisingly, however, protection is not confined to the transfected cells since there is an increase in viability of the whole monolayer as measured by the global indices of trypan blue exclusion and LDH release. This degree of protection cannot be explained by an enhanced resistance to ischemia within just the 5–12% of cells successfully transfected.

The protection seen in non-transfected cells implies a bystander effect. Our cultures of neonatal cardiocytes beat synchronously probably indicating intact gap junctions. The bystander effect could thus be secondary to cell to cell communication through an extracellular or low molecular weight intercellular signal.

**Investigation of the Bystander Effect**—To investigate further the possibility of a bystander effect, we co-cultured cardiocytes transfected with different plasmids. The cardiocyte monolayer within the wells of a standard 6-well plate were transfected with pCAGGS encoding \(\beta\)-gal alone. The cardiocyte monolayers within an insertable well were co-transfected with pCAGGS encoding \(\beta\)-gal and pCAGGS encoding wild type PKC-\(d\).

Myocyte cultures were exposed to 6 h of simulated ischemia 48–72 h after transfection. At this time point there is appreciable recombinant PKC expression and activity (see Fig. 3 and Table I). It is apparent from Fig. 4 that overexpression of the PKC-\(d\) deletion results in significant protection of the subpopulation of transfected cells as assessed by \(\beta\)-gal activity (panel A). Surprisingly, however, protection is not confined to the transfected cells since there is an increase in viability of the whole monolayer as measured by the global indices of trypan blue exclusion and LDH release. This degree of protection cannot be explained by an enhanced resistance to ischemia within just the 5–12% of cells successfully transfected.

The protection seen in non-transfected cells implies a bystander effect. Our cultures of neonatal cardiocytes beat synchronously probably indicating intact gap junctions. The bystander effect could thus be secondary to cell to cell communication through an extracellular or low molecular weight intercellular signal.

**Investigation of the Bystander Effect**—To investigate further the possibility of a bystander effect, we co-cultured cardiocytes transfected with different plasmids. The cardiocyte monolayer within the wells of a standard 6-well plate were transfected with pCAGGS encoding \(\beta\)-gal alone. The cardiocyte monolayers within an insertable well were co-transfected with pCAGGS encoding \(\beta\)-gal and pCAGGS encoding either the PKC-\(d\) deletion, PKC-\(d\) wild type, or lacking any insert. These separately transfected monolayers were only combined in a single tissue culture well immediately before adding ischemia buffer and entering the ischemia chamber. After 6 h of simulated ischemia, cell viability in each monolayer was assessed separately. Fig. 5 shows viability of the monolayer transfected only with pCAGGS-\(\beta\)-gal. Despite the larger volume of ischemia buffer increasing dilution, the plasmid used to transfecƟ the upper monolayer influenced the viability of the lower monolayer. The results of these experiments suggest that during ischemia a diffusible factor is released from cells expressing the PKC-\(d\) deletion mutant that is capable of acting upon and protecting

**Experimental Procedures** are given in Table IV. The rationale behind the co-transfection approach was that viability in the transfected subpopulation of cells could be measured using \(\beta\)-gal activity as demonstrated above. This relies on the widely accepted finding that cells taking up one plasmid will also take up the others available (26). This assumption forms the basis of a variety of investigative techniques involving plasmid co-transfection (27). These experiments were performed in a blinded fashion the operator only being aware of the assignment of pCAGGS-\(\beta\)-gal and pCAGGS encoding wild type PKC-\(d\).

Myocyte cultures were exposed to 6 h of simulated ischemia 48–72 h after transfection. At this time point there is appreciable recombinant PKC expression and activity (see Fig. 3 and Table I). It is apparent from Fig. 4 that overexpression of the PKC-\(d\) deletion results in significant protection of the subpopulation of transfected cells as assessed by \(\beta\)-gal activity (panel A). Surprisingly, however, protection is not confined to the transfected cells since there is an increase in viability of the whole monolayer as measured by the global indices of trypan blue exclusion and LDH release. This degree of protection cannot be explained by an enhanced resistance to ischemia within just the 5–12% of cells successfully transfected.

The protection seen in non-transfected cells implies a bystander effect. Our cultures of neonatal cardiocytes beat synchronously probably indicating intact gap junctions. The bystander effect could thus be secondary to cell to cell communication through an extracellular or low molecular weight intercellular signal.
untransfected cells or cells expressing β-gal alone.

