Modulation of protein phosphatase 2a by adenosine A1 receptors in cardiomyocytes: role for p38 MAPK

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Liu, Qinghang, and Polly A. Hofmann. Modulation of protein phosphatase 2a by adenosine A1 receptors in cardiomyocytes: role for p38 MAPK. Am J Physiol Heart Circ Physiol 285: H97–H103, 2003. First published March 20, 2003; 10.1152/ajpheart.00956.2002.—Adenosine A1 receptor activation causes protein phosphatase 2a (PP2a) activation in ventricular myocytes. This attenuates β-adrenergic functional effects in the heart (Liu Q and Hofmann PA. Am J Physiol Heart Circ Physiol 283: H1314–H1321, 2002). The purpose of the present study was to identify the signaling pathway involved in the translocation/activation of PP2a by adenosine A1 receptors in ventricular myocytes. We found that N6-cyclopentyladenosine (CPA; an adenosine A1 receptor agonist)-induced PP2a translocation was blocked by p38 MAPK inhibition but not by JNK inhibition. CPA increased phosphorylation of p38 MAPK, and this effect was abolished by pertussis toxin and inhibitors of the cGMP pathway. Moreover, CPA-induced p38 translocation was blocked by inhibition of the cGMP pathway. Guanylyl cyclase activation mimicked the effects of CPA and caused p38 MAPK phosphorylation and PP2a translocation. Finally, CPA-induced dephosphorylations of troponin I and phospholamban were blocked by pertussis toxin and attenuated by p38 MAPK inhibition. These results suggest that adenosine A1 receptor-mediated PP2a activation uses a pertussis toxin-sensitive G protein-guanylyl cyclase-p38 MAPK pathway. This proposed, novel pathway may play a role in acute modulation of cardiac function.

p38 mitogen-activated protein kinase; guanylyl cyclase; G protein

Protein phosphatase 2a (PP2a) is known to be involved in the regulation of cell growth and apoptosis and numerous cell signaling pathways in cardiac myocytes. Recently, it has been shown that the antiadrenergic effect of adenosine A1 receptor activation is mediated through PP2a activation (24). Moreover, the antiadrenergic effect of muscarinic receptor activation, similar in magnitude and effect to adenosine A1 receptor activation, involves activation of a protein phosphatase (14, 16). These studies suggest that modulation of PP2a by adenosine A1 and muscarinic receptors plays a role in acute changes of cardiac function. However, little is known about the signaling mechanisms of PP2a activation. The goal of the present study was to elucidate the signaling pathway from adenosine A1 receptor activation to the increased PP2a activity in cardiac myocytes.

PP2a is a type II serine/threonine phosphatase consisting of a catalytic “C” subunit (PP2a-C), a structural “A” subunit, and one of several regulatory “B” subunits. PP2a activities can be regulated by posttranslational modifications of the catalytic subunit by phosphorylation and carboxymethylation (6, 9, 13). In addition, localized PP2a activity can be altered by direct, transient translocation of the heterotrimeric holoenzyme in Hela cells and mast cells (25, 34). Our previous study (24) demonstrated that in cardiomyocytes PP2a activity can also be modulated through carboxymethylation and translocation. However, how PP2a translocation is regulated and what signaling pathway is involved are not well understood.

Recent studies suggest that PP2a can be activated by p38 MAPK in NIH 3T3 cells (39) and neutrophils (1). Activation of p38 MAPK by adenosine has been demonstrated in the perfused rat heart (15). Moreover, the adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) activates p38 MAPK in cultured smooth muscle cells, and this effect was shown to be pertussis toxin (PTX) sensitive (33). It is also known that PTX-sensitive G protein is involved in adenosine A1 receptor antiadrenergic effects and adenosine-induced cardiac preconditioning (3, 21). It remains unknown whether the modulation of PP2a by adenosine A1 receptor activation involves a G protein. Evidence is available indicating that PTX inhibits PP2a-like activity in PC12 cells (8), and angiotensin II receptor activation works through a G protein-dependent mechanism to increase PP2a activity in cultured neuronal cells (17). Thus we hypothesized that adenosine A1 receptor activation works through a G protein to activate p38 MAPK and, in turn, to activate PP2a in cardiomyocytes.

