

Protein kinase C-dependent activation of K_{ATP} channel enhances adenosine-induced cardioprotection

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Prior activation of protein kinase C (PKC) can precondition the cardiac cell against injury during subsequent ischaemia. By using cultured chick ventricular cell model for simulated ischaemia and preconditioning, the present study investigated the biochemical mechanism underlying the PKC-mediated preconditioning. A 5 min exposure to PMA enhanced the ability of pinacidil to mediate cardioprotection during a subsequent 90 min period of ischaemia, which is consistent with a sustained activation of the K_{ATP} channel initiated by PKC. The brief prior exposure to PMA was also associated with an enhanced ability of the adenosine A_1 or A_3 receptor agonist 2-chloro- N^6 -cyclopentyladenosine or N^6 -(3-iodobenzyl)adenosine-5'- N -methyluronamide to elicit a cardioprotective response during the subsequent ischaemia. In myocytes pretreated with PMA, the cardioprotection mediated

by receptor agonist was blocked by the concomitant presence of K_{ATP} -channel antagonists glibenclamide or 5-hydroxydecanoic acid during the ischaemia. Thus the K_{ATP} channel acts downstream of the adenosine A_1 and A_3 receptors in mediating the protective effect due to prior PMA exposure. K_{ATP} channel activation is responsible for the adenosine receptor-mediated effect. PMA treatment had no effect on other A_1 or A_3 receptor-mediated effects such as the inhibition of adenylate cyclase, ruling out a direct stimulation of the receptor or G-protein by PMA. The present results indicate that prior stimulation of PKC causes a sustained K_{ATP} channel activation, which in turn renders the myocyte more responsive to the protective action of adenosine A_1 and A_3 receptor agonists during the subsequent ischaemia.

INTRODUCTION

Brief ischaemia before a second sustained ischaemia can decrease the size of myocardial infarction. This cardioprotective phenomenon, known as pre-ischaemia conditioning or ischaemic preconditioning, has been demonstrated in isolated perfused hearts of a number of mammalian species including dog, guinea pig, pig, rabbit and rat [1–9]. Indirect evidence for such protective pre-ischaemia conditioning also exists in humans [10–13]. A number of mediators have been proposed for pre-ischaemia conditioning, including adenosine receptor, protein kinase C (PKC), and K_{ATP} channel [1,4,5,9,12–19]. Thus direct activation of adenosine receptor by adenosine, of PKC by phorbol ester, and K_{ATP} channel by pinacidil have all been demonstrated to precondition the heart against injury incurred during the subsequent ischaemia. The sequence of signalling events during initiation of preconditioning begins with activation of the adenosine receptor, causing stimulation of PKC, which, in turn, activates the K_{ATP} channel [15]. Direct stimulation of PKC can by-pass the receptor, activate the K_{ATP} channel and mimic the cardioprotective effect of ischaemic preconditioning [15]. Thus PKC-initiated preconditioning of the cardiac cell offers a unique experimental paradigm for investigating directly the biochemical signalling mechanism(s) mediating the actual cardioprotection during the subsequent ischaemia. A novel cardiac-cell model of simulated ischaemia and pre-ischaemia conditioning has been established that exhibits characteristics similar to those determined in the intact heart model of pre-ischaemia conditioning [15–17]. In this model, the K_{ATP} channel is the downstream effector from PKC. Activation of the channel, but not of the adenosine receptor, is necessary for the PKC-induced initiation

of preconditioning, whereas the activation of both the adenosine receptor and the channel is required in initiating the preconditioning process as well as in exerting the actual cardioprotection during the subsequent ischaemia [15]. By using this model, the objective of the present study was to determine the signalling mechanism by which prior activation of PKC renders the cardiac cell resistant to the subsequent ischaemia. The results demonstrate that a sequential activation of the adenosine A_1 or A_3 receptor and of the K_{ATP} channel mediates the resistance to ischaemia and that PKC causes a sustained activation of the K_{ATP} channel, which leads to a subsequent increase in the A_1 and A_3 agonist-induced cardioprotective effect during the subsequent ischaemia.

METHODS

Preparation of cultured ventricular myocytes and simulation of ischaemia

Ventricular cells were cultured from chick embryos 14 days *in ovo* and were cultivated in humidified air/ CO_2 (19:1) at 37 °C as described previously [15–17]. All experiments were performed on day 3 in culture, at which time cells exhibited spontaneous contraction. The medium was changed to a Hepes-buffered medium, termed HEPES/glucose-free medium, containing (in mM) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 5 Hepes and 2% (v/v) fetal bovine serum, pH 7.4 at 37 °C before exposing the myocytes to simulated ischaemia. Ischaemia was simulated by exposing the myocytes to 90 min of hypoxia and glucose deprivation in a hypoxic incubator (NuAire), in which O_2 was replaced by N_2 with the final O_2 concentration being less than

Abbreviations used: ADA, adenosine deaminase; CCPA, 2-chloro- N^6 -cyclopentyladenosine; CK, creatine kinase; 5-HD, 5-hydroxydecanoic acid; IB-MECA, N^6 -(3-iodobenzyl)adenosine-5'- N -methyluronamide; PKC, protein kinase C; PMS, phenazine methosulphate; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide.

