**A1-Adenosine Receptor–Mediated Inhibition of Isoproterenol-Stimulated Protein Phosphorylation in Ventricular Myocytes**

**Evidence Against a cAMP-Dependent Effect**

Ramesh C. Gupta, Joachim Neumann, Pam Durant, and August M. Watanabe

CAMP content and protein phosphorylation were determined in unlabeled and 32P-labeled guinea pig ventricular myocytes. Isoproterenol (10 nM, 37°C, 10 seconds) increased CAMP content (236%) and phospholamban (265%) and troponin I (135%) phosphorylation in ventricular myocytes. When isoproterenol (0–300 nM) and the A1-adenosine receptor agonist (-)-N6-phenylisopropyladenosine (PIA, 1 μM) or the A1- and A2-adenosine receptor agonist 5'- (N-ethylcarboxamido)-adenosine (NECA, 1 μM) were administered simultaneously, both adenosine receptor agonists attenuated phospholamban phosphorylation to approximately the same extent (40%). The EC50 value for isoproterenol to phosphophosphate phospholamban was 8±1 nM (n=3), which increased to 31±4 nM (n=3) in the presence of PIA or NECA. IC50 values for PIA or NECA to decrease the phosphorylation of phospholamban were 30 or 32 nM in 10 nM isoproterenol-stimulated cells and 80 or 85 nM in 30 nM isoproterenol-stimulated cells. Both adenosine receptor agonists failed to inhibit the phosphorylation of troponin I. However, acetylcholine (2 μM) in the presence of 10 nM isoproterenol inhibited phosphorylation of phospholamban as well as troponin I in ventricular cells. These effects were antagonized by 10 μM atropine. The effects of PIA and NECA on phosphorylation were antagonized by the A2-selective adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (1 μM) but not by the A2-selective adenosine receptor antagonist 9-chloro-2-(2-furanyl)-5,6-dihydropyrido[1,2,4]triazolo[1,5-c]quinazolin-5-imine (1 μM). PIA and NECA did not reduce CAMP levels in isoproterenol-stimulated cells. We conclude that phospholamban phosphorylation was inhibited by A1-adenosine receptor activation and that these effects on phospholamban phosphorylation cannot be explained by a reduction in CAMP levels. (Circulation Research 1993;72:65–74)

**Key Words** • adenosine • protein • phosphorylation • myocytes

Adenosine is a naturally occurring nucleoside and antagonizes the positive inotropic effects of β-adrenergic catecholamines in various isolated cardiac preparations, including isolated guinea pig ventricular myocytes. The effects of extracellular adenosine are mediated via specific receptors in the sarcolemma. Activation of A2-adenosine receptors leads to an inhibition, and activation of A1-adenosine receptors leads to a stimulation of adenylate cyclase activity in various tissues. In the heart, adenosine antagonizes the contractile responses via activation of A1-adenosine receptors, but their coupling to a second messenger system is still controversial. In initial studies on multi-

From the Department of Medicine (R.C.G., P.D.), Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis; Abteilung Allgemeine Pharmakologie (J.N.), Universität Krankenhaus Eppendorf, Hamburg, FRG; and Lilly Research Laboratories (A.M.W.), Eli Lilly and Co., Lilly Corporate Center, Indianapolis, Ind.


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Address for correspondence: Dr. Ramesh C. Gupta, Henry Ford Heart and Vascular Institute, Cardiovascular Research, 2799 West Grand Boulevard, Detroit, MI 48202-2689.

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nase, leading to phosphorylation of regulatory proteins like phospholamban (PLB), troponin I (TnI), and C protein. The phosphorylation of PLB in sarcoplasmic reticulum18,19 and TnI in myofilaments18,20 may regulate the rate of relaxation of the heart and thereby its mechanical performance.

