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INHIBITION OF PHOSPHORYLATION OF TROPONIN I IN RAT HEART BY ADENOSINE AND 5′-CHLORO-5′-DEOXYADENOSINE

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Abstract—We have investigated the effects of adenosine on protein phosphorylation in extracts of rat heart. Incubation of a myofibrillar fraction with [γ-^{32}P]ATP resulted in the phosphorylation of several proteins by endogenous protein kinases. The adenosine analog 5′-chloro-5′-deoxyadenosine inhibited the phosphorylation of a 29 kD protein in this preparation. The protein was idenitified as cardiac troponin I (cTnI) by two-dimensional gel electrophoresis, using purified cTnI as standard. Addition of the catalytic subunit of cAMP-dependent protein kinase to the myofibrillar fraction increased phosphorylation of cTnI; this increase was inhibited by 5′-chloro-5′-deoxyadenosine and adenosine. Phosphorylation of purified cTnI by the catalytic subunit was also inhibited by 5′-chloro-5′-deoxyadenosine. Under the conditions used, 50% inhibition of phosphorylation by either endogenous or exogenous kinase was observed at approximately 50 μM 5′-chloro-5′-deoxyadenosine or adenosine. The inhibition described here occurred independently of catecholamines. The effects of ADP, AMP, and adenine on cTnI phosphorylation are also described.

Hypoxia and ischemia of the heart result in an increase in the production of adenosine [1, 2]. Adenosine inhibits the positive chronotropic [3, 4] and positive inotropic [5] effects of catecholamines on the heart. While the mechanism of action of adenosine is unclear, it is believed that adenosine acts on heart through external receptors which inhibit the activation of adenylate cyclase [6, 7]. However, other reports claim that adenosine has no effect on intracellular cAMP production in isolated rat atria or papillary muscle [8] or on adenylate cyclase activity of membranes prepared from guinea pig ventricles [9]. Böhm *et al.* [8] and Scholz *et al.* [10] have suggested that adenosine may exert its effect directly on protein kinases. Adenosine has been shown to inhibit cAMP-dependent protein kinase in beef thyroid [11], bovine brain [12], and rat brain [13]. It also inhibits myosin light chain kinase and phosphatidylinositol kinase in calf aortic smooth muscle [14].

In the heart, catecholamines cause the activation of adenyl cyclase, a rise in the intracellular cAMP concentration, and activation of cAMP-dependent protein kinase (PK-A). One substrate of PK-A is phospholamban, the phosphorylation of which stimulates the calcium pumping action of sarcoplasmic Ca^{2+}-ATPase [15–17]. Another substrate of PK-A is the regulatory protein cardiac troponin I (cTnI) [18, 19]. Phosphorylation of cTnI leads to a decrease in the affinity of cTnC for Ca^{2+} [20], and an increase in the rate of dissociation of Ca^{2+} [21]. The result is an increase in the concentration of Ca^{2+} required for actomyosin ATPase activity [22–24] and for contraction [25]. Other heart proteins which may be phosphorylated in response to catecholamine stimulation are the 150 kD C-protein [26], a 15 kD sarcolemmal protein [27, 28], and the sarcolemmal Ca^{2+} channel [29]. The purpose of our study was to determine whether the mechanism of action of adenosine in heart includes modulation of the phosphorylation of certain proteins that regulate the contractile force.

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MATERIALS AND METHODS

Materials. Purified cardiac troponin I prepared from beef heart was provided by Dr. James Potter, Department of Pharmacology, University of Miami, FL [30]. The catalytic subunit of cAMP-dependent protein kinase was a gift of Dr. Susan Taylor, University of California at San Diego, CA [31]. 5′-Chloro-5′-deoxyadenosine was prepared by Dr. Susan Doctrow [14]. cAMP-dependent protein kinases, cAMP, adenosine, and *p*-toluene sulfonyl fluoride were from Sigma. Materials for gel electrophoresis and Triton X-100 were from Bio-Rad. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was obtained from Research Organics, Inc. Protosol and Econofluor were from New England Nuclear. Crude [γ-^{32}P]ATP was from ICN. All other materials were of reagent grade or of the highest quality available.