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Table 1 Tetrazolium-based myocyte viability assay

Cultured cardiac ventricular cells were exposed to 90 min of simulated ischaemia, preconditioned by a 5 min exposure to 1 μ M PMA before the 90 min ischaemia or maintained under normoxia as control. After these treatments, viable cells were quantified by the extent of reduction of the tetrazolium XTT into a water-soluble formazan, which in turn was measured as an increase in the D_{450} . D_{570} was similar in all three conditions, representing non-metabolized XTT and background cellular attenuation [21], and was subtracted from the D_{450} . The percentage of injured myocytes was calculated as the difference in attenuation from the control value divided by the control attenuation. Results represent means \pm S.E.M. for triplicate determinations and were typical of three other experiments. *Significant difference from the control attenuation (one-way ANOVA analysis followed by Student–Newman–Keuls multiple comparison test, $P < 0.01$).

Condition	$D_{450} - D_{570}$	Injured myocytes (%)
Control	0.383 \pm 0.017	0
Ischemia (90 min)	0.302 \pm 0.011*	21.1
Preconditioning by PMA (5 min)	0.341 \pm 0.010*	10.9

1 %, as described previously [15–17]. Determination of myocyte injury was made at the end of the simulated ischaemia. The extent of myocyte injury was quantified as the amount of creatine kinase (CK) released into the medium and as the percentage of cells killed. The amount of CK was measured as enzyme activity (units/mg), and increases in CK activity above the control level were determined. The percentage of cells killed was calculated as 100 times the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group, divided by number of cells in the control group.

The cell viability assay, represented by the percentage of cells killed, was performed as described previously [15–17]. After the prolonged exposure to simulated ischaemia, cells were detached by treatment with trypsin/EDTA in Hanks balanced salt solution, centrifuged at 300 g for 10 min and resuspended in culture medium for counting in a haemocytometer. Previous studies demonstrated that only live, Trypan Blue-excluding, cells sedimented and that the trypsin treatment, re-exposure to Ca^{2+} -containing medium or the 300 g centrifugation did not result in any significant damage to normoxia-exposed cells, whereas the trypsin/EDTA medium from cells exposed to 90 min of ischaemia contained a significant amount of CK activity and proteins [17]. Thus this cell viability assay distinguished the hypoxia-damaged from the control normoxia-exposed cells. In other studies, CK release was associated with a release of lactate dehydrogenase and proteins [15]. Parallel changes in the percentage of cells killed and CK released, obtained in the present as well as in previous studies, further validated this assay for the percentage of cells killed [15–17].

Biochemical assay for myocyte viability based on a soluble tetrazolium/formazan method

To quantify the extent of cell viability, another biochemical assay was developed that was based on the metabolic reduction of the tetrazolium compound 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) by viable cells to a water-soluble formazan product [20,21]. In summary, cultured myocytes were either exposed to 90 min of simulated ischaemia, maintained under normoxic condition or preconditioned by a 5 min treatment with the phorbol ester PMA or with ischaemia. Cells were detached by treatment with trypsin/EDTA in Hanks balanced salt solution, centrifuged at 300 g for 10 min, resuspended in the HEPES/

glucose-free medium and incubated in the presence of XTT (250 μ g/ml) and phenazine methosulphate (PMS) (1.9 μ g/ml) for 30 min. PMS was added to enhance the cellular reduction of XTT. After incubation with XTT and PMS, cells were homogenized with a Dounce homogenizer, and D_{450} and D_{570} were determined. D_{450} measured the reduced formazan; D_{570} represented the attenuation due to the presence of a varying number of cells and non-metabolized XTT [21] and was subtracted from the D_{450} . The supernatant from the 300 g centrifugation, after parallel incubation with XTT and PMS, did not give rise to any appreciable D_{450} or D_{570} .