It is thus possible that adenosine receptor agonists may influence ventricular function by modulating the phosphorylation of proteins. The effect of adenosine agonists on protein phosphorylation was studied in chick21 and rat22 hearts, but no correlation was established between changes in protein phosphorylation and cAMP levels. Those studies were carried out in whole-heart preparations, which are made up of different cell types. Therefore, the present study describes for the first time a method for measuring phosphorylation of both PLB and TnI in guinea pig ventricular myocytes. In this study, we show that the adenosine receptor agonist (±)-N6-phenylisopropyladenosine (PIA) and 5'-[(N-ethylcarboxamido)-adenosine (NECA) inhibit Iso-induced phosphorylation of PLB but not TnI. In contrast, acetylcholine in the presence of Iso inhibits phosphorylation of both proteins. These effects of adenosine agonists are independent of changes in cAMP levels. Therefore, we conclude that both adenosine agonists via the A1-adenosine receptor may stimulate PLB-specific phosphatase(s) in guinea pig ventricular myocytes.

**Materials and Methods**

**Isolation of Myocytes**

Myocytes were isolated as previously described,23 with minor modifications. Guinea pigs (500–600 g) of either sex were heparinized (500 units i.p.) and killed after 45–60 minutes by a sharp blow to the head. The heart and lungs were removed and perfused via the aorta (Langendorff preparation) with a modified Krebs-Henseleit bicarbonate buffer (KHB) solution at 37°C with a constant flow rate of 6.5 ml/min. The composition of KHB was (mM) NaCl 118, NaHCO3 27.1, KCl 3.8, KH2PO4 1.0, MgSO4 1.2, CaCl2 1.8, sodium pyruvate 2.5, and glucose 10. The solution was saturated with 95% O2–5% CO2, yielding a pH of 7.4. Coronary circulation was maintained during digestion by cutting the pulmonary artery close to its origin and occluding the pulmonary veins with a tie between the heart and lungs before removal of the lungs. A 15-minute perfusion with KHB was used to clear blood and synchronize digestion in four hearts. This was followed by perfusion with a solution containing (mM) NaCl 105, NaHCO3 20, KCl 4.8, KH2PO4 1.0, MgSO4 1.2, CaCl2 0.01, mannitol 5, taurine 10, glucose 10, and sodium pyruvate 5, and saturated with 95% O2–5% CO2 (low calcium buffer). Hearts were perfused for 8 minutes with low calcium buffer and then with 40 ml of 0.5 mg/ml collagenase in low calcium buffer for each heart. Hearts were immersed in the recirculating collagenase buffer, which was continuously bubbled with 95% O2–5% CO2. After a 40-minute digestion, ventricular muscle was minced into 37°C equilibrated solution composed of three parts KHB and one part low calcium buffer with enzyme. Minced tissue was disaggregated by use of a wide-mouthed pipette with a 2–3 mm opening. Disaggregated tissue was filtered through stainless-steel mesh (pore size, 400 μm). The resulting suspensions were diluted to 30 ml with KHB and centrifuged at 60g for 15 seconds. Myocytes in the pellet were resuspended in KHB and separated from tissue debris by filtration through a stainless-steel mesh (pore size, 200 μm). Rod-shaped myocytes were selected by repeated gravity sedimentation and elutriation, i.e., loaded into a glass column (29x0.8 cm) that was kept at an angle of approximately 5° from vertical. Sodium-HEPES buffer containing (mM) CaCl2 1.8, KCl 4.8, MgSO4 1.2, NaCl 132, HEPES 10, glucose 10, and sodium pyruvate 2.5 (pH 7.4) was oxygenated and then allowed to flow from the bottom of the column at a flow rate of 3–4 ml/min. Flow rate was periodically decreased to 2 ml/min, and cell aggregates were broken by repeated occlusion and release of the inlet line. Viability, expressed as the percentage of rod-shaped cells, was determined by counting at least 100 cells with a hemocytometer after myocytes were fixed in 10% formalin. Only cell suspensions with a viability of greater than 65% rod-shaped cells were used in this study.

