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Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D

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Abstract

Adenosine released during cardiac ischemia exerts a marked protective effect in the heart that is mediated by the A₁ and A₃ subtypes of adenosine receptors. The signaling pathways activated by these adenosine receptors have now been characterized in a chick embryo ventricular myocyte culture model of cardioprotection against ischemia. Selective A₁ and A₃ receptor agonists were shown to activate phospholipases C and D, respectively, to achieve their distinct cardioprotective effects. The specificity of the A₃ receptor–phospholipase D interaction was also demonstrated in chick embryo atrial myocytes (which do not express endogenous A₃ receptors) that had been transfected with a vector encoding the human A₃ receptor. Activation of both endogenous A₁ and A₃ receptors in ventricular myocytes resulted in a protective response greater than that induced by stimulation of either receptor alone. Agonists that activate both adenosine A₁ and A₃ receptors may thus prove beneficial for the treatment of myocardial ischemia.—Parsons, M., Young, L., Lee, J. E., Jacobson, K. A., Liang, B. T. Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D.

Keywords

ischemia; ventricular myocyte; PKC activity; cardioprotection

Adenosine is released in substantial amounts during myocardial ischemia and exerts a marked protective effect in the heart (1). Furthermore, adenosine released during a brief ischemic episode is able to protect the heart against injury during a subsequent period of prolonged ischemia, resulting in a reduction in infarct size (2–8). Brief exposure of the heart to adenosine, instead of to ischemia, induces a similar protective effect against subsequent ischemia-induced damage. Although both A₁ and A₃ subtypes of adenosine receptors

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mediate cardioprotection, the respective protective effects are distinct (9). The signaling mechanisms that underlie these distinct receptor-mediated responses have remained unknown.

A culture model for simulating ischemia and cardioprotection has been developed with embryonic chick cardiac ventricular myocytes (5, 9, 10, 11). This model exhibits adenosine-induced protective effects similar to those apparent in the intact heart (4, 6–8, 12, 13). With this cardiac cell model, the objective of the current study was to investigate whether the signaling mechanism underlying the adenosine A₁ receptor-mediated cardioprotective effect differs from that used by the adenosine A₃ receptor. Specifically, the study was aimed at testing the hypothesis that the distinct cardioprotective effects were mediated by differential coupling of adenosine A₁ and A₃ receptors to phospholipase C (PLC) and phospholipase D (PLD), respectively.

MATERIALS AND METHODS

Preparation of cultured cardiac atrial and ventricular myocytes and simulation of ischemia and ischemic preconditioning

Atrial or ventricular myocytes were cultured from chick embryos 14 days *in ovo*, maintained in culture, and used on day 3, when cells exhibited rhythmic beating as described previously (5, 10). In simulating ischemia and ischemic preconditioning, the medium was changed to a glucose-free HEPES-buffered medium containing (mM) 139 NaCl, 4.7 KCl, 0.5 MgCl₂, 0.9 CaCl₂, 5 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 2% fetal bovine serum, pH=7.4, 37°C before exposing the cells to simulated ischemia. Ischemia was simulated by incubating the myocytes in a hypoxic incubator (NuAire) for 90 min, during which O₂ was replaced by N₂ as described previously (5, 10). Determination of ischemia, when aliquots of the media were collected for creatine kinase activity measurement. This was followed by quantitation of the number of viable cells. Measurement of the basal level of cell injury was made after parallel incubation of control cells under normal % (room air) O₂. The extent of ischemia-induced myocyte injury was quantitated as increased number of cells killed and amount of creatine kinase (CK) released. The amount of CK was measured as enzyme activity (unit/mg), and increases in CK activity above the control level were determined. The percentage of cells killed was calculated as 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 the number of cells in the control group multiplied by 100%.