Preparation of myofibrillar protein fraction. Rat hearts were either used fresh or were purchased frozen from Pel Freez and thawed in a solution containing 20 mM HEPES buffer, pH 7.4, and 0.1 mM *p*-toluene sulfonyl fluoride, hereafter called *buffer*. All steps were performed on ice. The hearts were washed in cold *buffer*, cut into small pieces, and suspended in 3 vol. of *buffer*. The suspension was homogenized in a Polytron (Brinkmann, PCU-2) for 30 sec at medium speed, followed by a waiting period of 2 min. The homogenization was repeated

twice. The homogenate was centrifuged at 5000 *g* for 15 min. The pellet was resuspended in *buffer* using the Polytron, and the suspension was centrifuged at 500 *g* for 15 min. The pellet was resuspended in *buffer* containing 1% (v/v) Triton X-100. The suspension was incubated on ice for 1 hr and was then centrifuged at 70,000 *g* for 1 hr. The supernatant was discarded and the pellet was washed twice with buffer containing 1% Triton X-100. The resulting pellet was resuspended in a small volume of *buffer* containing 1% Triton X-100. The suspension was filtered through six layers of cheesecloth and divided into small aliquots for storage at −70°. Each aliquot was thawed only once before use. The yield was 163 mg of myofibrillar protein from 4.2 g of heart tissue.

Phosphorylation experiments. Phosphorylation reactions contained 50 mM HEPES buffer, pH 7.4, 2 mM $MgCl_2$, and 100 mM KCl. The protein fraction to be phosphorylated was added as described in the figure legends. The reaction was started by adding 200 μM [γ-^{32}P]ATP (500 Ci/mol). The mixture was incubated at 30° for 30 sec and the reaction was stopped by adding trichloroacetic acid (TCA) to a final concentration of 9% (w/v). The samples were centrifuged at 5000 *g* for 10 min. The soluble fraction was removed, and the insoluble pellet was washed twice with cold acetone. The pellet was dried and the samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [32]. The gels were dried onto filter paper and subjected to autoradiography. In some experiments the labeled bands were cut from the dried gels and incubated in 3% (v/v) Protosol in Econofluor at room temperature for 5 hr. The samples were then counted for ^{32}P in a Beckman model LS 6800 scintillation counter. In other experiments, autoradiograph bands were compared by densitometry using a microdensitometer (Joyce Leobl, Mark IIICS), and the relative intensities of the radioactive peaks were calculated. Proteins were also analyzed using non-equilibrium pH gradient gel electrophoresis in the first dimension and SDS–PAGE in the second [33–35]. ATPase activity was determined under the same conditions used for phosphorylation reactions. The extent of ATP hydrolysis was measured by neutralizing the TCA-soluble fraction and separating nucleotides in the mixture by thin-layer chromatography on PEI cellulose using 1.0 M LiCl as developing solvent. ATP, ADP, AMP and [^{32}P]inorganic phosphate ($^{32}P_i$) were used as standards. Radioactive spots corresponding to ATP and P_i were cut out and counted. The fraction of ATP hydrolyzed was used to calculate the average ATP concentration over the time interval of the reaction.

Assay procedures. Myokinase activity was assayed by the method of Whitesides *et al.* [36] with the following concentrations of reagents: 50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 100 mM KCl, and 1 mM ADP. Protein was estimated by the method of Bradford [37], with bovine serum albumin as standard.

RESULTS

When the myofibrillar fraction was incubated with 200 μM [γ-^{32}P]ATP, several proteins were phosphorylated (Fig. 1a). The phosphorylation of one of these proteins, a 29 kD protein, was especially sensitive to inhibition by 5′-chloro-5′deoxyadenosine (Fig. 1b). The phosphorylation of some of the other proteins was inhibited by higher concentrations of 5′-chloro-5′-deoxyadenonsine. The 5′-chloro-5′-deoxy analog of adenosine was used in these experiments, because it is not a substrate for either adenosine deaminase and, therefore, not likely to be metabolized in the reaction mixture. As shown below, 5′-chloro-5′-deoxyadenosine and adenosine had virtually the same inhibitory effects on the phosphorylation of the 29 kD protein. The low molecular weight substance whose phosphorylation was inhibited by 5′-chloro-5′-deoxyadenosine was phosphatidylinositol-4-phosphate (results not shown). Adenosine also inhibits phosphatidylinositol kinase of calf vascular smooth muscle [14].