The viability of the monolayer transfected with the plasmids encoding the PKC isotypes was no different from data shown in Fig. 4. The Relationship between PKC Activation during Simulated Ischemia and Protection—Protection in our model may be related to alterations in gene expression induced by PKC rather than directly to PKC pre-activation during prolonged simulated ischemia. This is because in classical preconditioning there is a short period of reperfusion between the preconditioning trigger and prolonged ischemia, while in our model there is 2 to 3 days between transfection and prolonged simulated ischemia. In an attempt to overcome these uncertainties, we examined whether protection was altered in cells transfected with constitutively active PKC-δ when a PKC inhibitor was present during prolonged simulated ischemia.

In the presence of 10 μM chelerythrine expression of activated PKC-δ continued to be protective, whereas protection was abolished by 1 μM staurosporine (see Table IV). The discrepancy between these inhibitors may be the result of the fact that the inhibitory effect of chelerythrine is possibly mediated through the PKC regulatory region (13) rather than the catalytic subunit. With a pseudosubstrate domain deletion the regulatory domain is likely to be in an activated configuration and chelerythrine therefore without effect.

In summary our data suggest that resistance to simulated ischemia is consistently enhanced in cardiomyocytes expressing PKC-δ rendered constitutively active by a limited deletion within the pseudosubstrate domain. Moreover these cardiomyocytes release a diffusible factor that increases the resistance of cells bathed in the same medium.

DISCUSSION

Our findings suggest that activation of PKC-δ consistently enhances the resistance of isolated neonatal rat cardiocytes to simulated ischemia. This finding is compatible with the observational data demonstrating PKC-δ translocation following brief periods of ischemia in the rat heart (16, 28, 29). However, the most surprising finding is the bystander protection conferred by the expression of constitutively active PKC-δ.

Classical Ischemic Preconditioning Versus Protection by Expression of Mutant PKC Isotypes—To achieve our research objectives, we had to adopt and characterize an unorthodox model of ischemic preconditioning. Ischemic preconditioning in the strictest sense refers to brief episodes of ischemia reducing subsequent infarction in vivo (1). Although models of ischemic preconditioning in isolated adult myocytes are established and accepted, these cells are relatively resistant to transfection and are difficult to maintain in culture. In addition most models rely upon an ischemic cell pellet and hypo-osmotic shock (30), techniques best suited to freshly isolated, unplated cardiomyocytes. Thus far there have been two reports of ischemic preconditioning of immature cardiomyocytes in a similar model (19, 31). These
though transfection efficiency in neonatal cardiocytes was low, their are protected. That even myocytes subjected to a sub-threshold ischemic trig-extracellular signal from the most ischemic areas would ensure conditioned whereas the epicardium is not. However, if the shorter durations of ischemia the endocardium should be pre-

This observation is counterintuitive since the depth of preconditioning appears to be an all or nothing phenomenon (35). This observation is counterintuitive since the depth of ischemia varies from endo- to epicardium, and thus with shorter durations of ischemia the endocardium should be pre-conditioned whereas the epicardium is not. However, if the bystander effect we observed occurs in vivo it would provide an explanation for the apparent spatial homogeneity since an extracellular signal from the most ischemic areas would ensure that even myocytes subjected to a sub-threshold ischemic trig-er are protected.

Protection with Constitutively Active Isoforms of PKC—Although transfection efficiency in neonatal cardiocytes was low, there was appreciable PKC accumulation on immunobots and an increased activity of “maximally activable” phosphorylation of a PKC substrate. Transfection with wild type PKC-δ cDNA caused similar levels of recombinant protein expression as transfection with PKC-δ differed only in a limited deletion of 10 amino acids within the pseudosubstrate domain. However, protection was only seen with the deletion mutant. In addition, although there was robust accumulation of a similarly basally activated PKC-α, this failed to cause consistent protection and a bystander effect was absent. The use of these structurally related proteins, differing only in a limited deletion within a single subdomain, suggests their effects on myocyte resistance to ischemia are specific and that PKC-δ activation causes cytoprotection in this model. Although activation of PKC-δ is sufficient to cause protection, it is unclear, despite the PKC inhibitor experiments we performed, whether PKC-δ activation is an absolute prerequisite for protection. In view of the bystander effect, to answer this question it would presumably be necessary to inhibit endogenous PKC-δ in greater than 95% of myocytes. Although this is possible with pharmacological inhibitors, these are nonspecific. Recently an alternative method using short peptide sequences to saturate isotype-specific receptors for activated PKCs has demonstrated the dependence of preconditioning on endogenous PKC-ε (19). In addition the similarities between the model used in this previous study (19) and that described here allow direct comparison of findings. Taken together they imply that PKC-δ is sufficient but not necessary to elicit preconditioning in isolated neonatal rat cardiocytes.