Determination of the signalling roles of PKC, adenosine receptors and K_{ATP} channel

The cardioprotective effect mediated by the various agents was investigated by exposing the cells in accordance with the following protocol. Cells were exposed to 0.1 μ M of PMA for 5 min. The medium was replaced with PMA-free medium and cells were incubated in this medium for 10 min at 37 °C before exposure to 90 min of simulated ischaemia. As a control, cells were incubated in medium containing the vehicle DMSO (0.1 %, v/v), instead of the PMA-containing medium, for 5 min. In studying the roles of adenosine A_1 and A_3 receptors and of the K_{ATP} channel in mediating the preconditioning effect of PMA, 2-chloro- N^6 -cyclopentyladenosine (CCPA), N^6 -(3-iodobenzyl)adenosine-5'- N -methyluronamide (IB-MECA) or pinacidil was added individually to the medium during the 90 min ischaemia. To eliminate the influence of endogenous adenosine, adenosine deaminase (ADA; 5 units/ml), was added during the 90 min ischaemia. To investigate whether the K_{ATP} channel acts downstream of the adenosine receptor in mediating the protection during the 90 min ischaemia, one of two structurally different K_{ATP} channel antagonists, glibenclamide or 5-hydroxydecanoic acid (5-HD) [1,22], was added individually to the medium containing 10 nM of CCPA or IB-MECA during the ischaemia.

Measurement of cAMP and adenosine levels

To examine the effect of PMA on the subsequent CCPA- or IB-MECA-mediated inhibition of the cAMP level by isoprenaline, cells were pre-exposed to 0.1 μ M PMA for 5 min; medium was then replaced with fresh PMA-free medium. Cells were incubated in this medium for a further 10 min period, at which time various concentrations of CCPA or IB-MECA were added to the medium, either in the presence or the absence of 1 μ M isoprenaline. After 10 min of incubation, cAMP was extracted and determined as described previously [17]. Adenosine levels in the culture medium were quantified by using a radioimmunoassay with antibodies specific for adenosine and with ^{125}I -adenosine as a tracer (Yamasa Corporation, Tokyo, Japan) as described previously [17]. In analysing the statistical significance of differences in a multiple-group comparison, a one-way ANOVA followed by a Student–Newman–Keuls multiple comparison test was performed. In analyses of the difference between two individual groups, a t -test was used.

Materials

The K_{ATP} channel modulators glibenclamide, 5-HD, pinacidil, CCPA and IB-MECA were from Research Biochemicals International (Natick, MA, U.S.A.). The PKC activator PMA was obtained from Calbiochem (San Diego, CA, U.S.A.). Embryonated chick eggs were from Spafas Inc. (Storrs, CT, U.S.A.).

RESULTS

Biochemical mechanism of PKC-mediated cardioprotection: role of adenosine receptor and K_{ATP} channel

The prior activation of PKC by PMA conferred subsequent protection against ischaemia-induced injury; the inactive 4 α -phorbol 12,13-didecanoate or 4 α -phorbol 12-myristate 13-acetate had no such preconditioning effect [15]. By using another cell viability assay based on the metabolic reduction of XTT in live cells [20,21], it was shown that the prior activation of PKC by