**Labeling of Myocytes With 32P-Labeled Orthophosphate**

Approximately 70 mg protein of the freshly isolated myocytes (or 5 ml gravity-settled suspension) was incubated at 37°C for 40 minutes with 20 mCi 32P-labeled orthophosphate in 25 ml sodium-HEPES buffer. Myocytes were then washed three times with sodium-HEPES buffer, and then the gravity-settled cells were diluted fivefold in the same buffer.

**Phosphorylation of Proteins**

Phosphorylation of 32P-labeled myocytes was carried out in a final volume of 300 μl. Equal volumes of the drug and myocytes were mixed together and incubated at 37°C. The drug solution was prepared in sodium-HEPES buffer containing 200 μM sodium metabisulfite and 10 units/ml adenosine deaminase (ADA), unless otherwise stated, and preincubated at 37°C for 2 minutes before mixing with myocytes. At the indicated periods of time, reaction was stopped by adding 150 μl sodium dodecyl sulfate (SDS)–Stop solution (30 mM Tris-HCl [pH 7.8], 3 mM EDTA, 1 mM dithiothreitol, 15% glycerol [vol/vol], 6% SDS [wt/vol], and a trace of bromophenol blue). Samples were boiled by incubation in a boiling water bath for 10 minutes before electrophoresis, unless otherwise stated. An aliquot of 40 μl corresponding to 40–50 μg protein was applied to each lane. Gels were dried and then exposed to Dupont Cronex x-ray film. Bands corresponding to PLB and troponin inhibitor were cut out from the dried gels and then counted using Dupont Formula-963 in a liquid scintillation counter.

**Optimum Conditions for Phosphorylation of Myocytes**

The stability of myocytes was tested by incubating at room temperature and 37°C in sodium-HEPES buffer. Myocytes were viable up to 6 hours if they were stored either at 25–27°C or 37°C in sodium-HEPES buffer (data not shown). When myocytes were labeled with inorganic phosphate (32P) and phosphorylation of PLB and TnI were quantitated as described above, PLB and TnI phosphorylation remained unchanged until 2-hour storage of myocytes (data not shown). Therefore, 32P-labeled myocytes were used within 2 hours of isolation.
Further, we examined the effects of varying concentrations (0–3%) of dimethylsulfoxide (DMSO) on PLB and TnI phosphorylation, since adenosine analogues such as PIA and NECA were dissolved in DMSO. Addition of DMSO up to 0.2% in 32P-labeled myocytes did not affect PLB and TnI phosphorylation, but raising the concentration further caused an increase in both PLB and TnI phosphorylation (data not shown). Whenever drugs soluble in DMSO were used in the experiment, the same concentration of the solvent, but not more than 0.1%, was also included in the control experiments. Adherence to these conditions was very important in order to reproduce the effects of adenosine analogues on protein phosphorylation in 32P-labeled guinea pig ventricular myocytes.

**cAMP Analysis in Ventricular Myocytes**

Drugs and the diluted myocytes were incubated together at 37°C as described in "Phosphorylation of Proteins." At the end of the incubation, 300 μl of 0.2 M HCl was added to stop the reaction and lyse the cells, and samples were frozen at −20°C. For cAMP assay, samples were incubated in a boiling water bath for 10 minutes and then cooled down and centrifuged at 3,000 rpm for 20 minutes. The supernatant was used for the assay of cAMP, which was measured by radioimmunoassay, as published previously. To check the release of cAMP that was elevated in response to β-adrenergic stimulation, myocytes were incubated (10 minutes) with 1 μM Iso and then centrifuged at 5,000 rpm for 10 minutes. cAMP levels were assayed in the whole-cell suspension and in the cell-free suspension (supernatant). Less than 0.16% of total cAMP was detected in the myocyte-free supernatant. Iso caused a 2.4-fold increase in total cAMP levels, but less than 0.2% of the total cAMP was released in the myocyte-free supernatant (data not shown). Thus, myocytes used in this study were able to hold the Iso-stimulated cAMP levels inside the cells.

**Protein Determination**

For protein determination, an aliquot of 150 μl of the cell suspension was mixed with 500 μl of 1N NaOH. The amount of proteins was analyzed by the Lowry method.