Unfortunately, constitutively active mutants of PKC-ε were not examined within this article. When the work described within this article was conceived, there were clear indications that PKC-δ translocated in response to brief periods of ischemia, whereas less was known of other isotypes.

Study Limitations—The principal limitation of the study is the surrogate nature of the model and study design. As already outlined, the purpose of the study was to address the PKC hypothesis by specific manipulation of the activity of a single PKC isotype. This was most easily achieved in cultured cardiocytes. With this in vitro system we attempted as closely as possible to mimic changes occurring during ischemia in vivo. However, the differences between true ischemia and simulated ischemia and between in vivo and in vitro end points of injury questionably reduce the relevance of our findings.

A number of different end points have been used to measure viability after simulated ischemia. Following transfection with constitutively active PKC-δ, these end points give consistent results indicative of protection. After transfection with PKC-δ wild type the end points consistently demonstrate lack of protection. However the conclusions after transfection with constitutively active PKC-α are inconsistent and difficult to interpret. In addition although PKC-α immunoreactivity was marked, maximally activable PKC activity was less than with the PKC-δ constructs. These uncertainties prevent any definite conclusion regarding the presence or absence of protection with PKC-α.

The constitutively active PKC isotypes used will almost certainly have triggered the immediate early gene response associated with hypertrophy in transfected cells. This in turn may have altered the resistance of myocytes to simulated ischemia. Although this may have been the scenario in transfected cells, it certainly was not in the “naïve” cells used in the co-culture experiments.

Conclusion—In cultured rat neonatal cardiocytes it is possible to increase resistance to a prolonged period of simulated ischemia by prior exposure to a brief period of simulated ischemia. This protection can be blocked by two structurally diverse PKC inhibitors and mimicked by transfection and expression of PKC-δ rendered basally active by a limited deletion within the pseudosubstrate domain. The depth of protection afforded by this strategy exceeds that expected on the basis of the efficiency of transfection. This observation is supported by co-culture experiments which indicate that transfected cardiocytes are capable of protecting neighboring untransfected cells by the release of an extracellular signal during simulated ischemia.

Although these experiments were performed in a cell culture model, it is likely that they have relevance to preconditioning in vivo. In future studies it may be possible to address this issue by transferring to, and expressing in, the in vivo myocardium basally active PKC isotypes. Strategies of this type may eventually lead to clinically relevant techniques that mimic ischemic preconditioning.

Acknowledgments—We gratefully acknowledge the support of the British Heart Foundation, Medical Research Council, and Wellcome Trust. The expression plasmids used in this study were constructed from materials generously provided by Dr. Peter Parker, Imperial Cancer Research Fund Laboratories, London. Patient Fib, London WC2A 3PX. We thank Dr. Gavin Brooks, Cardiovascular Research, St. Thomas’ Hospital, London SE1 7EH, for guidance and technical advice and Dr. Clare Dollery, Department of Cardiology, UCL Hospitals NHS Trust, London WIN 5AA, for the suggestion of using insertable wells to examine the bystander effect.
REFERENCES

1. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) Circulation 74, 1124–1136