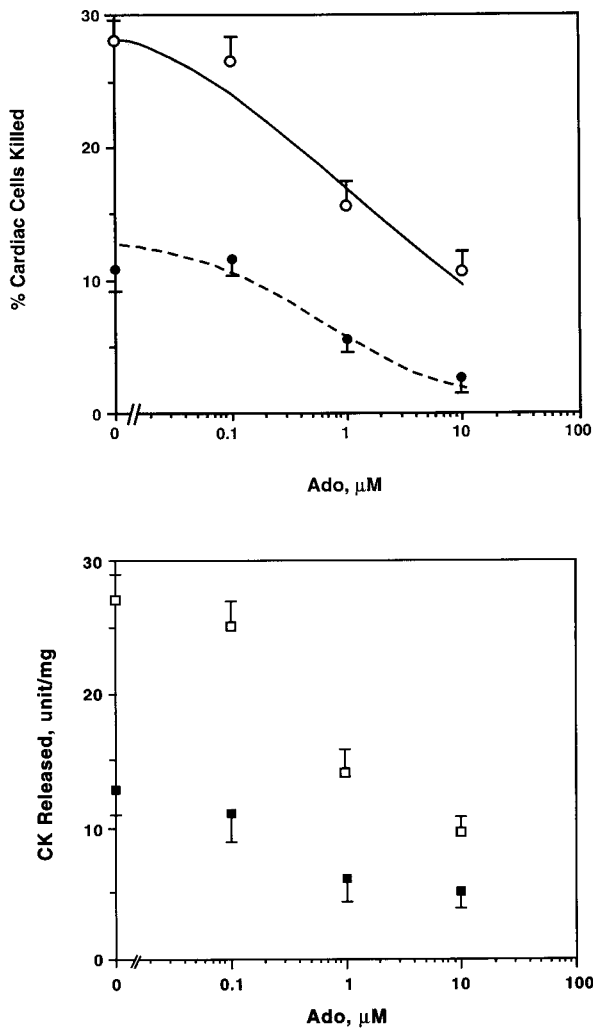


Figure 1 Enhanced adenosine-mediated cardioprotection in PMA-treated cells

Ventricular cells were cultured from chick embryos 14 days *in ovo* and exposed to simulated ischaemia as previously described [15,16]. For PMA treatment (●, ■), cardiac cells were incubated in the presence of 0.1 μM PMA [in 0.1% (v/v) DMSO] for 5 min, followed by replacement with PMA-free medium and incubation under room air at 37 °C for 10 min. For the control group (○, □), cardiac cells were exposed to 0.1% (v/v) DMSO for 5 min before a 10 min incubation in DMSO-free medium. Both groups of cells were then incubated in the presence of the indicated concentrations of adenosine (Ado) during the exposure to 90 min of simulated ischaemia. The percentage of cardiac cells killed (upper panel) and the amount of CK released (lower panel) were determined at the end of the 90 min of ischaemia. The percentage of cells killed are means \pm S.E.M. for five experiments. In the control group the percentage of cells killed and the amount of CK released were greater than those in PMA-preconditioned cells at each adenosine concentration tested (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.01$).

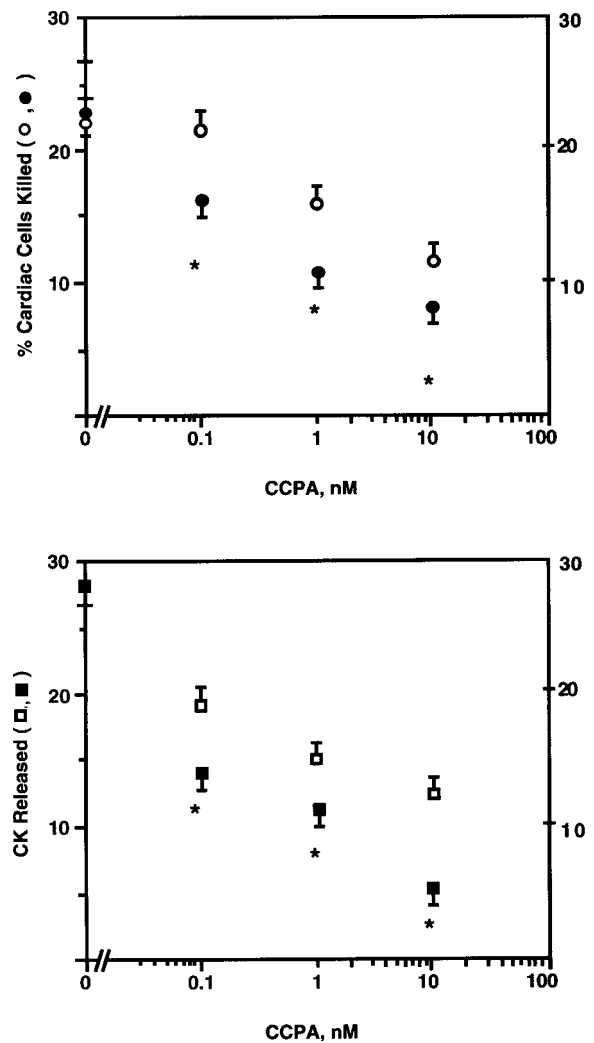


Figure 2 Enhanced adenosine A_1 receptor-mediated protection in PMA-preconditioned cells

Cardiac ventricular cells were prepared and pretreated with either PMA (●, ■) or DMSO (○, □; control) as described in the legend to Figure 1. Cells were then incubated in the presence of ADA plus the various concentrations of CCPA during the 90 min of simulated ischaemia. The percentage of cells killed (upper panel) and the amount of CK released (lower panel) were determined and represent means \pm S.E.M. for four experiments. *At each CCPA concentration, the percentage of cells killed and the amount of CK released were lower in the cells pre-exposed to PMA than those not pre-exposed to PMA (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.05$).

PMA also resulted in less injury incurred during the 90 min ischaemic exposure (Table 1).

The presence of ADA during the 90 min ischaemia completely abolished the preconditioning effect of PMA [the percentage of cells killed in PMA-pretreated myocytes was 10.5 ± 0.7 (mean \pm S.E.M., $n = 5$), compared with PMA pretreatment followed by the addition of ADA during 90 min of ischaemia, for which the percentage killing was $27 \pm 2\%$ (mean \pm S.E.M., $n = 5$)]. These results are consistent with the notion that, for PMA to exert its preconditioning effect, activation of the adenosine receptor is required for mediation of the actual cardioprotection during the subsequent ischaemia [15]. The present study further investigated the biochemical mechanism by which PKC activation enhances