**SDS–Polyacrylamide Gel Electrophoresis**

Slab gel electrophoresis was performed as described using 12% polyacrylamide gels (1.5 mm thick) composed of 0.1% SDS, 0.1 M Tris (pH 8.8), 10% glycerol, 0.225 M glycine, and 0.02% polyacrylamide and polymerized with 4% (vol/vol) tetramethylene diamine and 0.04% ammonium persulfate. The stacking gel was composed of 4% polyacrylamide, 0.8% bis-acrylamide, 0.125 M Tris (pH 6.8), and 0.1% SDS and polymerized as above. The electrode buffer consisted of 0.05 M Tris, 0.01% SDS, and 0.225 M glycine. Approximately 40–50 μg solubilized protein obtained from the myocytes was placed on the gel. A current of 20 mA was applied until the dye entered the separating gel, and then the current was increased to 40 mA. Electrophoresis was continued for 20 minutes after the bromophenol blue tracing dye reached the gel bottom. The gels were stained with Coomassie blue R-250, destained, and dried. For autoradiography, the dried gels were exposed to Dupont Cronex x-ray film at −20°C. Molecular weight standards were phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (42,700), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400).

**Data Analysis**

Student's t test for paired or unpaired samples was used for all data. A value of p < 0.05 was considered statistically significant. Concentrations of drugs that produced 50% inhibition (IC50) or 50% of the maximal stimulation (EC50) were determined graphically in each experiment.

**Materials**

ADA (EC 3.5.4.4), (−)-isoproterenol bitartrate, DMSO, cAMP, and cGMP were obtained from Sigma Chemical Co., St. Louis, Mo. Collagenase (lot 107909), PIA, and NECA were purchased from Boehringer-Mannheim, Indianapolis, Ind. Carrier-free 32P, was purchased from New England Nuclear, Boston. SDS–polyacrylamide gel reagents, including low molecular weight standards (14,000–100,000), and protein reagents were supplied by Bio-Rad Laboratories, Richmond, Calif. 9-Chloro-2-(2-furanyl)-5,6-dihydro-1,2,4, triazolo(1,5-c)quinazolin-5-imine (CGS 15943) was a kind gift from CIBA-GEIGY Corp., Pharmaceutical Division, Summit, N.J., and 1,3-dipropyl-8-cycloptenylxanthine (DPCPX) was purchased from Research Biochemicals Inc., Natick, Mass. All other chemicals were of analytical grade.

**Results**

**Identification of Proteins Phosphorylated in Myocytes**

In Figure 1, Iso stimulated phosphorylation of 31-kd and 11-kd proteins. The 31-kd protein appears to be TnI, since this protein migrated the same distance on the gel as did an authentic sample of TnI. The identity of the 11-kd protein was confirmed to be PLB by the mobility shift from a protein of 27 kd before boiling (data not shown). Since adenosine is released from the heart during stimulation by catecholamines, the effect of different concentrations (0–10 units/ml) of ADA was examined on phosphorylation of PLB and TnI. ADA degrades endogenously formed adenosine into inactive inosine. Increases in PLB phosphorylation were noticed up to the addition of 5 units/ml ADA. TnI phosphorylation remained unchanged (Figure 2). To exclude interference from endogenous adenosine, all subsequent experiments included 5 units/ml ADA (unless otherwise stated).

**Effect of Adenosine Agonists on Phosphorylation of Proteins**

The time course for the effect of Iso on PLB and TnI phosphorylation is shown in Figure 3. The effect of Iso is rapid. Phosphorylation of PLB and TnI increased maximally within 1 minute. Further incubation did not change protein phosphorylation. 32P-labeled myocytes were incubated with 10, 30, or 300 nM Iso alone or Iso plus PIA (1 μM) or NECA (1 μM) for 10 seconds (37°C), as shown in Figure 4. PIA and NECA did not reduce PLB and TnI phosphorylation in the absence of Iso (data not shown). However, both adenosine agonists reduced the Iso-induced phosphorylation of PLB to the
The effect of isoproterenol (Iso) on phosphorylation of proteins in guinea pig ventricular myocytes. TnI, troponin I; PLB, phospholamban. Ctr, control. P32-labeled myocytes were incubated in the absence (Ctr) and presence of 300 nM Iso at 37°C for 10 minutes. Reaction was stopped, and then the samples were boiled before electrophoresis (2% Poriz gel) and autoradiography.