Activation of G protein increases cGMP in ventricular myocytes (12, 30). This has been extensively studied in the ability of muscarinic receptors to activate G protein and cause an increase in cellular cGMP (12, 30). Adenosine A1 receptor activation has been shown to increase cGMP accumulation in atrial myocytes (37) and atrioventricular nodal cells (26, 43) but not in ventricular myocytes (2, 5). However, the adenosine A1 receptor agonist R-phenylisopropyladenosine, at relatively high
concentrations, can inhibit cGMP phosphodiesterase in ventricular myocytes (28). Thus we investigated the possible link between cGMP and adenosine A1 receptor activation in rat ventricular myocytes. Finally, cGMP-dependent activation of p38 MAPK has been shown in isolated cardiomyocytes and fibroblasts (4, 20), and a link between cGMP and protein phosphatase has been suggested in cardiomyocytes (29, 35). Taken together, these studies led us to hypothesize that the adenosine A1 receptor induces cardiac PP2a translocation/activation through a G1-cGMP-p38 MAPK signaling pathway in ventricular myocytes.

**MATERIALS AND METHODS**

*Enzymatic isolation of rat ventricular myocytes.* Experiments using animals were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Sciences Center.

Ventricular myocytes were isolated according to the protocol of Lester et al. (22) with slight modifications. Briefly, hearts were removed from female Wistar rats (200–220 g) anesthetized by isoflurane inhalation. The aorta was cannulated, and the heart was mounted on a Langendorff apparatus. Residual blood in the heart 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, 1 KH2PO4, 5 pyruvate, and 11 glucose. The heart was then perfused with Ringer solution containing type II collagenase (1 mg/ml, Worthington Biochemical; Lakewood, NJ) for 12–15 min. After collagenase perfusion, the heart was removed from the perfusing apparatus, trimmed of atria and great vessels, cut into small pieces, and incubated in fresh Ringer solution without collagenase for 10 min. The cells were then dissociated by gentle trituration, filtered through a nylon mesh, and resuspended in oxygenated Ringer solution containing 1.25 mM CaCl2 and 0.1% BSA. Cell suspensions containing <60% rod-shaped viable ventricular myocytes were discarded.

*Preparation of cell fractions.* Cell fractions of ventricular myocytes were prepared by the digitonin permeabilization method described by Whisler et al. (40) with some modifications. Briefly, after the various treatments, ventricular myocytes were centrifuged, and cell pellets were resuspended in fresh Ringer solution without collagenase for 10 min. The cells were then dissociated by gentle trituration, filtered through a nylon mesh, and resuspended in oxygenated Ringer solution containing 1.25 mM CaCl2 and 0.1% BSA. Cell suspensions containing <60% rod-shaped viable ventricular myocytes were discarded.

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*PP2a translocation.* PP2a translocation studies were performed as previously described (24). Ventricular myocytes were pretreated with vehicle, 5 or 25 μM SB-203580, 1 μM SP-600125, 100 μM LY-83583, 1 μM NS-2028, or 50 μM 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic mono-phosphothioate-Rp-isomer (Rp-8-pCPT-cGMPS) for 20 min, followed by 1 μM CPA for 5 min. In another set of experiments, the cells were treated with 100 μM dibutyryl-cGMP for 0, 1, 5, 10, and 30 min. All cells were then centrifuged and fractionated into cytosolic and particulate fractions (see Preparation of cell fractions). Fractions were incubated with 0.1 M NaOH at 30°C for 30 min to fully demethylate PP2a-C (6). After NaOH treatment, samples were neutralized with HCl and heated at 95°C for 5 min. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting was carried out using an antibody to PP2a-C (1:1,000 dilution, catalog number A3682, Sigma; St. Louis, MO) and visualized with enhanced chemiluminescence (Perkin Elmer Life Sciences; Boston, MA). Blots were also stained with Coomassie blue or Ponceau S for an assessment of protein loading. Densities of the PP2a-reactive band were determined with NIH Image software (public domain). Data were normalized to protein load and corresponding controls in each experiment.