The percentage of cells killed was determined as follows. Cells treated under the various conditions were allowed to recover at 37°C for 1 h before being sedimented at 300 *g* for 10 min. The pellet was then resuspended in 1 ml of the medium used to incubate cells during the preconditioning protocol. Resuspended cells were drawn up and placed in the hemocytometer for quantitation. The concentration of cells counted was then converted to the total number of cells in 1 ml. Two additional lines of evidence are provided to support the validity of the assay for quantitating cells injured. First, there was minimal variability of proteins from one culture plate to another (a typical culture has 0.9±0.03 mg protein per 60 mm dish, ± SD, *n*=20 dishes). The CK enzyme activity was then normalized to the total

cellular protein (total amount of protein on the plate in the absence of ischemia or other experimental intervention). Second, parallel changes in percentage of cells killed and CK released during preconditioning by adenosine or by the brief hypoxia or during the various interventions further validated this assay for percentage of cells killed. Overall, the data indicate that the assay for quantitating percentage of cells killed can separate out hypoxia-damaged from control normoxia-exposed cells.

Preconditioning of the cardiac myocyte was achieved by exposing the myocyte to either simulated ischemia or adenosine receptor agonist for 5 min as described previously (5, 10). In preconditioning induced by 5 min simulated ischemia, myocytes were then incubated under an intervening normoxic condition before being exposed to 90 min of simulated ischemia. In preconditioning induced by adenosine receptor agonist, myocytes were incubated for 10 min with agonist-free medium, and then exposed to simulated ischemia for 90 min. Myocytes not subjected to preconditioning were exposed to 90 min ischemia only (non-preconditioned cells).

Measurement of diacylglycerol level and determination of phospholipase C, phospholipase D, and protein kinase C activity

Quantitation of diacylglycerol (DAG) was carried out as described previously (14, 16). In brief, cultured myocytes were labeled with [^3H] myristate (49 Ci/mmol, 2 $\mu\text{Ci/ml}$) for 24 h and exposed to receptor agonist for the times indicated. Lipids were extracted by the method of Bligh and Dyer (17). The formation of [^3H] DAG in cells was quantitated by separation of the labeled DAG from other phospholipids on thin-layer chromatography (petroleum ether/diethylether/acetic acid, 70/30/1, v/v/v on a Whatman K6 Silica Gel 60A plate) and scintillation counting of the ^3H label in spots migrating to the same position as unlabeled DAG. For measurement of PLD activity, myocytes labeled with [^3H] myristate for 24 h were exposed to receptor agonist in the presence of 0.5% (v/v) ethanol. The formation of [^3H] phosphatidylethanol (PEt) was determined by scintillation counting after its separation from other phospholipids on thin-layer chromatography (chloroform/methanol/ H_2O , 65/25/3, v/v/v on the Whatman K6 Silica Gel 60A plate) as described (15, 16). The level of [^3H] PEt represents an index of the PLD activity. The position of DAG or PEt was determined visually by placing the thin layer plate in an iodine chamber, and their levels were expressed as a percentage of total lipids. Data were expressed as the percentage increase in the amount of DAG or PEt relative to that for unstimulated cells.

The PLC activity was determined as the increase in the total inositol phosphates after receptor agonist stimulation according to a previously described method (19, 20). Myocytes were labeled with *myo*-[^3H]inositol (21 Ci/mmol, 5 $\mu\text{Ci/ml}$) for 24 h and stimulated for 30 min with the indicated concentrations of receptor agonist. The sum of inositol 1-phosphate, inositol 1,4-bisphosphates, and inositol 1, 4, 5-trisphosphate represented the total inositol phosphates. Data were expressed as the percentage increase in the amount of inositol phosphates relative to that for unstimulated cells. Protein kinase C activity in the intact myocyte was determined as described previously (21). In brief, ventricular myocytes were labeled with carrier-free [^{32}P]orthophosphate (100 $\mu\text{Ci/ml}$) for 4 h and then incubated in the absence or presence of adenosine A_1 or A_3 receptor agonist for 10 min. The phosphorylation

of the PKC substrate MARCKS (myristoylated alanine-rich C kinase substrate) was then examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described (21). The position of the 60 kDa MARCKS protein and of molecular size standards (in kilodaltons) are indicated.