No reduction in phosphorylation of TnI was noticed. Furthermore, a decrease in PLB phosphorylation was evident in the presence of low concentrations (10 or 30 nM) but not in high concentrations (300 nM) of Iso in ventricular cells (Figure 4). The EC50 values for Iso to phosphorylate PLB and TnI were approximately similar (Figure 5, 8±1 nM, n=3). Addition of PIA or NECA shifted the curve of phosphorylation of PLB to the same extent, toward EC50 values of 31±4 nM (Figure 5, n=3). Both PIA and NECA failed to reduce TnI phosphorylation significantly in Iso-stimulated myocytes (Figures 4 and 5) under any conditions. For comparison, the effects of acetylcholine on phosphorylation of PLB and TnI were determined in the same preparation of the ventricular cells isolated from the guinea pig heart using the same lot of collagenase. Acetylcholine attenuated 10 nM Iso-stimulated phosphorylation of PLB and TnI. These effects were antagonized by atropine (10 μM), suggesting a muscarinic cholinergic effect (Figure 6). Hence, acetylcholine, compared with PIA and NECA, has different effects on phosphorylation in Iso-stimulated ventricular myocytes. The effect of acetylcholine on TnI also shows that our experimental methods can indeed detect changes in the phosphorylation of TnI. The effect of adenosine agonists on protein phosphorylation is observed in a short incubation of cells with drugs. In Figure 7, incubation of 32P-labeled cells and drugs (10 nM Iso alone or Iso plus PIA or NECA) for 10 seconds led to a decrease in PLB phosphorylation of 37% by PIA and 47% by NECA (Figure 7A). Incubation for a longer duration reduces the adenosine-mediated inhibition in Iso-enhanced PLB phosphorylation (Figures 7B and 7C). PIA or
NECA reduced PLB phosphorylation by only 20% or 22% at 1 minute (Figure 7B) and by 10% or 12% at 10 minutes (Figure 7C). Both adenosine agonists failed to reduce PLB phosphorylation in 300 nM Iso-stimulated cells under any conditions (Figures 7A’–7C’). Furthermore, incubation for different periods did not produce any effects of PIA or NECA on Iso-induced phosphorylation of TnI (Figures 7A–7C).

![Graph showing the effects of (-)-N^6-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxamido)-adenosine (NECA) in the presence of varying concentrations of isoproterenol (Iso) on phosphorylation of phospholamban and troponin I (TnI) in guinea pig ventricular myocytes. (PIA) and 5'-N-ethylcarboxamido)-adenosine (NECA) in the presence of varying concentrations of isoproterenol (Iso) on phosphorylation of phospholamban and troponin I (TnI) in guinea pig ventricular myocytes. 

**Figure 4.** Autoradiogram depicting the effects of (-)-N^6-phenylisopropyladenosine (PIA) and 5'-N-ethylcarboxamido)-adenosine (NECA) on isoproterenol (Iso)-stimulated phosphorylation of phospholamban (PLB) and troponin I (TnI) in guinea pig ventricular myocytes. ^32P-labeled myocytes were incubated with 10, 30, or 300 nM Iso without or with 1 μM PIA or 1 μM NECA at 37°C for 10 seconds. Reaction was stopped, and samples were boiled before electrophoresis and autoradiography.

