Antiadrenergic effects of adenosine A$_1$ receptor-mediated protein phosphatase 2a activation in the heart

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Protein phosphatase type 1 (PP1) and type 2a (PP2a) account for $>90\%$ of all serine/threonine dephosphorylation reactions (5). Muscarinic receptor-dependent decrease in catecholamine-induced cAMP signaling occurs through activation of PP2a (18). Furthermore, PP2a can dephosphorylate the catalytic subunit of PKA and reduce its activity (25). These observations led us to test the hypothesis that PP2a is involved in the ability of adenosine to antagonize the positive inotropic effect of β-adrenergic stimulation in rat hearts.

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In vitro studies indicate that PP2a-C is regulated by posttranslational modifications including phosphorylation (4, 14) and methylation (2). Phosphorylation of PP2a-C leads to inhibition of PP2a activity (4, 14). Methylation does not appear to directly influence the enzymatic activity of PP2a but is important for regulatory subunit binding (2, 37). Regulatory subunit binding to the catalytic subunit and translocation of the heterotrimer alter local PP2a activity (26, 38). These observations led us to test the hypothesis that adenosine A1 receptor activation can alter the methylation state, subcellular localization, and activity of PP2a in cardiac myocytes.

MATERIALS AND METHODS

Isolated heart preparation and experimental protocol. Hearts were removed from female Wistar rats (250–300 g) anesthetized by isoflurane inhalation. The hearts were cannulated in ice-cold modified Krebs-Henseleit solution, mounted on a Langendorff perfusion apparatus, and immersed in a temperature-controlled organ bath containing oxygenated modified Krebs-Henseleit buffer. Modified Krebs-Henseleit buffer contained (in mM) 4.7 KCl, 118 NaCl, 1.2 MgSO4, 1.3 CaCl2, 25 NaHCO3, 11 glucose, and 1.2 KH2PO4. The pH of this solution was 7.4 when it was gassed with 95% O2-5% CO2 at 37°C. A balloon connected to a pressure transducer (model BLPR, World Precision Instruments, Sarasota, FL) was inserted into the left ventricle and inflated until a maximum left ventricular developed pressure (LVDP) was observed. Pressures were continually monitored, and values were stored on a computer. Hearts were externally paced at 300 beats/min with a voltage twice that of threshold.

All hearts were equilibrated with noncirculated oxygenated, modified Krebs-Henseleit solution at 37°C for 30 min. After equilibration, hearts were perfused with modified Krebs-Henseleit solution containing either 1 μM cantharidin or DMSO for 30 min. Hearts were then exposed to 10 μM isoproterenol (Iso) for 5 min, followed by Iso plus the adenosine A1 receptor agonist 3'-cyclopentyladenosine (CPA; 1 μM) for 5 min. Agonists were then washed out of the heart with Krebs-Henseleit solution for 20 min. To determine the effect of the experimental protocol on the outcome, hearts were then rechallenged with Iso followed by Iso plus DMSO.

Enzymatic isolation of ventricular myocytes. Ventricular myocytes were isolated by using the protocol of Lester et al. (24) with slight modifications. In brief, hearts were cannulated and mounted on a Langendorff perfusion apparatus and residual blood was washed out with Ringer solution containing 0.5 mM EGTA for 5 min. Ringer solution contained (in mM) 25 HEPES (pH 7.4), 1.2 MgCl2, 4.8 KCl, 118 NaCl, 2 KH2PO4, 5 pyruvate, 11 glucose, and 1 insulin. The heart was then perfused with Ringer solution containing 1 mg/ml type II collagenase (Worthington Biochemical, Lakewood, NJ) for 15−15 min. After collagenase perfusion, the ventricles were cut into small pieces, rinsed in fresh Ringer solution without collagenase, and dissociated by gentle trituration. The resulting cells were resuspended in oxygenated Ringer solution containing 1.3 mM CaCl2 and 0.1% bovine serum albumin. Final ventricular cell preparations containing <50% rod-shaped viable myocytes were discarded.

Preparation of cell fractions. Cell fractions of ventricular myocytes were isolated by the digitonin permeabilization method of Whisler et al. (42). Briefly, isolated cells were treated with 1 μM CPA, CPA plus 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPX), or DPX alone for 5 min and centrifuged. Cell pellets were resuspended in ice-cold 0.05% digitonin permeabilization buffer containing (in mM) 40 Tris·HCl (pH 7.4), 5 β-mercaptoethanol, 2 EDTA, 1 sodium fluoride, 1 sodium orthovanadate, 1 leupeptin, and 2 phenylmethylsulfonyl fluoride. The cells were gently mixed in permeabilization buffer for 10 min on ice and centrifuged at 12,000 g for 15 min at 4°C. The supernatant from this centrifugation was designated the cytosolic fraction. The pellet was dissolved with vigorous vortexing in the same buffer supplemented with 1% Triton X-100 and left on ice for 20 min. Subsequent centrifugation at 12,000 g for 15 min at 4°C produced a supernatant containing PP2a solubilized by Triton X-100 that was designated the particulate fraction. PP2a content remaining in the insoluble pellet was negligible as determined by Western blot analysis (data not shown).