Gene transfer into cardiac myocytes

Cardiac atrial myocytes were transfected with pcDNA3 or the recombinant pcDNA3 vector using a newly modified calcium phosphate precipitates method (22). Human cDNAs encoding the adenosine A₃ receptor (hA₃R) were subcloned into the eukaryotic expression vector pcDNA3, termed pcDNA3/hA₃R. Cardiac myocytes were maintained in culture for 24 h prior to being exposed to the calcium phosphate/DNA precipitates for 6 h at 37°C. Media were replaced with fresh growth media and the myocytes were cultured for an additional 48 h. The expression of human adenosine A₃ receptor cDNAs as functional proteins was determined by the ability of transfected human adenosine A₃ receptor to mediate inhibition of isoproterenol-stimulated adenylyl cyclase, as determined previously (9, 23).

Materials

The adenosine analog 2-chloro-N⁶-cyclopentyladenosine (CCPA), and the protein kinase C inhibitor chelerythrine were from Research Biochemicals International (Natick, Mass.). Cl-IB-MECA was synthesized according to a previously described procedure (24). In brief, methyl 1,2,3-tri-O-acetyl-β-D-ribofuronate was condensed with silylated 2-chloro-N⁶-(3-iodobenzyl)adenine in dry 1,2-dichloroethane using trimethylsilyl triflate as a catalyst (2 h at room temperature followed by 48 h reflux). The β-anomer (methyl 1-[2-chloro-N⁶-(3-iodobenzyl)-adenin-9-yl]-2,3-di-O-β-D-ribofuronate) was isolated using silica gel column chromatography and treated with methylamine in THF overnight at 50°C in a sealed tube to provide Cl-IB-MECA (methyl 1-[2-chloro-N⁶-(3-iodobenzyl)-adenin-9-yl]-β-D-ribofuronamide). 1-(6-((17β-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) was obtained from BioMol (Plymouth Meeting, Pa.). Diacylglycerol and phosphatidylethanol were from Avanti Polar Lipids (Alabaster, Ala.). Propranolol was obtained from Sigma Chemical Co. (St. Louis, Mo.). The vector pcDNA3 was obtained from Invitrogen (Carlsbad, Calif.). [³H]myristate, *myo*-[³H]inositol, [³²P]orthophosphate were from New England Nuclear (Boston, Mass.). Embryonated chick eggs were from Spafas, Inc. (Storrs, Conn.).

RESULTS

Role of protein kinase C in the cardioprotective effects of adenosine A₁ receptor- and A₃ receptor-selective agonists

Previous study has shown that protein kinase C (PKC) inhibitor was able to block the preconditioning-like effect of adenosine (21). Since this effect of adenosine is mediated by its receptors, the objective here was to determine whether a PKC inhibitor could block the cardioprotective effect induced by adenosine A₁ receptor- or A₃ receptor-selective agonists. Figure 1A shows that chelerythrine abolished the preconditioning-like effect of an A₁ (CCPA, 2-chloro-N⁶-cyclopentyladenosine) or A₃ (Cl-IB-MECA, 2-chloro-N⁶-(3-

iodobenzyl)adenosine-5'-N-methyluronamide) receptor agonist, indicating that PKC acts downstream from each receptor in mediating this effect. The link between adenosine receptors and PKC was further supported by the observation that activation of either A₁ or A₃ receptors induced both accumulation of the PKC activator DAG (Fig. 1B) and PKC activation itself (Fig. 1C) in intact myocytes. However, the effect of the A₃ receptor agonist Cl-IB-MECA on the abundance of DAG was more pronounced and longer lasting than that of the A₁ receptor agonist CCPA. Because PLD stimulation produces a more sustained increase in DAG level, these data suggested that the A₃ receptor is coupled to PLD, whereas the A₁ receptor is coupled to PLC.