*Measurement of p38 MAPK phosphorylation.* Assessment of the phosphorylation state of p38 MAPK in ventricular myocytes was accomplished by Western blotting. Isolated ventricular myocytes were pretreated with 2.0 μg/ml PTX for 3 h or for 30 min with 25 μM SB-203580, 100 μM LY-83583, 1 μM NS-2028, or 50 μM Rp-8-pCPT-cGMPS. Cells were then exposed to 1 μM CPA or vehicle for 5 min and lysed in SDS sample buffer. In another set of experiments, cells were treated with 100 μM dibutyryl-cGMP for 0, 1, 5, 10, and 30 min and lysed in SDS sample buffer. Samples were heated for 5 min at 95°C, separated by SDS-PAGE, and transferred to PVDF membranes. Western blotting was performed with polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (1:1,000 dilution, catalog number 9211, New England Biolabs; Beverly, MA) and horseradish peroxidase-conjugated secondary antibody (1:16,000 dilution, catalog number A0545, Sigma). Phospho-p38 MAPK-reactive bands were visualized with enhanced chemiluminescence. Signals were quantitated with NIH Image software and normalized for protein load and corresponding controls in each experiment.

*Determination of cardiac protein phosphorylation.* Changes in the phosphorylation state of cardiac proteins were determined by autoradiography as described previously (24). Isolated myocytes were incubated in Ringer solution containing 200 μCi/ml [32P]orthophosphate (NEN) and 1 mM CaCl2 for 1 h at room temperature. Ventricular myocytes were pretreated with 2.0 μg/ml PTX for 3 h or 25 μM SB-203580 for 30 min. Cells were then untreated or treated with 1 μM CPA, CPA plus 10 nM isoproterenol (Iso), or Iso alone for 5 min. All drug solutions were prepared in 1 mM CaCl2-Ringer solution containing 100 μM sodium metabisulfate to protect Iso from oxidation and 10 U/ml adenosine deaminase to avoid inference from endogenous adenosine. Reactions were quenched by the addition of SDS sample buffer. All samples were heated for 5 min at 95°C, and proteins were resolved by 17% SDS-PAGE. Gels were stained with Coomassie blue, dried between cellophane, and subjected to autoradiography. Western blotting was performed with polyclonal anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (1:1,000 dilution, catalog number 9211, New England Biolabs; Beverly, MA) and horseradish peroxidase-conjugated secondary antibody (1:16,000 dilution, catalog number A0545, Sigma). Phospho-p38 MAPK-reactive bands were visualized with enhanced chemiluminescence. Signals were quantitated with NIH Image software and normalized for protein load and corresponding controls in each experiment.

*Statistical analysis.* All data were analyzed by two-way ANOVA and the appropriate post hoc test. All values are expressed as means ± SE, and P < 0.05 was chosen to indicate statistical significance.

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RESULTS

p38 MAPK inhibition blocks adenosine A<sub>1</sub> receptor-induced PP2a-C translocation. The effect of the adenosine A<sub>1</sub> receptor agonist CPA on the subcellular distributions of PP2a-C was determined in ventricular myocytes in the presence and absence of the p38 MAPK inhibitor SB-203580 or the JNK inhibitor SP-600125. CPA caused a significant increase in the level of PP2a-C in the particulate fraction that was blocked by 5 μM SB-203580 but not by 1 μM SP-600125 (Fig. 1). SB-203580 and SP-600125 in the absence of CPA had no effect on PP2a-C subcellular distribution.