The effects of PIA or NECA on phosphorylation of PLB were concentration dependent (Figure 8A). In 10 nM Iso-stimulated cells, IC^50 values for PIA or NECA were 30 or 32 nM and increased to 80 and 85 nM in 30 nM Iso-stimulated cells (Figure 8A). As observed earlier, adenosine agonists did not decrease PLB phosphorylation in unstimulated (Figures 5 and 8) or 300 nM Iso-stimulated (Figures 4, 5, 7, and 8) cells. However, TnI phosphorylation remained unaffected (Figure 8B). The reduction of phosphorylation of PLB by PIA or NECA in 10 nM Iso-stimulated cells (Figure 9, top panel) was antagonized by 1 μM DPCPX, an A_t-adenosine antagonist (Figure 9, middle panel), but not by CGS 15943, an A_2-adenosine antagonist (Figure 9, bottom panel). These results clearly imply that the effects of adenosine agonists are mediated by activation of A_t-adenosine receptors but not by A_2-adenosine receptors.

**Effect of Adenosine Agonists on cAMP Levels**

Incubation (37°C, 10 seconds) of isolated guinea pig ventricular myocytes with 10 or 300 nM Iso caused 2.3- or 4.5-fold increases in cAMP levels (Figure 10). Simultaneously applied adenosine receptor agonists PIA (1 μM) or NECA (1 μM) did not decrease cAMP levels (Figure 10). Incubation of cells with Iso (10 nM) plus PIA (1 μM) for different times (from 10 seconds to 10 minutes) did not affect cAMP levels as compared with ISO alone (Figures 11A–11C).

**Discussion**

The present study is the first to report that adenosine agonists via activation of A_t-adenosine receptors attenuated isoproterenol-stimulated phosphorylation of PLB but not TnI in isolated ventricular cardiac myocytes. This reduction occurred without changes in cAMP levels. Attention was focused on the phosphorylation of PLB of cardiac sarcoplasmic reticulum and TnI of myofilaments because their states of phosphorylation in
vivo are believed to regulate the rate of relaxation of heart cells after β-adrenergic stimulation.17

Our present study clearly demonstrates that Iso phosphorylated two proteins in a calcium-tolerant preparation of 65–70% rod-shaped endothelial-free ventricular cardiac cells isolated from the guinea pig heart. These phosphoproteins had molecular weights of 31 and 11 kd, as determined on autoradiograms of 12% SDS slab gel. Phosphorylation of similar proteins has also been reported by other investigators in heart cells. The 31-kd protein was identified as TnI28 and the 11-kd protein as PLB29 by immunoblotting with monoclonal antibodies to cardiac TnI and cardiac PLB. Since in the present study the 31-kd protein comigrated with the purified TnI, the 11-kd protein was formed from the 27-kd protein after boiling30 (which is a characteristic of PLB), and both proteins were phosphorylated in response to Iso, they were therefore tentatively labeled as TnI and PLB, respectively. In addition, a polypeptide of 27 kd, which was not phosphorylated by Iso and whose mobility on SDS gel was not changed upon boiling, was also present. To quantitate the extent of phosphorylation, TnI (31-kd) and PLB (11-kd) bands were excised from the gel, and radioactivity was counted. Effects of Iso on protein phosphorylation were concentration dependent and rapid. EC_{50} values (50% of the maximal phosphorylation) of PLB and TnI for Iso were similar (8–10 nM). These results are in agreement with a recent report in rat myocardial cells.31 In those studies, Iso phosphorylated several proteins with molecular weights of 12 or 24

![Autoradiogram depicting the effects of acetylcholine (Ach), isoproterenol (Iso), and atropine (Atr) on phosphorylation of phospholamban (PLB) and troponin I (TnI) in guinea pig ventricular myocytes. 32P-labeled myocytes were incubated without or with Iso (10 nM). Ach and Atr were present at 2- and 10-μM concentrations. After incubation at 37°C for 10 seconds, samples were boiled before electrophoresis and autoradiography.](image)