Determination of cardiac protein phosphorylation. Changes in cardiac protein phosphorylation were determined by 32P autoradiography. Isolated ventricular myocytes were incubated with [32P]orthophosphate in Ringer solution containing 1 mM CaCl2 for 1 h at room temperature. The cells were then incubated in the presence or absence of the protein phosphatase inhibitor okadaic acid (OA; 1 μM) during the final 30 min of labeling. Cells were then pretreated with 10 nM Iso for 5 min, followed by no additional agonists (Iso alone), Iso plus 1 μM CPA, or Iso plus CPA plus 1 μM DPX for 5 min. Additional groups included cells treated with CPA or DPX alone for 5 min. All drug solutions were prepared in 1 mM CaCl2-Ringer solution containing 100 μM sodium metabisulfite to protect Iso from oxidation and 10 U/ml adenosine deaminase to decrease the effect of endogenous adenosine. The reactions were quenched by addition of SDS-sample buffer. All samples were heated for 5 min at 95°C, and proteins were separated by SDS-PAGE with a 17% resolving gel and a 5% stacking gel. Gels were stained with Coomassie blue, and dried gels were subjected to autoradiography with X-OMAT film (Eastman Kodak, Rochester, NY) with exposure times ranging from 12 to 48 h. Digital images of X-rays and gels were obtained and densities were determined with Image software (NIH, public domain). Data were normalized to protein load and to untreated (control) myocyte response.

Analysis of PP2a translocation. Isolated ventricular myocytes were untreated or treated with 1 μM CPA, CPA plus 1 μM DPX, or DPX alone for 5 min. Cells were then fractionated into cytosolic and particulate fractions (see Preparation of cell fractions). All fractions were incubated in 0.1 N NaOH for 30 min at 30°C (2). This alkali treatment fully demethylates PP2a and addresses the concern that methylation state may have an effect on PP2a immunoreactivity (2, 11). After NaOH incubation samples were neutralized with HCl and heated at 95°C for 5 min. Proteins were separated by SDS-PAGE with a 12% resolving gel and a 5% stacking gel and transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, PVDF membranes were blocked in PBS-3% milk for 30 min and incubated with an antibody to PP2a-C (1:1,000 dilution, catalog no. 65-421; Upstate Biotechnology) overnight at 4°C. Membranes were then incubated for 2 h at room temperature with peroxidase-conjugated secondary antibody (1:4,000 dilution, catalog no. A3682; Sigma). Blots were also stained with Coomassie blue for an assessment of the extent of protein transferred for each sample. Densities of the PP2a reactive band were determined with Image. Data were normalized to protein load and corresponding controls in each experiment.

Determination of carboxymethylation state of PP2a. Isolated ventricular myocytes were untreated (control) or treated with 1 μM CPA, CPA plus 1 μM DPX, or DPX alone for 5 min. Cells were then lysed in a 1% Triton X-100 buffer.
RESULTS

CPA treatment significantly decreased the positive inotropic effect of Iso in isolated, paced rat hearts (Fig. 1A, Table 1). To ensure that experimental protocol, in and of itself, did not contribute to this effect, hearts were rechallenged with Iso followed by Iso plus DMSO (vehicle for CPA). There was no significant difference in LVDP between Iso and Iso plus DMSO treatments (Fig. 1, Table 1). CPA at 1 μM had no effect on LVDP in the absence of Iso stimulation (data not shown). The antiadrenergic effect of CPA on LVDP was blocked in hearts pretreated with the protein phosphatase inhibitor cantharidin for 30 min (Fig. 1B, Table 1). Pretreatment of hearts with 1 μM cantharidin had no significant effect on baseline LVDP or the magnitude of the positive inotropic effect of Iso.