Adenosine A<sub>1</sub> receptor-G<sub>i</sub>-cGMP pathway activates p38 MAPK in ventricular myocytes. The ability of CPA to activate p38 MAPK was determined using phospho-p38 MAPK-specific antibodies. As shown in Fig. 2, CPA stimulated p38 MAPK phosphorylation. The response was maximal at 5 min and declined thereafter. Phosphorylation of p38 MAPK remained elevated at 30 min (longest time tested).

To establish whether CPA-induced p38 MAPK activation involves PTX-sensitive G<sub>i</sub> protein and guanylyl cyclase, we determined the effect of PTX, the soluble guanylyl cyclase inhibitor NS-2028, and the cell-permeable inactive cGMP analog Rp-8-pCPT-cGMPS on CPA-induced p38 MAPK phosphorylation. PTX and SB-203580 abolished the effect of CPA on p38 MAPK phosphorylation (Fig. 3A). NS-2028 and Rp-8-pCPT-cGMPS also blocked the effect of CPA to phosphorylate p38 MAPK (Fig. 3B). Additional studies revealed that the guanylyl cyclase inhibitor LY-83583 (100 μM) also blocked the effect of CPA on p38 MAPK phosphorylation (data not shown). PTX, SB-203580, LY-83583, NS-2028 or Rp-8-pCPT-cGMPS in the absence of CPA had no effect on p38 MAPK phosphorylation. To further confirm the role of guanylyl cyclase in CPA-induced p38 MAPK activation, we treated cardiomyocytes with the membrane-permeable cGMP analog dibutyryl-cGMP. As shown in Fig. 4, dibutyryl-cGMP stimulated p38 MAPK phosphorylation as early as 1 min, peaked at 10 min, and remained elevated for at least 30 min.

Adenosine A<sub>1</sub> receptor-induced PP2a-C translocation requires guanylyl cyclase activation. To investigate whether guanylyl cyclase is involved in CPA-induced PP2a-C translocation, the effect of CPA on the subcellular distribution of PP2a-C was examined in the presence of the soluble guanylyl cyclase inhibitor NS-2028 and the cell-permeable inactive cGMP analog Rp-8-pCPT-cGMPS. Both NS-2028 and Rp-8-pCPT-cGMPS blocked CPA-induced PP2a-C translocation, whereas NS-2028 or Rp-8-pCPT-cGMPS in the absence of CPA had no effect (Fig. 5). The effect of dibutyryl-cGMP on PP2a-C subcellular distribution was also investigated. Dibutyryl-cGMP caused a translocation of PP2a-C to the particulate fraction of ventricular myocytes (Fig. 6). This translocation occurred as early as 1 min, peaked at ~10 min, and lasted for at least 30 min.

Adenosine A<sub>1</sub> receptor activation dephosphorylates cardiac proteins through PTX-sensitive G protein and p38 MAPK. We (24) have previously demonstrated that CPA induced a dephosphorylation of the cardiac regulatory proteins phospholamban (PLB) and troponin I (TnI) in the presence and absence of β-adrenergic stim-
ulation and that the PP2a inhibitor okadaic acid blocked this effect. In the present study, we investigated the role of PTX-sensitive G protein and p38 MAPK in CPA-induced dephosphorylation of PLB and TnI by $^{32}$P autoradiography. CPA caused a decrease in both basal and ISO-stimulated PLB and TnI phosphorylation in ventricular myocytes. Ventricular myocytes were pretreated to inhibit $G_i$ proteins (PTX), p38 MAPK (SB), cGMP signaling (NS and Rp), or vehicle (Con). Cells were then treated with the adenosine $A_1$ receptor agonist CPA or vehicle. *$P < 0.05$ compared with Con.