**Figure 6.**

![Time course showing the effects of (-)-N⁶-phenylisopropyladrenosine (PIA) and 5'-N-ethylcarboxamido-adenosine (NECA) on isoproterenol (Iso)-stimulated phosphorylation of phospholamban (PLB) and troponin I (TnI) in guinea pig ventricular myocytes. 32P-labeled myocytes were incubated with 10 nM Iso (panels A–C) or 300 nM Iso (panels A’–C’) without or with 1 μM PIA or 1 μM NECA at 37°C for 10 seconds (panels A and A’), 1 minute (panels B and B’), or 10 minutes (panels C and C’). Samples were electrophoresed and autoradiographed, and PLB and TnI were quantitated. Percent inhibition by PIA or NECA of Iso-stimulated phosphorylation equals 100% of Iso plus PIA or NECA activity remaining, considering Iso alone (control) as 100%. Data are represented as mean±SEM of three observations.](image)

**Figure 7.**
kd, 28 kd, and 150 kd, which were tentatively labeled as PLB, TnI, and C protein, respectively. Phosphorylation of all of these proteins was rapid and reached maximum within 2 minutes, and EC_{50} values for Iso were also very close to those reported in the present study. The time course of Iso-induced phosphorylation of PLB and TnI correlated closely with those increases in cardiac contractility by Iso.\textsuperscript{2,19} To our knowledge this is the first study that reports phosphorylation of PLB and TnI in isolated guinea pig ventricular myocytes in response to Iso treatment. All the results taken together suggest that Iso led to the formation of cAMP, which activated cAMP-dependent protein kinase. Hence, two physiological substrates, PLB and TnI, were phosphorylated.

Since the heart produces adenosine and its formation is stimulated by \(\beta\)-adrenergic catecholamines,\textsuperscript{27} ADA (5 units/ml) was added to exclude interference from endogenous adenosine. Under optimum assay conditions, nonhydrolyzable adenosine agonists PIA (A_2 specific) and NECA (potent at A_1 and A_2 but not A_3 selective or specific) attenuated Iso-stimulated PLB phosphorylation; the effect was pronounced at low Iso concentrations (3–30 nM), and it was not present at higher Iso concentrations (300 nM). Furthermore, PIA or NECA increased the EC_{50} value for Iso to phosphorylate PLB. These results are in close agreement with the findings reported in chick heart,\textsuperscript{21} in which PIA reduced PLB phosphorylation and adenylate cyclase activity. In those studies, phosphorylation of TnI was not reported. In perfused rat hearts,\textsuperscript{22} adenosine reduced the phosphorylation of proteins of molecular weights of 155, 92, 30, 38, 22, and 20 kd. In those studies, neither cAMP levels nor the effects of NECA and adenosine receptor antagonists on protein phosphorylation were studied. In addition, those studies were performed in whole ventricles rather than in isolated myocytes. Our results on protein phosphorylation are in close agreement with the contractile data in myocytes.\textsuperscript{2} PIA and NECA in the presence of Iso reduced contractile responses to the same extent. Decrease in protein phosphorylation by adenosine agonists can be explained by cAMP-dependent and cAMP-independent mechanisms. When cyclic nucleotide (cAMP) levels were determined under the conditions wherein protein phosphorylation was significantly reduced by adenosine agonists, no changes in cAMP were noticed. However, a previous study reported a slight decrease in cAMP levels by PIA in guinea pig ventricular myocytes.\textsuperscript{2} This discrepancy may be due to the difference in conditions, such as using electrically stimulated cells and different incubation times (10 minutes). In the present study, nonstimulated myocytes were incubated with drugs for only 10 seconds. Several other laboratories, however, noticed either a decrease or increase in cAMP content in the whole