The effect of adenosine A₁ receptor activation on cardiac protein phosphorylation was determined by ³²P autoradiography. Iso increased PLB phosphorylation ~2.5-fold over control, and this effect was antagonized by CPA treatment in isolated rat ventricular myocytes (Figs. 2 and 3). The antiadrenergic effect of CPA on Iso-stimulated PLB phosphorylation was abolished by 1 μM DPX (adenosine A₁ receptor antagonist). DPX alone did not affect PLB phosphorylation compared with control. Notably, CPA exposure by itself significantly decreased basal PLB phosphorylation in ventricular myocytes (Fig. 3A). Changes in phosphate incorporation into troponin I (TnI) caused by Iso, CPA, and DPX were similar to those of PLB (Fig. 3B).

Additional autoradiography studies examined the effect of CPA on cardiac protein phosphorylation in the presence of a PP2a inhibitor. As shown in Figs. 4 and 5, the effect of CPA to decrease phosphorylation of PLB and TnI is abolished in myocytes pretreated with 1 μM OA. OA also caused a significantly decreased basal PLB phosphorylation compared with control. Notably, CPA exposure by itself significantly decreased basal PLB phosphorylation in ventricular myocytes (Fig. 3A). Changes in phosphate incorporation into troponin I (TnI) caused by Iso, CPA, and DPX were similar to those of PLB (Fig. 3B).

Table 1. Anti-β-adrenergic effect of adenosine A₁ receptor activation on left ventricular developed pressure in the presence and absence of protein phosphatase inhibitor cantharidin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle</th>
<th>1 μM Cantharidin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LVDP, mmHg</td>
<td>% LVDP</td>
</tr>
<tr>
<td>Control</td>
<td>88.7 ± 4.2</td>
<td>100</td>
</tr>
<tr>
<td>Iso</td>
<td>181.3 ± 10.8*</td>
<td>204</td>
</tr>
<tr>
<td>Iso + CPA</td>
<td>145.4 ± 6.4*</td>
<td>164</td>
</tr>
<tr>
<td>Control 2</td>
<td>86.4 ± 4.9</td>
<td>97</td>
</tr>
<tr>
<td>Iso 2</td>
<td>169.0 ± 8.4*</td>
<td>196</td>
</tr>
<tr>
<td>Iso + DMSO</td>
<td>174.4 ± 7.8*</td>
<td>202</td>
</tr>
</tbody>
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Values are means ± SE for 5 hearts. Isoproterenol (Iso; 10 nM) is a β-adrenergic receptor agonist, whereas N⁶-cyclopentyladenosine (CPA; 1 μM) is an adenosine A₁ receptor agonist. DMSO (0.001%) is the CPA vehicle. LVDP, left ventricular developed pressure. *P < 0.05 compared with Control; †P < 0.05 compared with Iso.

Statistical analysis. All data were analyzed by two-way analysis of variance and Student’s t-test. All values are expressed as means ± SE, and P < 0.05 was chosen to indicate statistical significance.

Fig. 1. Representative examples of the anti-β-adrenergic effect of adenosine A₁ receptor activation on function of isolated rat hearts pretreated with either vehicle (A) or the protein phosphatase inhibitor cantharidin (B). Hearts were stimulated with the β-adrenergic receptor agonist isoproterenol (Iso; 10 nM), followed by Iso + the adenosine A₁ receptor agonist N⁶-cyclopentyladenosine (CPA; 1 μM). Hearts were then washed and rechallenged with Iso followed by Iso + DMSO (vehicle for CPA).
To establish whether PP2a is modulated by adenosine A₁ receptor activation, the carboxymethylation state of PP2a-C was determined in ventricular myocytes treated with an agonist and an antagonist to the adenosine A₁ receptor. The extent of carboxymethylation was assessed with an antibody that specifically detects the demethylated form of PP2a-C. As shown in Figs. 6 and 7, a 5-min exposure to CPA caused a decrease in demethylated PP2a-C in ventricular myocytes. Total cellular PP2a-C content was not significantly different between groups as determined from the NaOH-treated/fully demethylated samples. The effect of CPA on the carboxymethylation state of PP2a-C was blocked by DPX, whereas DPX alone had no effect on carboxymethylation state of PP2a-C.

The subcellular distribution of PP2a-C was determined in ventricular myocytes in the presence and absence of CPA. There was a significant increase in the level of PP2a-C in the particulate fraction with a reciprocal decrease in the PP2a-C content in the cytosolic fraction on CPA stimulation (Fig. 8). The particulate fraction contains PP2a-C that is Triton-solubilized from both membrane- and myofilament-associated proteins (see MATERIALS AND METHODS). The effect of CPA on PP2a translocation was blocked by the adenosine A₁ receptor antagonist DPX. DPX alone had no effect on carboxymethylation state of PP2a-C.