**DISCUSSION**

Adenosine $A_1$ receptor activation causes PP2a translocation and activation, leading to an antiadrenergic effect in the heart (24). In the present study, we investigated the signaling pathway involved in the translocation/activation of PP2a by adenosine $A_1$ receptors in ventricular myocytes. We found that CPA-induced PP2a translocation was blocked by the p38 MAPK inhibitor SB-203580 (Fig. 1). CPA also increased phosphorylation of p38 MAPK, and this effect was abolished by PTX, the soluble guanylyl cyclase inhibitor NS-2028, and the protein kinase G inhibitor Rp-8-pCPT-cGMPS (Figs. 2 and 3). The guanylyl cyclase activator dibutyryl-cGMP caused p38 MAPK phos-
phorylation and PP2a translocation (Figs. 4 and 6), mimicking the effect of CPA. CPA-induced PP2a translocation was also blocked by NS-2028 and Rp-8-pCPT-cGMPS (Fig. 5). Finally, we showed that CPA-induced dephosphorylations of TnI and PLB were blocked by PTX and attenuated by SB-203580 (Fig. 7 and 8). These results suggest that CPA-induced PP2a translocation requires p38 MAPK activation and involves both a PTX-sensitive G′ protein and guanylyl cyclase.

The regulatory mechanisms controlling cellular PP2a activity are not fully understood. Evidence suggests that PP2a can be regulated by posttranslational modifications of PP2a-C and regulatory subunits, association of PP2a-C with regulatory proteins, and subcellular localization of the PP2a holoenzyme (18). Translocation of PP2a to specific subcellular locations provides a means of increasing PP2a activity in a local microenvironment with specific PP2a substrates. In the present study, we confirmed that adenosine A1 receptor activation caused translocation of PP2a to the particulate fraction in ventricular myocytes (24). In addition, CPA-induced PP2a translocation could be blocked by the p38 MAPK inhibitor SB-203580. SB-

![Fig. 6. Effect of 100 μM dibutyryl-cGMP on localization of PP2a-C to the particulate fraction in ventricular myocytes. A representative Western blot for PP2a-C in the particulate fraction (top) and cumulative quantitative results (bottom) are presented. Data are expressed relative to untreated myocytes (i.e., time = 0 min) and are means ± SE of 4 independent experiments. *P < 0.05 compared with untreated cells.](http://ajpheart.physiology.org/)

![Fig. 7. 32P incorporation into phospholamban (PLB; A) and troponin I (TnI; B) in the presence (+) and absence (−) of 2 μg/ml PTX. Ventricular myocytes were pretreated with PTX (a G′ protein inhibitor) or vehicle, followed by 1 μM CPA (an adenosine A1 receptor agonist) in the presence or absence of the β-adrenergic receptor agonist isoproterenol (Iso). Data are means ± SE from 5 isolations. *P < 0.05 compared with Con without PTX; #P < 0.05 compared with Iso without PTX.](http://ajpheart.physiology.org/)

![Fig. 8. 32P incorporation into PLB (A) and TnI (B) in the presence (+) and absence (−) of p38 MAPK inhibition. Ventricular myocytes were pretreated with 25 μM SB (a p38 MAPK inhibitor) or vehicle, followed by 1 μM CPA (an adenosine A1 receptor agonist) in the presence or absence of the β-adrenergic receptor agonist Iso (100 nM). Data are means ± SE from 5 isolations. *P < 0.05 compared with Con without SB; #P < 0.05 compared with Iso without SB.](http://ajpheart.physiology.org/)
203580 can inhibit other kinases, such as JNK (10). To confirm that the linkage between the adenosine A1 receptor and PP2a translocation is through p38 MAPK, the effect of CPA on PP2a translocation was examined in the presence of two doses of SB-302580 (5 and 25 μM) and with the JNK inhibitor SP-600125. SB-302580 at 5 and 25 μM both blocked CPA-induced PP2a translocation, whereas SP-600125 had no effect. This suggests a role of p38 MAPK in the modulation of PP2a translocation. This finding is consistent with the study of Westermarck et al. (39) showing that p38 MAPK activation stimulates PP1/2a activity and inhibits the phosphorylation of MEK1/2 in human skin fibroblasts, and work by Avdi et al. (1) demonstrating p38 MAPK-dependent PP2a activation in human neutrophils. How p38 MAPK affects PP2a translocation/activation is still unknown. However, phosphorylation of the PP2a regulatory subunit has been shown to promote in vitro activity (41), and the regulatory subunit is known to target PP2a to specific subcellular localizations and determines substrate specificity (7, 27, 36). Thus it is possible that CPA induces PP2a translocation through p38 MAPK-dependent phosphorylation of specific PP2a regulatory subunits.