The 29 kD protein was identified as cTnI on the

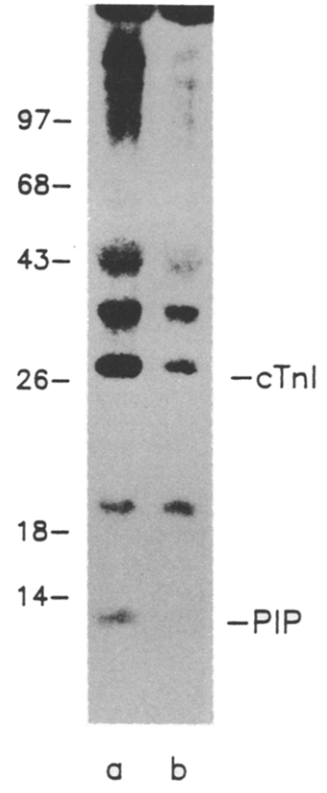

Fig. 1. Phosphorylation of the myofibrillar fraction by endogenous protein kinases. The myofibrillar fraction (2.0 mg/mL) was incubated with [γ-^{32}P]ATP and the reaction was stopped with TCA as described in Materials and Methods. The TCA precipitate was analyzed by SDS–PAGE [12% (w/v) acrylamide] using 133 μg protein/lane. More than twenty proteins were visualized by Coomassie blue staining (not shown). The gel was dried onto filter paper, and the autoradiograph is shown. Molecular weight standards are expressed in kilodaltons (kD). The positions of the 29 kD protein (identified below as cTnI) and phosphatidylinositol-4-phosphate (PIP) are indicated. Key: (a) control phosphorylation; and (b) phosphorylation in the presence of 100 μM 5′-chloro-5′-deoxyadenosine.