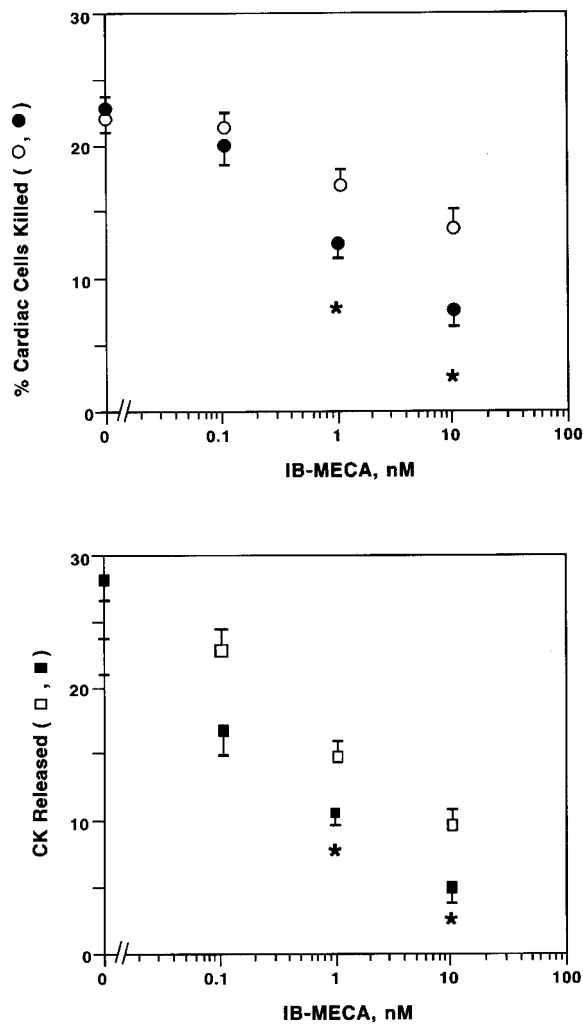


Figure 3 Enhanced adenosine A_3 receptor-mediated protection in PMA-preconditioned cells

Cardiac ventricular cells were prepared and pretreated with either PMA (●, ■) or DMSO (○, □; control) as described in the legend to Figure 1. Cells were then incubated in the presence of ADA plus various concentrations of IB-MECA during the 90 min of simulated ischaemia. The percentage of cells killed (upper panel) and the amount of CK released (lower panel) were determined and represent means \pm S.E.M. for four experiments. *At 1 or 10 nM IB-MECA, the percentage of cells killed and the amount of CK released were lower in the cells pre-exposed to PMA than those not pre-exposed to PMA (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.01$).

the subsequent protection against ischaemia-induced cell injury. Specifically, it tested the hypothesis that prior PKC activation sensitizes the cardiac cell to the cardioprotective effect of the adenosine receptor agonist and the K_{ATP} channel opener. Prior treatment of the cardiac cell with PMA enhanced the protective effect of exogenous adenosine during the sustained ischaemia, as evidenced by the decreased number of cardiac cells killed and the lower level of creatine kinase released in PMA-treated compared with control cells (Figure 1). Because the level of adenosine released during ischaemia was similar in cells pretreated with 0.1 μ M PMA (150 ± 43 nM; mean \pm S.E.M., $n = 5$) or in cells not pre-exposed to PMA (125 ± 40 nM; mean \pm S.E.M., $n = 4$, $P > 0.05$, t -test), the results suggest that the PMA-induced increase in the extent of cardioprotection at each adenosine concentration was not due to a PMA-mediated increase in the release of

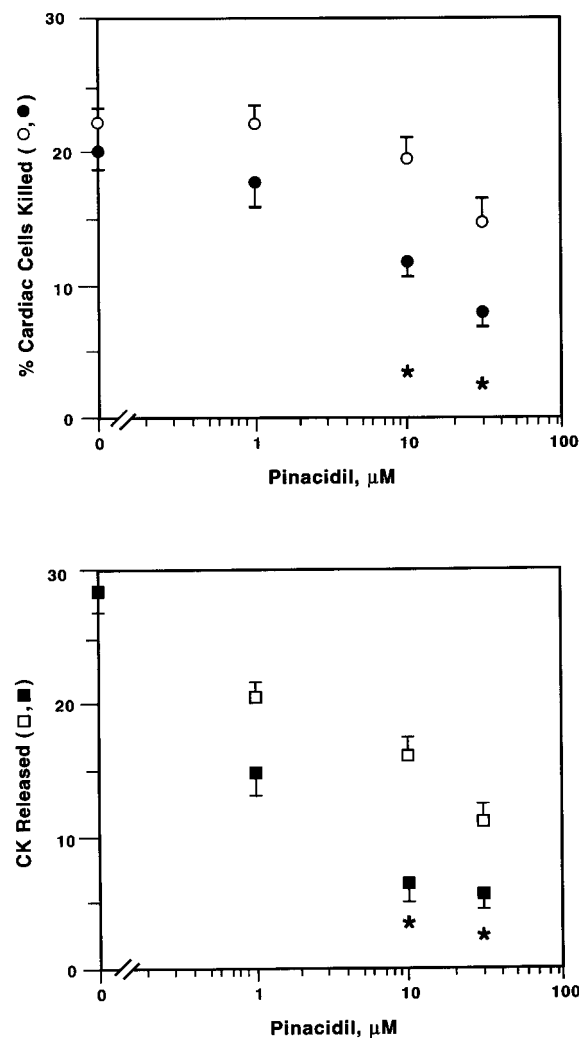


Figure 4 Effect of prior PMA treatment on pinacidil-induced protection during prolonged simulated ischaemia