Although rapid phosphorylation and activation of p38 MAPK by adenosine has been demonstrated in the perfused rat heart (15), it is not known whether p38 MAPK is activated by adenosine A1 receptors in ventricular myocytes. The present study demonstrated that adenosine A1 receptor activation with CPA caused a rapid phosphorylation of p38 MAPK in cardiomyocytes, and this effect was blocked by PTX. This suggests the involvement of a PTX-sensitive G protein. This result is consistent with similar observations in smooth muscle cells (33). Moreover, Gβ protein-dependent p38 MAPK activation has been demonstrated in cultured cardiomyocytes with M2 muscarinic receptor or β2-adrenergic receptor stimulation (11). No other studies have demonstrated a role of Gβ proteins in the modulation of PP2a activation in cardiomyocytes, although Gβ-dependent PP2a activation occurs in neural cells and PC12 cells (8, 17).

In the present study, CPA-induced p38 MAPK activation and PP2a translocation were blocked by the soluble guanylyl cyclase inhibitor NS-2028 and LY-83583 and the protein kinase G inhibitor Rp-8-pCPT-cGMPS. This suggests a role for cGMP. However, adenosine does not appear to increase total cGMP levels in ventricular myocytes (2, 5). One possible explanation of this apparent discrepancy is that adenosine A1 receptor activation increases cGMP in select subcellular compartments, and this increase is not measurable by the whole cell cGMP assay. The present study also demonstrated that activation of guanylyl cyclase with dibutylryl-cGMP mimicked the effects of CPA to increase p38 MAPK phosphorylation and caused PP2a translocation. Together, these findings suggest that adenosine A1 receptor activation, via Gi protein and guanylyl cyclase, activates p38 MAPK. Activation of p38 MAPK appears to promote PP2a translocation in ventricular myocytes.

Our previous study (24) demonstrated that CPA treatment of ventricular myocytes caused a significant decrease in basal as well as β-adrenergic-stimulated phosphorylation of TnI and PLB. This effect was adenosine A1 receptor and PP2a dependent (24). Others have also demonstrated that TnI and PLB are substrates of PP2a (19, 32, 42). Adenosine A1 receptor-dependent PP2a activation, by itself, reduced myocardial protein phosphorylation with no significant changes in cardiac function (24). In the presence of β-adrenergic stimulation, adenosine A1 receptor-dependent PP2a activation and subsequent dephosphorylation of myocardial proteins leads to an attenuation of the β-adrenergic functional effects (24). A decrease in the contractile response of myocardial preparations has also been reported with G0 protein, cGMP, and p38 MAPK activation (3, 23, 38). Thus modulation of cardiac function by adenosine A1 receptor-dependent PP2a activation may involve concerted activation of G0 protein, cGMP, and p38 MAPK.

In summary, the present study strongly suggests that cardiac regulatory proteins can be dephosphorylated by PP2a through an adenosine A1 receptor-dependent G0-cGMP-p38 MAPK pathway. This novel pathway suggests a role for PP2a in the acute modulation of cardiac function.

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REFERENCES