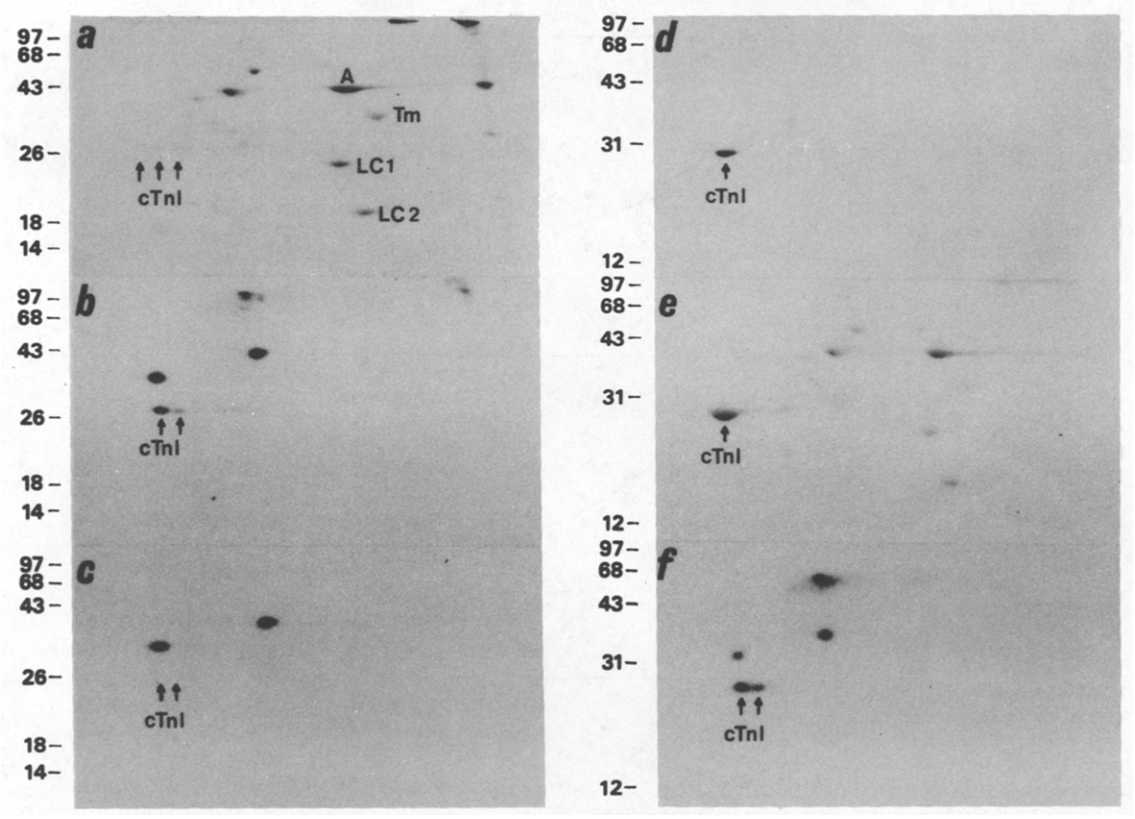

Fig. 2. Two-dimensional gel electrophoresis of myofibrillar fraction. First dimension (from left to right): non-equilibrium pH gradient gel electrophoresis; second dimension (downward): SDS–PAGE. Basic proteins are the left, acidic to the right. Molecular weight standards are expressed in kilodaltons. Key: (a) Coomassie blue stain of 75 μg of the phosphorylated myofibrillar fraction. Identifications are by comparison with a similar gel containing added, purified cTnI, and the gels published by Murakami and Uchida [35]: LC1 and LC2, myosin light chains 1 and 2; Tm, tropomyosin; A, actin. (b) Autoradiograph of the gel in panel a, showing two radioactive spots of cTnI. (c) Autoradiograph of a gel similar to that in panel a, except that 500 μM 5′-chloro-5′-deoxyadenosine was present in the phosphorylation mixture. (d) Coomassie blue stained gel of 5 μg of purified, non-phosphorylated cTnI. (e) Coomassie blue stained gel of a sample containing 5 μg of non-phosphorylated purified cTnI and 75 μg phosphorylated myofibrillar fraction. (f) Autoradiograph of panel e.

basis of molecular weight, pI, and comparison with similar gels of dog cardiac myofibrils published by Murakami and Uchida [35]. Two-dimensional electrophoresis, with non-equilibrium pH gradient gel electrophoresis in the first dimension and SDS–PAGE in the second dimension, was used to analyze the phosphorylated myofibrillar proteins. Purified cTnI was used as standard. The myofibrillar fraction and purified cTnI were analyzed separately and together (Fig. 2). The phosphorylated 29 kD protein in the myofibrillar fraction comigrates with cTnI [35]. cTnI can appear as three spots, because it occurs in unphosphorylated, monophospho, and diphospho forms [38]. The 29 kD protein in the myofibrillar appeared as three faint spots on Coomassie blue staining and as two spots on the autoradiograph, suggesting that it too can exist as unphosphorylated, monophospho, and diphospho forms. When the phosphorylated myofibrillar fraction was combined with unphosphorylated, purified cTnI (Fig. 2, e and f), the dark spot (cTnI) observed with Coomassie blue staining was in the same

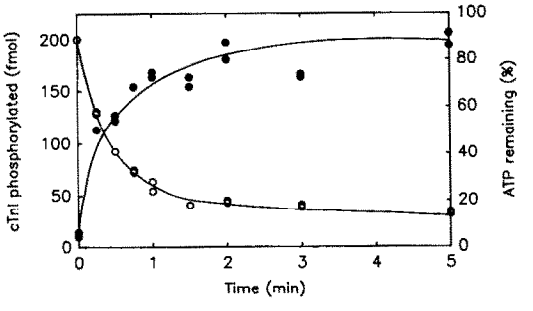

Fig. 3. cTnI phosphorylation and ATPase activity in the myofibrillar fraction as a function of time. Phosphorylations were as described under Materials and Methods, but for the times indicated. The TCA-insoluble fraction was separated by SDS–PAGE, an autoradiograph was prepared, and bands corresponding to cTnI were cut out and counted. ATPase activity was measured as described in Materials and Methods. Key: (●) cTnI phosphorylated; and (○) percent ATP remaining (100% = 20 nmol ATP).