Cardiac ventricular cells were prepared and pretreated with either PMA (●, ■) or DMSO (○, □; control) as described in the legend to Figure 1. Cells were then incubated in the presence of ADA plus the indicated concentrations of pinacidil during the 90 min of simulated ischaemia. The percentage of cells killed (upper panel) and the amount of CK released (lower panel) were determined and represent means \pm S.E.M. for four experiments. *At 10 or 30 μ M pinacidil, the percentage of cells killed and the amount of CK released were lower in the cells pre-exposed to PMA than those not pre-exposed to PMA (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.01$).

adenosine. Instead, these results are consistent with a PKC-mediated increase in the ability of adenosine to achieve cardioprotection. To provide further evidence for this notion, the effect of prior PKC activation on A_1 or A_3 receptor-mediated protection during the subsequent ischaemia was determined. To perform this study, the effect of endogenously released adenosine at the A_1 and A_3 receptors was eliminated by the inclusion of ADA during the 90 min of ischaemia in cells pre-exposed to PMA. The role of A_1 and A_3 receptors was then determined by studying whether an A_1 receptor agonist or an A_3 receptor agonist, added concomitantly with ADA, could restore the protective effect due to prior PMA treatment. Results summarized in Figures 2 and 3 showed that prior treatment with PMA enhanced the extent of cardioprotection mediated by each receptor agonist; both CCPA

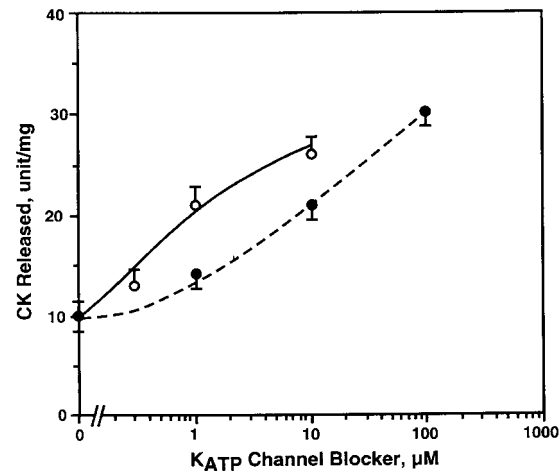
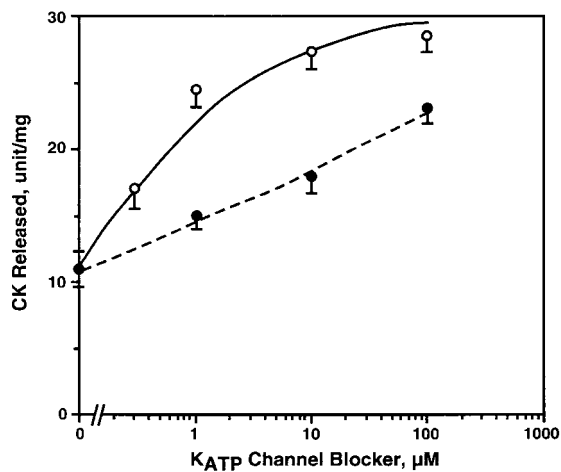
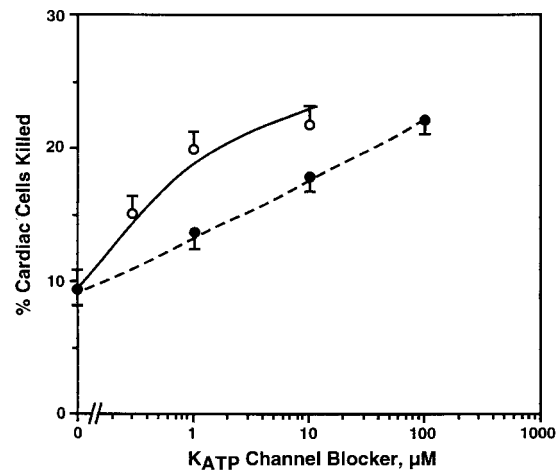
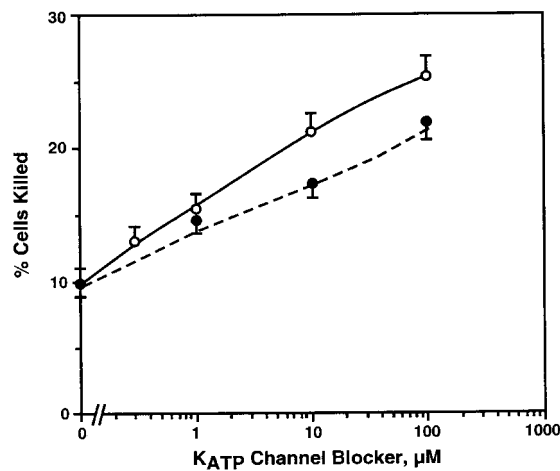