Coupling of the adenosine A₁ receptor to PLC and of the adenosine A₃ receptor to PLD

The coupling of each receptor subtype to these phospholipases was investigated directly by measuring the effects of the selective agonists on the production of both inositol phosphates (products of PLC-mediated hydrolysis of inositol phospholipids) and phosphatidylethanol (a product of PLD-mediated hydrolysis of phospholipids in the presence of ethanol). The A₁ receptor agonist CCPA induced a markedly greater increase in the production of inositol phosphates than did the A₃ receptor agonist Cl-IB-MECA (Fig. 2A), whereas the increase in PEt production induced by Cl-IB-MECA was much larger than that induced by CCPA (Fig. 2B). Thus, the A₁ and A₃ receptors appeared selectively coupled to PLC and PLD, respectively.

The cardioprotective effects of adenosine A₁ and A₃ receptors are mediated by PLC and PLD, respectively

The next objective was to investigate whether activation of PLC and of PLD mediates the cardioprotective effects induced by A₁ and A₃ receptors, respectively. The data showed that this is in fact the case. First, the PLC-selective inhibitor U-73122 blocked most of the CCPA-induced accumulation of DAG (Fig. 3A). In parallel, U-73122 was also able to abolish the A₁ agonist-induced activation of PLC (not shown). On the other hand, U-73122 exerted only moderate inhibitory effects on the accumulation of DAG (Fig. 3A) and activation of PLD (not shown) induced by Cl-IB-MECA. Second, U-73122 abolished the cardioprotective effect of CCPA but had no effect on that of Cl-IB-MECA (Fig. 3B). These data are thus consistent with the hypothesis that PLC mediates the cardioprotective effect of the A₁ receptor but does not play a role in A₃ receptor-induced protection.

The role of PLD in the cardioprotective effects of adenosine A₁ and A₃ receptor agonists was similarly investigated by selective inhibition of DAG formation by this enzyme. Ethanol or butanol inhibits the formation of phosphatidic acid (and therefore that of DAG) mediated by PLD as a result of PLD-catalyzed transphosphatidylation of the alcohol (14–16, 18). Ethanol inhibited the A₃ agonist-induced accumulation of DAG (Fig. 3C) and abolished A₃ agonist-induced cardioprotection (Fig. 3D). In contrast, neither the DAG accumulation (Fig. 3C) nor the cardioprotective response (Fig. 3D) induced by the A₁ receptor was affected by ethanol. Similar data were obtained with butanol in place of ethanol (not shown).

Additional evidence that PLD selectively mediates the cardioprotective effect of the A₃ receptor was provided by the observation that propranolol, which inhibits the conversion of

phosphatidic acid to DAG (15, 18), blocked the A₃ agonist-induced accumulation of DAG (Fig. 3C) as well as the cardioprotection afforded by this agonist (Fig. 3D). In contrast, propranolol had virtually no effect on DAG accumulation (Fig. 3C) or cardioprotection (Fig. 3D) induced by CCPA. Thus, the adenosine A₃ receptor signals selectively through PLD to induce its cardioprotective effect, whereas the A₁ receptor acts via PLC to achieve cardioprotection.

The A₃ receptor is likely present on the cardiac cell and not on nonmyocytes such as the fibroblast for the following reasons. First, the A₃ receptor agonist CI-IB-MECA is able to inhibit the isoproterenol-stimulated increase in the contractility of these chick cardiac myocyte (data not shown). Since fibroblasts are not excitable cells and do not contract, the A₃ agonist effect on contractility is likely due to myocyte A₃ receptor activation. Second, it is unlikely that adenosine receptors of fibroblasts play an important role in cardioprotection because the principal nonmyocytes in these cultures, the fibroblasts, express predominantly the A_{2B} receptor, and because the A₃ agonist CI-IB-MECA at the concentrations used does not cause significant stimulation of the A_{2B} receptor.