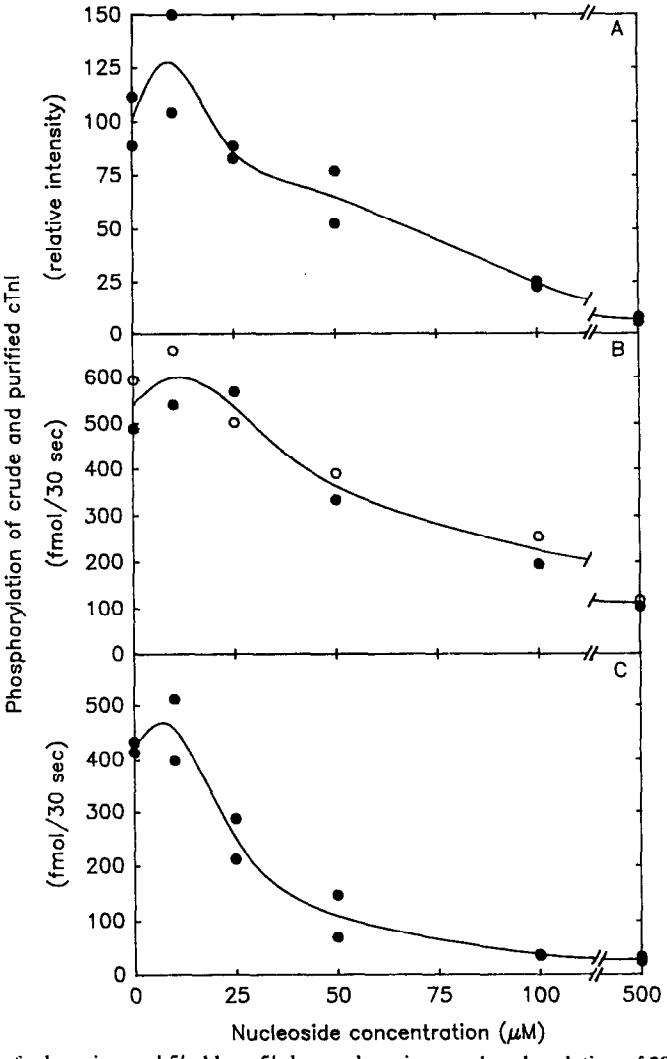

Fig. 4. Effects of adenosine and 5'-chloro-5'-deoxyadenosine on phosphorylation of 29 kD protein and purified cTnI. Phosphorylation reactions, SDS–PAGE, and autoradiography were performed as described under Materials and Methods. (A) Phosphorylation of the myofibrillar fraction (2.0 mg/mL) in the presence of various concentrations of 5'-chloro-5'-deoxyadenosine. The intensities of the bands on the autoradiograph corresponding to the 29 kD protein were compared by densitometry. The control values were arbitrarily set at 100. (B) Phosphorylation of the myofibrillar fraction (2.0 mg/mL) in the presence of 1 μg/mL catalytic subunit of PK-A and various concentrations of 5'-chloro-5'-deoxyadenosine (●) or adenosine (○). The bands of cTnI were cut from the gels and counted as described in Materials and Methods. (C) Phosphorylation of purified cTnI (0.1 mg/mL) by 1 μg/mL catalytic subunit of PK-A in the presence of various concentrations of 5'-chloro-5'-deoxyadenosine. Analyses were as described in B.

horizontal row and displaced slightly from two radioactive spots, which were absent when 5'-chloro-5'-deoxyadenosine was included in the phosphorylation mixture. Lastly, phospho-cTnI from crude and purified preparations also comigrated on one-dimensional gels (data not shown). We conclude that the 29 kD protein is cTnI.