2. Van Winkle, D. M., Thornton, J., and Downey, J. M. (1991) Coron. Artery Dis. 2, 613–619

3. Cohen, M. V., Yang, X. M., and Downey, J. M. (1994) Circ. Res. 74, 998–1004

4. Przyklenk, K., and Kloner, R. A. (1995) Br. Heart J. 74, 575–577

5. Cohen, M. V., and Downey, J. M. (1993) Lancer 341, 6

6. Sugden, P. H., and Bogoyevitch, M. A. (1995) Cardiovasc. Res. 30, 478–492

7. Wall, T. M., Sheehy, R., and Hartman, J. C. (1994) J. Pharmacol. Exp. Ther. 270, 681–689

8. Liu, Y., Gao, W. D., O'Rourke, B., and Marban, E. (1996) Circ. Res. 78, 443–454

9. Light, P. E., Sabir, A. A., Allen, B. G., Walsh, M. P., and French, R. J. (1996) Circ. Res. 79, 399–406

10. Vahlhaus, C., Schulz, R., Post, H., Onallah, R., and Heusch, G. (1996) Circ. Res. 79, 575–577

11. Li, Y., and Kloner, R. A. (1995) Am. J. Physiol. 268, H426–H431

12. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498

13. Brooks, G., and Hearse, D. J. (1996) Circ. Res. 79, 627–630

14. Johnson, J. A., Gray, M. O., Karliner, J. S., Chen, C.-H., Mochly-Rosen, D. (1996) Circ. Res. 78, 1086–1099

15. Speechly-Dick, M. E., Moana, M. M., and Yellon, D. M. (1994) Circ. Res. 75, 586–590

16. Mitchell, M. B., Meng, X., Ao, L., Brown, J. M., Harken, A. H., and Banerjee, A. (1995) Circ. Res. 76, 73–83

17. Genot, E. M., Parker, P. J., and Cantrell, D. A. (1995) J. Biol. Chem. 270, 9833–9839

18. Decock, J. B. J., Gillepie-Brown, J., Parker, P. J., Sugden, P. H., and Fuller, S. J. (1994) FEBS Lett. 356, 275–278

19. Webster, K. A., Diaccher, D. J., and Bishopric, H. H. (1995) J. Mol. Cell. Cardiol. 27, 453–458

20. Alton, E. W., Caplen, N. J., Smith, S. N., Steel, D. M., Munkonge, F. M., Jeffery, P. R., Geddes, D. M., Hart, S. L., Williams. R., Dorin, J. R., and Porteous, D. J. (1993) Nature Genet. 5, 135–142

21. Cumming, D. V. E., Heads, R. J. H., Watson, A., Latchman, D. S., and Yellon, D. M. (1996) J. Mol. Cell. Cardiol. 28, 2343–2349

22. Niwa, H., Yamanura, K. I., and Miyazaki, J. I. (1991) Gene (Amst.) 106, 193–200

23. Marber, M. S., Demmel, R., Chi, S. H., Sayen, M. R., Yellon, D. M., and Dillmann, W. G. (1995) J. Clin. Invest. 95, 1440–1446

24. Brooks, G., Wilson, R. E., Dooley, T. P., Goss, M. W., and Hart, I. R. (1991) Cancer Res. 51, 3281–3288

25. Ikonomides, J. S., Shirai, T., Niu, V., Li, R. K., Mickle, D. A. G., and Weisel, R. D. (1996) in Ischaemia: Preconditioning and Adaptation (Marber, M. S., Yellon, D. M., eds) pp. 131–162, Bios Scientific Publishers Ltd., Oxford

26. Kariya, K., Karns, L. R., and Simpson, P. C. (1991) J. Biol. Chem. 266, 10923–10926

27. Gorman, C. M. (1995) in DNA Cloning, A Practical Approach (Clever, D. M., ed), pp. 143–190, IRL Press at Oxford University Press, Oxford

28. Kawamura, S., Yoshida, K., Mizukami, Y., and Matsuzaki, M. (1996) Circulation 94, Suppl. I, I-392

29. Gho, B. C. G., van den Doel, M. A., Duval, S. Y., Bezstarosti, K., Schoemaker, R. G., Lamers, J. M. J., and Verdouw, P. D. (1996) Circulation 94, Suppl. I, I-392

30. Armstrong, S., and Ganote, C. E. (1994) Cardiovasc. Res. 28, 1700–1706

31. Arstall, M. A., Zhao, Y., Kennedy, S. P., Buchholz, A. R., Kelly, R. A., and Smith, T. W. (1996) Circulation 94, Suppl. I, I-423

32. Gray, M. O., Karlner, J. S., and Mochly-Rosen, D. (1997) J. Biol. Chem. 272, 30945–30951

33. Kitakaze, M., Node, K., Minamino, T., Komamura, K., Funaya, H., Shinosaki, Y., Chujo, M., Mori, H., Inoue, M., and Kamada, T. (1996) Circulation 93, 781–791

34. Van Wylen, D. G. L. (1994) Circulation 90, 2283–2289

35. Zhao. J., Renner, O., Latchman, D. S., Marber, M. S. (1997) Circulation 96, Suppl. I, I-450

36. Koning, M. M., De Zeeuw, S., Nieuwkop, S., De Jong, J. W., and Verdouw, P. D. (1994) Ann. N. Y. Acad. Sci. 723, 333–336