PLD mediates the cardioprotective effect induced by activation of human adenosine A₃ receptors expressed in chick atrial myocytes

Atrial myocytes cultured from embryonic chick hearts lack native A₃ receptors (9). The cardioprotection induced by a brief period of ischemia in these cells is thus of shorter duration than that apparent with ventricular myocytes (Fig. 4A) and is characteristic of an A₁ receptor response (9). Transfection of chick atrial myocytes with a vector encoding the human adenosine A₃ receptor confers a sustained cardioprotective response to A₃ agonists (9). The acquisition of this sustained cardioprotective response is associated with the appearance of A₃ agonist-induced increases in both DAG abundance and PLD activity (Fig. 4B). The A₃ agonist-induced accumulation of DAG was abolished by ethanol but was unaffected by U-73122 (Fig. 4C), suggesting that the human adenosine A₃ receptor selectively couples to PLD when expressed in chick atrial myocytes. The cardioprotection mediated by the human adenosine A₃ receptor was also abolished by ethanol or propranolol (Fig. 4D) but was not affected by U-73122 (data not shown). These data show that the human A₃ receptor signals selectively through PLD to achieve its cardioprotective effect.

Additive cardioprotective effects of adenosine A₁ and A₃ receptor agonists

The specificity of the A₁ receptor–PLC and A₃ receptor–PLD linkages and the consequent distinct biological effects mediated by each receptor suggested that concomitant activation of both receptors may result in a response greater than that triggered by the activation of either receptor alone. Indeed, the extent of cardioprotection induced by the combination of A₁ and A₃ receptor agonists was greater than that apparent with an equivalent concentration of either agonist alone (Fig. 5). These data indicate a productive, additive interaction between the two adenosine receptors that elicits a potent cardioprotective response in the cardiac myocyte.

DISCUSSION

Adenosine is a potent cardioprotective agent capable of reducing myocardial injury either as the trigger and mediator of ischemic preconditioning or as an injury-limiting agent when it is present even after ischemia has begun (1–5, 11, 23). Although activation of adenosine receptors has been shown to mediate these cardioprotective effects of adenosine, the cellular and molecular mechanisms downstream of the receptor remain incompletely understood. Both the A₁ and A₃ adenosine receptors can trigger and mediate the cardioprotective effect of ischemic preconditioning. However, the preconditioning effect mediated by the A₃ receptor is more sustained than that induced by activation of the A₁ receptor (9). The objective of the current study was to investigate the signaling mechanism that acts downstream of A₁ or A₃ receptors in mediating these distinct preconditioning effects. Although PLD has been implicated in ischemic preconditioning (25), the coupling of PLD to a specific adenosine receptor and its role in mediating the preconditioning effect induced by adenosine are unknown. A previously established cardiac myocyte model of ischemic preconditioning (5, 9, 10) was used to test the hypothesis that the distinct cardioprotective effects of adenosine are mediated by differential coupling of A₁ and A₃ receptors to PLC and PLD, respectively. Multiple lines of evidence are provided to support this hypothesis.

First, activation of the adenosine A₃ receptor causes a more pronounced and sustained stimulation of the DAG accumulation than does the activation of A₁ receptors, consistent with a selective coupling of the A₃ receptor to stimulation of PLD. This coupling was determined directly by showing that activation of the A₃ receptor causes a much more pronounced stimulation of the PLD activity than does the A₁ receptor. The coupling of the adenosine A₃ receptor to PLD activation has also been observed in a rat mast cell line (26). In contrast, the A₁ receptor mediates a greater increase in the PLC activity than does the A₃ receptor. Thus, the A₁ receptor is selectively coupled to PLC, whereas the A₃ receptor is coupled to PLD. Second, activation of PLC is responsible for the A₁ receptor-mediated cardioprotective effect. The PLC inhibitor U-73122 blocked the A₁ agonist-induced activation of PLC, accumulation of DAG, and cardioprotective effect. In contrast, U-73122 had no effect on the cardioprotective effect mediated by the A₃ receptor. Although the selectivity of U-73122 for PLC vs. PLD is unclear, U-73122 could abolish the PLC activation. The present data show that U-73122 clearly has a different effect on the A₁ receptor–PLC linkage than it does on the A₃ receptor–PLD linkage. The data obtained with U-73122 support the hypothesis that PLC mediates the cardioprotective response to A₁ receptor agonist, but not to the A₃ receptor agonist.