A time course of cTnI phosphorylation and ATPase activity in the myofibrillar fraction is shown in Fig. 3. As expected for a myofibrillar protein fraction, ATPase activity was very high. Thirty seconds was chosen as a reasonable time point for further experiments, because at that point the rate of phosphorylation is still approximately linear. When linearity was critical, 15-sec time points were used. The effect of 5'-chloro-5'-deoxyadenosine concentration on the inhibition of cTnI phosphorylation is shown in Fig. 4A. Fifty percent inhibition was observed at about 50 μM 5'-chloro-5'-deoxyadenosine. The slight increase in phosphorylation at 10 μM 5'-chloro-5'-deoxyadenosine was almost always observed.

PK-A has been shown to phosphorylate cTnI *in vivo* and *in vitro* in at least two positions [38]. The

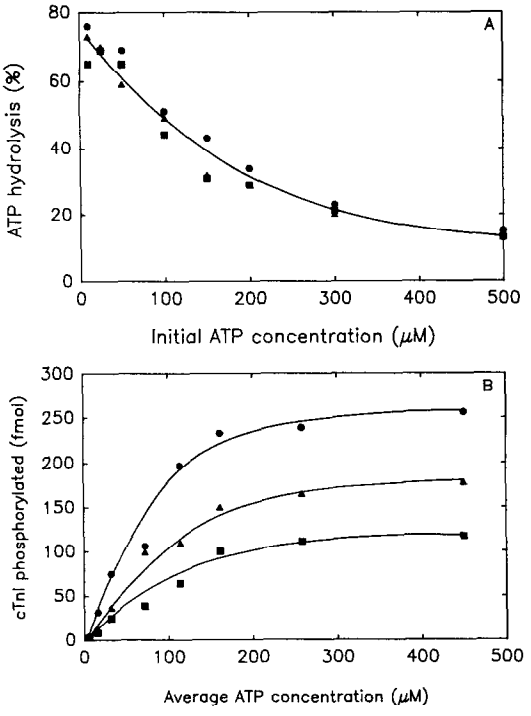

Fig. 5. Effect of ATP concentration on ATPase activity and cTnI phosphorylation in myofibrillar fraction in the presence and absence of 5′-chloro-5′-deoxyadenosine. The phosphorylation reactions were performed as described in Materials and Methods, except that the reaction time was 15 sec. The final volume was 0.1 mL. (A) The TCA-soluble fraction was neutralized and then separated by thin-layer chromatography, and the ATP and inorganic phosphate spots were cut out and counted. ATP hydrolysis is expressed as a percent of the initial ATP which remained at the end of the phosphorylation period. Initial ATP concentrations can be read from the graph. For example, for an initial ATP concentration of 100 μM, 50% of the ATP or 5 nmol was hydrolyzed after 15 sec. (B) Phosphorylated proteins in the TCA precipitate were separated on SDS–PAGE. Radioactive cTnI was located on the gel by autoradiograpy, cut out, and counted. (A and B) 5′-Chloro-5′-deoxyadenosine was present at the following concentrations: (●) none, (▲) 25 μM, and (■) 100 μM.

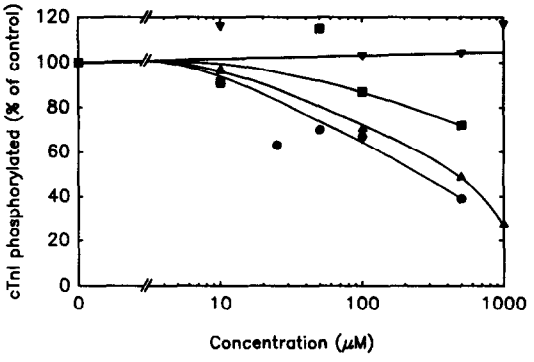

Fig. 6. Effects of 5′-chloro-5′-deoxyadenosine, AMP, ADP, and adenine on phosphorylation of purified cTnI by catalytic subunit. Purified cTnI (50 μg/mL) was phosphorylated for 1 min in the presence of 1.0 μg/mL catalytic subunit of PK-A and various concentrations of 5′-chloro-5′-deoxyadenosine (●), ADP (▲), AMP (▼), and adenine (■). TCA precipitation and SDS–PAGE were performed as described in Materials and Methods. Radioactive bands were cut out of the gel and counted. Four separate experiments were performed. The control value (100%) for the 5′-chloro-5′-deoxyadenosine experiment was 242 fmol cTnI phosphorylated; for the ADP experiment, 150 fmol; for the AMP experiment, 158 fmol; and for the adenine experiment, 282 fmol. Each point is the mean of two determinations.