DISCUSSION

The present study addresses the role of PP2a in the antiadrenergic effect of adenosine A₁ receptor activation in isolated hearts and ventricular myocytes from rats. We found that adenosine A₁ receptor activation significantly reduces the β-adrenergic-induced increase in LVDP and that this effect is blocked by PP2a inhibition (Table 1). Consistent with these observations β-adrenergic-induced PLB and TnI phosphorylations are reduced by adenosine A₁ receptor activation, and activation of the adenosine A₁ receptor in the absence of β-adrenergic stimulation causes a significant decrease in the phosphorylation states of PLB and TnI (Fig. 3). These dephosphorylations are blocked by selective PP2a inhibition (Fig. 5). Adenosine A₁ receptor-dependent increases in carboxymethylation state (Fig. 7) and translocation of PP2a-C (Fig. 8) directly support a role for adenosine A₁ receptors in the modulation of PP2a activity in ventricular myocytes.

Fig. 2. Representative SDS-polyacrylamide gel and corresponding autoradiograph demonstrating the anti-β-adrenergic effect of adenosine A₁ receptor activation on the phosphorylation states of phospholamban (PLB) and troponin I (TnI) in myocytes. [³²P]orthophosphate-labeled ventricular myocytes were treated with the β-adrenergic receptor agonist Iso (10 nM) for 5 min, followed by Iso, Iso + 1 μM CPA (adenosine A₁ receptor agonist), or Iso + CPA + 1 μM 8-cyclopentyl-1,3-dipropylxanthine (DPX; adenosine A₁ receptor antagonist) for 5 min. Additional groups included cells treated with CPA or DPX alone.

Fig. 3. Cumulative results of ³²P incorporation into PLB (A) and TnI (B) after various treatments. Ventricular myocytes were treated as in Fig. 2. Data are means ± SE from 8 isolations. *P < 0.05 compared with control (Con); #P < 0.05 compared with Iso alone.
these findings are consistent with the hypothesis that the antiadrenergic effect of adenosine A1 receptor activation involves activation of PP2a.

The precise cellular mechanism underlying the antiadrenergic effect of adenosine has been controversial. Inhibition of adenylate cyclase activity via inhibitory G protein-coupled adenosine A1 receptor was thought to be the primary mediator of this antiadrenergic effect (13, 33, 35). However, some studies demonstrated a dissociation between reductions in contractile responses and changes in cAMP-PKA (16, 17, 44). Narayan et al. (28) demonstrated that the antiadrenergic effects of adenosine A1 receptor stimulation on systolic intracellular Ca2+ concentration and cell shortening were blocked by phosphatase inhibition in rat cardiac myocytes. These observations plus those of the present study indicate that activation of a protein phosphatase is involved in the antiadrenergic effect of adenosine A1 receptor activation.

In vitro studies demonstrate that cantharidin inhibits PP2a (IC50 of 0.13 μM) >10-fold over PP1 (IC50 of 2.7 μM; Ref. 30). Cantharidin at 1 μM (concentration used in present studies) reduces the activity of purified phosphatase catalytic subunits by 80–90% for PP2a and by 15–35% for PP1 (19, 30). In vivo studies suggest that the efficiency of cantharidin to inhibit phosphatases is slightly reduced compared with that in in vitro studies because of the lipophobic nature of cantharidin (10). Thus 1 μM cantharidin will primarily inhibit PP2a, whereas PP-1 will be marginally affected. It should also be noted that cantharidin is an economically feasible tool to study the functional effects of PP2a in perfused hearts.

In the present study CPA treatment of isolated ventricular myocytes caused a significant decrease in basal phosphorylation of PLB and TnI that was blocked by the adenosine A1 receptor antagonist DPX. Consistent with this observation, Strang et al. (36) demonstrated that CPA reduces the basal level of phosphorylation of TnI and C protein in rat ventricular myocytes. β-Adrenergic stimulation is known to induce cardiac protein phosphorylation via activation of the adenylate cyclase-cAMP-PKA pathway. In the present study, CPA significantly reduced Iso-stimulated phosphorylation of both PLB and TnI and this effect was blocked by DPX. This observation is consistent with
the study of George et al. (13) demonstrating that 
R-phenylisopropyladenosine (R-PIA) inhibited Iso-
stimulated TnI and C protein phosphorylation in rat
ventricular myocytes but is at apparent odds with the
study of Gupta et al. (16) demonstrating that Iso-
induced phosphorylation of TnI was not inhibited by
R-PIA in guinea pig ventricular myocytes. In the
present study the CPA-dependent dephosphorylations
of TnI and PLB phosphorylation were blocked by 1
mM OA. OA is a well-characterized phosphatase inhibitor
with an IC50 for PP2a of 0.2 nM and an IC50 for PP1 of
20 nM in vitro (40). A number of studies demonstrated
that OA at a concentration up to 1 mM selectively
inhibits PP2a activity, with PP1 only marginally af-
ected in various cell types (8, 11, 26, 43). Previous
studies also demonstrated that TnI and PLB can be
substrates of PP2a (22, 27, 31). Thus our results using
PLB and TnI as endogenous substrates of PP2a and 1
mM OA as a selective PP2a inhibitor are consistent
with the hypothesis that adenosine A1 receptor stimu-
lation antagonizes β-adrenergic-stimulated protein
phosphorylation via activation of PP2a.