In establishing the third line of evidence, the role of PLD was also determined by selective inhibition of DAG formation by this enzyme. Ethanol or butanol can inhibit the formation of DAG mediated by PLD because of PLD-catalyzed transphosphatidylation of the alcohol. Ethanol or butanol inhibited the A₃ agonist-stimulated accumulation of DAG and cardioprotective effect. In contrast, the alcohol had no effect on the A₁ agonist-stimulated DAG accumulation or cardioprotective response. Additional evidence for this concept was obtained by the finding that propranolol, which inhibits the conversion of phosphatidic acid to DAG, blocked the A₃ agonist-induced DAG accumulation and cardioprotective response. On the other hand, propranolol had no effect on the A₁ agonist-induced DAG accumulation

or cardioprotective response. Although propranolol is also a β -adrenergic blocker, there was no exogenous or endogenous catecholamine during the experimental conditions. Most likely, the effect of propranolol on A₁ and A₃ receptor-mediated cardioprotection is due to its selective inhibitory effect on the A₃ receptor-PLD coupling.

Finally, atrial cardiac myocytes cultured from embryonic chick hearts, which lack native A₃ receptor, were used to determine the specificity of adenosine A₃ receptor-PLD coupling. The role of PLD in coupling to human adenosine A₃ receptor and in mediating the cardioprotective effect induced by the human adenosine A₃ receptor was investigated. Previous study showed that these myocytes can be efficiently transfected and that transfection of atrial myocytes with human A₃ receptor cDNA conferred a sustained cardioprotective response to A₃ agonist (9). The current study shows that transfection with human adenosine A₃ receptor cDNA results in the appearance of an A₃ agonist-induced increase in both DAG level and PLD activity. Ethanol blocked the DAG accumulation and the cardioprotective effect mediated by the human adenosine A₃ receptor. In contrast, the PLC inhibitor U-73122 had no effect on the DAG accumulation or the cardioprotective effect induced by activation of the human A₃ receptor. Thus, the human adenosine A₃ receptor is selectively coupled to PLD in achieving its cardioprotective effect.

In conclusion, the present data show that the distinct cardioprotective effects of adenosine acting at the A₁ and A₃ subtypes of adenosine receptors are mediated by selective coupling of these receptors to phospholipases C and D, respectively. The different signaling mechanisms that underlie the distinct A₁ and A₃ receptor-mediated protective responses raised the possibility that the two receptor pathways may interact in a productive manner to elicit an additive cardioprotective effect. The present data indicate that this is indeed the case. The concomitant presence of both A₁ and A₃ receptor agonists induced a greater cardioprotective effect than that produced by an equivalent concentration of either agonist alone. The present findings are important for our understanding of the basic biology of cardioprotection, an important self-adaptive mechanism intrinsic to the heart. Highly relevant to this investigative effort, our data indicate that agonists capable of activating both A₁ and A₃ receptors are thus likely to provide protection from ischemia at lower doses than those required for selective agonists and therefore should have fewer potentially adverse side effects. Agonists that act at both A₁ and A₃ adenosine receptors may prove therapeutically beneficial in the treatment of ischemic heart disease.

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Figure 1. Coupling of adenosine A₁ and A₃ receptors to stimulation of DAG accumulation and PKC activity in intact myocytes. **A)** Effect of the PKC inhibitor chelerythrine (Research Biochemical International) on cardioprotection induced by A₁ or A₃ receptor agonists. Ventricular myocytes were isolated from chick embryos after 14 days *in ovo* and cultured as described (5, 9). The ability of the A₁ receptor agonist CCPA or the A₃ receptor agonist CI-IB-MECA (24) to induce cardioprotection was determined by a preconditioning protocol as described (5, 10). In brief, myocytes were exposed to 10 nM CCPA or CI-IB-MECA for 5