Figure 5 Glibenclamide and 5-HD blocked the CCPA-mediated cardioprotection in PMA-preconditioned cardiac cells

Cardiac ventricular cells were prepared and pretreated with either PMA or DMSO as described in the legend to Figure 1. Cells were then incubated in the presence of ADA and CCPA (10 nM) or in the presence of ADA, CCPA and the indicated concentrations of glibenclamide (○) or 5-HD (●). The percentage of cells killed (upper panel) and the amount of CK released (lower panel) were determined and represent means \pm S.E.M. for four experiments. At all concentrations of either K_{ATP} channel blockers tested, the percentage of cells killed and the amount of CK released were significantly greater than those obtained in cells not exposed to the blocker (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.05$).

Figure 6 Glibenclamide and 5-HD blocked the IB-MECA-mediated cardioprotection in PMA-preconditioned cardiac cells

Cardiac ventricular cells were prepared and pretreated with either PMA or DMSO as described in the legend to Figure 1. Cells were then incubated in the presence of ADA and IB-MECA (10 nM) or in the presence of ADA, IB-MECA and the indicated concentrations of glibenclamide (○) or 5-HD (●). The percentage of cells killed (upper panel) and the amount of CK released (lower panel) were determined and represent means \pm S.E.M. for four experiments. At 1, 10 and 100 μ M, glibenclamide or 5-HD was able to cause a significantly greater percentage of cells killed and amount of CK released than in cells not exposed to the K_{ATP} channel blocker (one-way ANOVA followed by Student–Newman–Keuls multiple comparison test, $P < 0.05$).

and IB-MECA caused fewer cardiac cells to be killed and less CK to be released in cells pretreated with PMA than in cells not pre-exposed to PMA.

Sequential activation of the adenosine receptor and the K_{ATP} channel mediates the cardioprotective effect of prior PMA treatment during the sustained ischaemia

Because the K_{ATP} channel is an effector distal to the adenosine receptor, it is possible that PKC enhances the A₁ or A₃ agonist-mediated cardioprotection by activating the K_{ATP} channel. According to this notion, PKC activates the K_{ATP} channel and renders the channel more responsive to stimulation by the receptor agonist during the subsequent ischaemia. Consequently the cardioprotective effect mediated by the receptor is enhanced in PMA-preconditioned cardiac cells. Three lines of evidence

support this hypothesis. First, prior treatment with PMA enhanced the ability of pinacidil to protect the cardiac cell against ischaemia-induced injury (Figure 4), providing direct evidence that prior stimulation of PKC caused an activated K_{ATP} channel that became more responsive to the channel opener during the subsequent ischaemia. Secondly, if this hypothesis is correct, K_{ATP} channel antagonists should block the PKC-induced increase in the receptor-mediated cardioprotection. The present results showed that this is indeed so. In cells pre-exposed to PMA, the simultaneous presence of either glibenclamide or 5-HD with CCPA during the ischaemia abolished the A₁ agonist-mediated cardioprotection (Figure 5). A concentration of either K_{ATP} channel inhibitor as low as 1 μ M resulted in the reversal of A₁ receptor-mediated protection. Similarly, the PKC-mediated in-

crease in the A_3 agonist-induced protection was also blocked dose-dependently by glibenclamide or 5-HD (Figure 6).

Lastly, another possible explanation for the PMA-induced increase in the adenosine receptor-mediated protection is that PMA caused a direct activation of the adenosine A_1 or A_3 receptor. If PMA can activate the adenosine A_1 or A_3 receptor or G_i directly, it should also enhance the ability of A_1 or A_3 receptors to mediate other functional responses. However, prior PMA treatment did not affect the ability of CCPA or IB-MECA to inhibit isoprenaline-stimulated cAMP accumulation. The percentage inhibition of the isoprenaline-stimulated increase in cAMP level by 10 nM CCPA, the maximally effective concentration, was $14 \pm 3\%$ in control (DMSO-treated) cells and $16 \pm 3\%$ in PMA-treated cells. For IB-MECA, the maximal percentage of inhibition of cAMP, which was produced by 10 nM A_3 agonist, was $15 \pm 2\%$ in control cells and $13 \pm 2\%$ in PMA-treated cells (means \pm S.E.M., $n = 4$; $P > 0.1$, paired t -test). The A_1 or A_3 agonist-induced cAMP inhibition curves were superimposable in both the control and PMA-preconditioned cells (results not shown). These results argue against a direct PKC-mediated activation of the adenosine A_1 or A_3 receptor and further support the notion that PKC enhances the A_1 or A_3 agonist-mediated cardioprotection by activating the K_{ATP} channel.