**Figure 8.** Concentration response curves for the effects of (-)-N^6-phenylisopropyladenosine (PIA) and 5’-(N-ethylcarboxamido)-adenosine (NECA) in the presence of different concentrations of isoproterenol (Iso) on phospholamban (PLB, panel A) and troponin I (TnI, panel B) phosphorylation in guinea pig ventricular myocytes. P-labeled myocytes were incubated without Iso (○, ●) or with 3 nM Iso (△, ▲), 10 nM Iso (□, ■), or 300 nM Iso (▽, ▼) in the presence of varying concentrations (0–3 \(\mu\)M) of PIA (open symbols) or NECA (close symbols). At 10 seconds, reaction was stopped, and PLB and TnI were quantitated. Data are represented as the mean of two observations.
myocytes. The failure of adenosine agonists to reduce phosphorylation of TnI suggests either that two different phosphatases, one specific to PLB and the other to TnI, were involved or that some modification of adenosine receptors occurred during isolation of myocytes using this collagenase. The latter possibility is unlikely, since adenosine and muscarinic receptors appear to be functionally present in myocytes. The former assumptions are further supported in a study in which a potent inhibitor of protein kinases A and C, staurosporine, antagonized the stimulatory action of cAMP on the spontaneous beating of the myocytes, which was accompanied by dephosphorylation of PLB but not of TnI.22 At pCa 7–8, dephosphorylation of PLB was accelerated both by Ca2+ and staurosporine, but that of TnI took place only in the presence of Ca2+ ion (pCa < 6.5).

The most important aspects of our findings are reductions of phosphorylation of PLB but not of TnI by adenosine agonists. In contrast, acetylcholine, a muscarinic agonist, reduces phosphorylation of both proteins. Further, the decrease in protein phosphorylation is independent of cAMP levels. Reduction in protein phosphorylation by adenosine agonists in Iso-stimulated cells is antagonized by DPCPX, an A1-specific adenosine receptor antagonist, but not by CGS 15943, an A2-specific adenosine receptor antagonist. Also, the effects of both PIA and NECA on cardiac contractility are antagonized by DPCPX.34 These results suggest that A2-adenosine receptors upon activation reduce protein phosphorylation, but they appear not to be coupled to adenylate cyclase effector systems, since their activation by adenosine agonists does not reduce cAMP levels in Iso-stimulated cells.

Interestingly, there is increasing evidence that the negative inotropic effects of acetylcholine on the Iso-stimulated force of contraction are independent of changes in cAMP.

Figure 9. Bar graphs showing the effects of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) or 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo[1,5-c]quinazolin-5-imine (CGS) on (-)-N6-phenylisopropyladenosine (PIA) or 5′-(N-ethylcarboxamido)-adenosine (NECA)–induced inhibition in isoproterenol (Iso)–stimulated phospholamban (PLB) phosphorylation in guinea pig ventricular myocytes. [32P]-labeled myocytes were incubated (37°C, 10 seconds) without Iso (control [Ctrl]) or with Iso (10 nM), Iso + PIA (1 μM), or Iso + NECA (1 μM) in the absence (none) or presence of DPCPX (10 μM) or CGS (10 μM). PLB in the samples was analyzed. Data are represented as mean ± SEM of three observations.

Figure 10. Graph showing the effect of adenosine agonists on cAMP levels in guinea pig ventricular myocytes. Isolated myocytes were incubated without or with 10 or 300 nM isoproterenol (Iso) alone in the absence (control, ○) or presence of 1 μM (-)-N6-phenylisopropyladenosine (Δ) or 1 μM 5′-(N-ethylcarboxamido)-adenosine (●). After 10 seconds, the reaction was stopped, and cAMP was determined. Data are represented as mean ± SEM of three observations.
levels. They are accompanied by an increased activity of phosphatase. Because both adenosine and acetylcholine reduced the ISO-stimulated force of contraction in ventricular tissue, it is not unreasonable to assume a similar cAMP-independent mechanism of action, e.g., activation of protein phosphatase via inhibitory guanine nucleotide binding protein (Gi) by the A1-adenosine receptor.

In summary, the effects of adenosine analogues on phosphorylation of PLB in the presence of ISO in guinea pig ventricular myocytes cannot be explained by cAMP-dependent mechanisms, but cAMP-independent mechanisms, such as cAMP-independent phosphatase(s), lead to a dephosphorylation of proteins involved in regulating ventricular contractility.

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