min, incubated for 10 min with agonist-free medium, and then exposed to simulated ischemia for 90 min. The extent of myocyte injury was quantitated at the end of the 90 min period by determination of the percentage of cells killed and the amount of creatine kinase (CK) released (units per milligram of cell protein). Cells were exposed to chelerythrine during the 5 min incubation with CCPA or CI-IB-MECA. Data are means $\pm$ SE of values from five independent experiments. *B*) Adenosine receptor agonist-induced cellular accumulation of DAG. Ventricular myocytes were labeled with [3 H]myristate (49 Ci/mmol, 2 μ Ci/ml) for 24 h, exposed to 30 nM CCPA or CI-IB-MECA for 150 s, and then incubated in agonist-free medium for the indicated times, after which the amount of DAG in cells was determined by thin-layer chromatography. Data were expressed as the percentage increase in the amount of DAG relative to that for unstimulated cells, and are means $\pm$ SE of values from five independent experiments. *C*) Activation of PKC by adenosine receptor agonists. Ventricular myocytes were labeled with carrier-free [32 P]orthophosphate (100 μ Ci/ml) for 4 h and then incubated in the absence (lanes 1 and 3) or presence of 30 nM CCPA (lane 2) or CI-IB-MECA (lane 4) for 10 min. The phosphorylation of the PKC substrate MARCKS was then examined by SDS-polyacrylamide gel electrophoresis and autoradiography as described (21). The position of the 60 kDa MARCKS (myristoylated alanine-rich C kinase substrate) protein and of molecular size standards (in kilodaltons) are indicated. Lanes 1 and 2 and lanes 3 and 4 correspond to different gels. Similar results were obtained in four independent experiments.

Figure 2.

Differential activation of PLC and PLD by adenosine A₁ and A₃ receptors in cultured cardiac ventricular myocytes. *A*) Activation of PLC by A₁ receptors. Ventricular myocytes were labeled with *myo*-[³H]inositol (21 Ci/mmol, 5 μ Ci/ml) for 24 h and then stimulated for 30 min with the indicated concentrations of CCPA or CI-IB-MECA. The production of total inositol phosphates was determined by the sum of inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-trisphosphate as described (19, 20) and served as a measure of PLC activity. Data are expressed as the percentage increase in the amount of inositol phosphates relative to that for unstimulated cells, and are means $\pm$ SE of values from five independent experiments. *B*) Activation of PLD by A₃ receptors. Myocytes were labeled with [³H]myristate (49 Ci/mmol, 2 μ Ci/ml) for 24 h and then exposed for 5 min to the indicated concentrations of CCPA or CI-IB-MECA in the presence of 0.5% (v/v) ethanol. The formation of [³H] phosphatidylethanol was determined by scintillation counting after its separation from other phospholipids on thin-layer chromatography as described (18) and represents an index of PLD activity. Data are expressed as the percentage increase in the amount of phosphatidylethanol relative to that for unstimulated cells and are means $\pm$ SE of values from five independent experiments.

Figure 3.

Selective mediation of the cardioprotective effects of A₁ and A₃ receptor agonists by PLC and PLD, respectively. *A*) Effects of the PLC inhibitor U-73122 on DAG accumulation induced by A₁ and A₃ receptor agonists. Cardiac ventricular myocytes were labeled with [³H]myristate for 24 h and then exposed for 5 min to 30 nM CCPA or Cl-IB-MECA in the absence or presence of 10 μM U-73122, after which the amount of DAG was measured. Data are means ± SE from five independent experiments. **P* < 0.05 vs. corresponding value for agonist alone (Student's *t* test). The percentage of inhibition by U-73122 of DAG accumulation induced by CCPA (67 ± 3%) was significantly greater than that for DAG accumulation induced by Cl-IB-MECA (38 ± 4%) (*P* < 0.01, Student's *t* test). *B*) Effects of U-73122 on cardioprotection induced by A₁ and A₃ receptors. U-73122 (10 μM) was