DISCUSSION

The cardioprotective effect of pre-ischaemia conditioning, also known as ischaemic preconditioning, has been shown in virtually every species studied, including human. Although the infarct-limiting effect of this phenomenon is striking, the underlying mechanism remains incompletely understood. Specifically, although the adenosine receptor, PKC and the K_{ATP} channel have all been implicated in mediating the protective effect of pre-ischaemia conditioning, the biochemical mechanism by which they are linked is not clear. By using an established cardiac cell model for simulated ischaemia and pre-ischaemia conditioning [15–17], a previous study has shown that prior activation of the PKC can protect the cardiac cell against injury produced by subsequent ischaemia [15]. Activation of both the adenosine receptor and the K_{ATP} channel was necessary to exert the actual protection during the prolonged ischaemia. The present study utilized PKC-mediated preconditioning to elucidate the sequence of activation of the receptor and the channel as well as the mechanism linking these two effectors in producing cardioprotection during the subsequent ischaemia.

Three lines of biochemical evidence support the notion that PKC, by activating the K_{ATP} channel, induces a subsequent increase in the adenosine receptor-mediated cardioprotection. First, the prior activation of PKC enhances the ability of pinacidil, present during the subsequent ischaemia, to protect the cardiac cell against injury. The results are consistent with the notion that the K_{ATP} channel remained activated during the subsequent prolonged ischaemia, which led to a greater pinacidil-mediated protection against cell injury. Secondly, prior PKC activation increases the subsequent A_1 receptor- or A_3 receptor-mediated protection and this protection is completely blocked by K_{ATP} channel antagonist. Thus the K_{ATP} channel acts downstream of the receptor in exerting the actual cardioprotection during the prolonged ischaemia. Thirdly, the prior activation of PKC has no effect on the ability of the receptor to mediate other functional responses such as the inhibition of cAMP accumulation. These results ruled out a direct activation of the receptor or G_i by PMA. Additionally, either glibenclamide or 5-HD was able to block the CCPA- and IB-MECA-induced protection in myocytes

not pre-exposed to PMA (results not shown), indicating that the protective effect of receptor agonists during ischaemia is also mediated via the K_{ATP} channel and does not require prior activation of PKC by PMA. The most plausible explanation for the increase in adenosine receptor-mediated cardioprotection in PMA-preconditioned cells is a PKC-induced K_{ATP} channel activation, which then renders the receptor more capable of mediating cardioprotection.

The present results are compatible with others [23,24] demonstrating a PKC-mediated activation of the K_{ATP} channel in isolated rabbit and human ventricular cells. Although the present study did not directly measure the effect of PMA on the electrophysiological activity of the K_{ATP} channel, cellular and biochemical evidence strongly supports the notion that stimulation of PKC can activate the K_{ATP} channel in these ventricular cells. The preconditioning effect of PMA was abolished by either glibenclamide or 5-HD whether the channel blocker was present during the 5 min exposure to PMA or during the 90 min ischaemia [15]. Further, prior PMA exposure enhanced the ability of the K_{ATP} channel opener pinacidil to protect the cardiac cell during the subsequent ischaemia (Figure 4). This PMA-induced increase in the pinacidil-mediated cardioprotection was abolished by glibenclamide or 5-HD (results not shown).

An important consideration in interpreting the present results is that significant differences exist in the expression of PKC isoforms between fetal, neonatal and adult cardiac myocytes [25,26]. Therefore the question arises whether the PKC-mediated preconditioning in cultured fetal cardiac cells is similar to the PKC-mediated preconditioning in adult cardiac cells. However, many of the PKC isoforms are present during cardiac development and a major difference is that the abundance of PKC isoforms decreases with age [26]. For example, the isoenzyme PKC- ϵ , implicated in mediating preconditioning [27], is expressed in fetal, neonatal human and rat heart [26] as well as in adult rat heart [25]. Further, the central role of PKC in preconditioning the cultured fetal cardiac cells reported here is similar to the role of PKC in preconditioning the adult rabbit and human cardiac myocytes [12,14,19]. Although the exact PKC isoform that mediates the preconditioning response in the cultured fetal cardiac cells is not clear, the objective here was to investigate the biochemical mechanism by which PKC mediates the cardiac preconditioning response by taking advantage of the stable, beating, reproducible and relatively homogenous nature of these cells.

Taken together, the results of the present study indicate that PKC mediates the activation of the K_{ATP} channel, which in turn renders the cardiac cell more responsive to the protective effect of adenosine. This enhanced responsiveness probably mediates the cardioprotective effect of PKC-induced preconditioning. The study provides further evidence for the notion that K_{ATP} channel is an effector downstream of PKC in initiating [15] as well as mediating the preconditioning effect. The exact mechanism by which activation of the channel causes cardioprotection remains unclear but deserves further study.

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