PP2a activation by adenosine A1 receptor stimula-
tion may involve a G protein. It has been reported that
G protein-coupled muscarinic receptor activates PP2a
in cardiac myocytes (18). Furthermore, angiotensin II
increases PP2a activity in cultured neuronal cells
through a G protein-dependent mechanism (20) and
dephosphorylates calcineurin, which mediates the ac-
duction of PP2a-like activity (3). How PP2a is activated by a G protein remains unknown.

Modulation of PP2a activity can occur through phos-
phorylation and carboxymethylation of the COOH ter-
minal of PP2a-C. PP2a-C carboxymethylation can
have a number of biological effects, including a poten-
tial direct effect on the catalytic activity of PP2a and
modulation of the regulatory subunit binding and sub-
sequent targeting of the PP2a holoenzyme to specific
subcellular localizations (2, 11, 23). Physiological reg-
ulation of this reversible carboxymethylation of PP2a
has not been extensively studied. Carboxymethylation
of PP2a-C is increased by cAMP in Xenopus eggs (12),
and has been shown to modulate insulin secretion in
pancreatic β-cells (23). In the present study, CPA treat-
ment decreased the level of demethylated PP2a-C with
no change in total cellular PP2a-C level. This suggests
that the carboxymethylation state of PP2a-C is in-
Fig. 6. Representative Western blots demonstrating the effect of
adenosine A1 receptor activation on the carboxymethylation state of
the PP2a catalytic subunit. Relative content of demethylated PP2a
catalytic subunits (PP2a-C) was assessed with an antibody that
specifically recognizes the demethylated form of PP2a-C. Ventricular
myocytes were untreated (Con) or treated with 1 μM CPA, CPA + 1
μM DPX, or DPX alone for 5 min. Cells were then lysed and incu-
bated with or without 0.1 N NaOH. NaOH treatment fully demeth-
ylates PP2a-C (total PP2a-C).

Fig. 7. Cumulative results on the carboxymethylation state of
PP2a-C in myocytes after various treatments as described in Fig. 6.
Data are expressed relative to total PP2a-C and are means ± SE of
8 independent experiments. *P < 0.05.

Fig. 8. Effect of adenosine A1 receptor activation on the cytosolic and
particulate localization of the PP2a catalytic subunit. Representative
Western blots of cytosolic (A) and particulate (B) fractions and
cumulative results (C) are presented. Ventricular myocytes were
exposed to DMSO (Con; vehicle for CPA and DPX), 1 μM CPA,
CPA + 1 μM DPX, or DPX alone for 5 min. Before Western blot
determination of PP2a content, all samples were treated with 0.1 N
NaOH to fully demethylate PP2a and allow a determination of total
PP2a-C. Data are expressed relative to control in the speci-
ied fraction and are means ± SE of 5 independent experiments. *P <
0.05 compared with corresponding controls.
increased by adenosine $A_1$ receptor activation. In addition, we found a decrease in the PP2a-C content in the cytosolic fraction with a reciprocal increase in the particular fraction on CPA treatment. This data is consistent with the hypothesis that adenosine $A_1$ receptor activation leads to carboxymethylation of PP2a-C that allows the binding of the regulatory/targeting subunit of PP2a, subsequent translocation of the PP2a holoenzyme to cardiac proteins such as PLB and TnI, and an increase in localized PP2a activity.

Activation of PP2a via adenosine $A_1$ receptor activation may also antagonize $\beta$-adrenergic stimulation by modulating elements in the PKA pathway. In vitro studies indicate that PP2a dephosphorylates the PKA catalytic subunit and decreases PKA activity (25). Consistent with this observation is the study of George et al. (13) demonstrating that R-PIA causes a 41% decrease in PKA activity without changing iso-elicited increases in cAMP levels. Thus adenosine $A_1$ receptor-dependent PP2a activation antagonizes $\beta$-adrenergic stimulation through dephosphorylating/inactivating PKA and/or by directly dephosphorylating end substrates.

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