included or not during the 5 min exposure of ventricular myocytes to 10 nM CCPA or Cl-IB-MECA, and cardiac injury was assessed after ischemia as described in the legend to Fig. 1A. Data are means $\pm$ SE of values from five independent experiments. * P <0.01 vs. corresponding values in the presence of U-73122 (Student's t test). *C*) Effects of ethanol and propranolol on DAG accumulation induced by A₁ or A₃ receptor agonists. DAG accumulation in ventricular myocytes exposed to 30 nM CCPA or Cl-IB-MECA in the absence or presence of 0.5% ethanol or 100 μ M propranolol was determined as in panel *A*. Data are means $\pm$ SE of values from five independent experiments. * P <0.01 vs. corresponding value for agonist alone (Student's t test). *D*) Effects of ethanol and propranolol on cardioprotection induced by A₁ or A₃ receptors. Ethanol (0.5%) or propranolol (100 μ M) was included or not during the 5 min exposure of ventricular myocytes to 10 nM CCPA or Cl-IB-MECA, and cardiac injury was assessed after ischemia as described in panel *B*. Data are means $\pm$ SE of five independent experiments. * P <0.01 vs. corresponding value for agonist alone (Student's t test).

Figure 4.

PLD-mediated cardioprotection induced by activation of human adenosine A₃ receptors. *A*) Comparison of ischemia-induced cardioprotection between chick atrial and ventricular myocytes. Atrial and ventricular myocytes, both cultured from chick embryos after 14 days *in ovo*, were subjected to hypoxia for 5 min and then incubated for the indicated intervals under normoxia before exposure to ischemia for 90 min. The percentage of dead cells was determined at the end of the 90 min period. Data are means $\pm$ SE of values from four independent experiments. *B*) PLD activation and DAG accumulation induced by human A₃ receptors in chick atrial myocytes. Atrial myocytes were transfected with pcDNA₃ or with pcDNA₃ containing the full-length cDNA for the human adenosine A₃ receptor (pcDNA₃/hA₃R). Forty-eight hours after transfection, the ability of the A₃ agonist Cl-IB-

MECA (30 nM) to induce PLD activation or accumulation of DAG was determined as described in the legends to Figs. 2B and 3A, respectively. The amounts of DAG and phosphatidylethanol (PEt) are expressed as a percentage of total lipids and are means $\pm$ SE of triplicates from an experiment that was repeated an additional four times with similar results. * P <0.01 vs. corresponding value for pcDNA3/hA₃R-transfected cells incubated in the absence of CI-IB-MECA (Student's t test). *C*) Effects of ethanol and U-73122 on DAG accumulation induced by activation of human A₃ receptors in chick atrial myocytes. The effects of ethanol and U-73122 on the CI-IB-MECA-induced accumulation of DAG in atrial myocytes that had been transfected with pcDNA3/hA₃R (or pcDNA3) were investigated as described in the legends to Figs. 3A and 3C, respectively. Data are means $\pm$ SE of triplicates from an experiment that was repeated four additional times with similar results. * P <0.01 vs. corresponding value for pcDNA3/hA₃R-transfected cells incubated in the absence of ethanol or U-73122 (Student's t test). The inhibitory effect of ethanol ($90 \pm 0.6\%$) in the cells expressing human A₃ receptors was significantly greater than that of U-73122 ($12 \pm 8\%$) (P <0.05, Student's t test). *D*) Effects of ethanol and propranolol on the cardioprotection mediated by human A₃ receptors in chick atrial myocytes. The effects of ethanol and propranolol on the cardioprotective effect of CI-IB-MECA in atrial myocytes that had been transfected with pcDNA3/hA₃R (or pcDNA3) were investigated as described in the legend to (Fig. 3D) except that the time interval between drug exposure and the 90 min ischemia was 30 min. * P <0.01 vs. corresponding value for pcDNA3/hA₃R-transfected cells incubated in the absence of ethanol or propranolol (Student's t test).

Figure 5.

Comparison of the cardioprotective effects of adenosine A_1 and A_3 receptor agonists when presented alone or together. Ventricular myocytes were exposed to either CCPA, CI-IB-MECA, or both agonists for 5 min and the resulting cardioprotection against ischemia was assessed as described in the legend to Fig. 1A. The concentrations shown for incubations with both CCPA and CI-IB-MECA refer to the total concentration of both agonists (which were present in equal concentrations). Data are means $\pm$ SE of five independent experiments. * $P < 0.01$ vs. corresponding value for either agonist alone (Student's t test). Similar data were obtained with CK release as the index of cardioprotection (not shown).