catalytic subunit of PK-A was used to phosphorylate cTnI in the myofibrillar fraction and purified cTnI (Fig. 4, B and C). 5′-Chloro-5′-deoxyadenosine inhibited the phosphorylation in both cases. Adenosine and 5′-chloro-5′-deoxyadenosine had similar inhibitory effects (Fig. 4B). In addition, the extent of the inhibition was similar for phosphorylation by endogenous kinases and added catalytic subunit of PK-A (Fig. 4, A and B).

The fraction of ATP hydrolyzed by the myofibrillar protein decreased with increasing ATP concentrations (Fig. 5A). 5′-Chloro-5′-deoxyadenosine (25 or 100 μM) had no significant effect on this ATPase activity. A reaction time of 15 sec was chosen in this experiment to minimize problems of non-linearity as a function of time at low ATP concentrations. The inhibition of phosphorylation of cTnI by 5′-chloro-5′-deoxyadenosine as a function of ATP concentration was measured in the same experiment (Fig. 5B). 5′-Chloro-5′-deoxyadenosine inhibited the phosphorylation of cTnI at all ATP concentrations tested. The phosphorylation of cTnI by the catalytic subunit of PK-A deviated from Michaelis–Menten kinetics and a K_M for ATP and a K_I for 5′-chloro-5′-deoxyadenosine could not be determined.

The effects of ADP, AMP, and adenine on phosphorylation of purified cTnI by the catalytic subunit of PK-A are shown in Fig. 6. ADP and adenine inhibited cTnI phosphorylation, whereas AMP showed no significant effects. The potency order was adenosine = 5′-chloro-5′-deoxyadenosine > ADP > adenine. It was reported previously that ADP, AMP, adenosine, and adenine inhibit phosphorylation of histones by brain PK-A [12, 39]. Possibly these authors observed an inhibition by AMP because of the presence of 5′-nucleotidase in their phosphorylation mixture. In the case of ADP, the presence of myokinase could cause an apparent inhibition because of the dilution of the specific activity of the radioactive ATP. Assays showed that the purified cTnI and the catalytic subunit of PK-A used by us contained very low levels of myokinase, which were sufficient to generate only 0.8 and 1.9 μM ATP, respectively, during the time of the experiments.

DISCUSSION

Endogenous protein kinases present in the myofibrillar fraction phosphorylated cTnI upon addition of ATP. Phosphorylation of cTnI by PK-A

[40–44], but not by protien kinase C [45, 46], is believed to be important physiologically, as stimulation of perfused hearts by catecholamines results in phosphorylation of cTnI. Addition of cAMP to our preparation stimulated phosphorylation of cTnI (data not shown). The extent of the inhibition of cTnI phosphorylation in the myofibrillar fraction by 5′-chloro-5′-deoxyadenosine was similar for endogenous protein kinases and for exogenous catalytic subunit of PK-A (Fig. 4, A and B). Thus, the inhibitory effects of adenosine and its 5′-chloro analog observed with the myofibrillar protein fraction were probably due at least in part to the inhibition of endogenous PK-A. Other kinases present may also be inhibited by adenosine. Protein kinase C [45, 46], cGMP-dependent protein kinase [47, 48], and phosphorylase *b* kinase [49] have been shown to phosphorylate cTnI *in vitro*, but not in intact heart or myocytes.

cTnI is phosphorylated in response to stimulation of the intact heart by catecholamines, but the long-term phosphorylation state of cTnI correlates poorly with the contractility of the myocardium [19, 50–52]. Immediately upon stimulation, intracellular [cAMP] rises, cTnI is phosphorylated, and the contractility of the heart increases. Within 2 min, cAMP levels and the contractile force drop to prestimulation values, but the extent of phosphorylation of cTnI remains high [19, 51]. Phosphorylation of cTnI may be involved in mediating the positive chronotropic effects of catecholamines [53]. Phosphorylation of cTnI results in accelerated relaxation by decreasing the affinity of cTnC for Ca^{2+} [20] and by increasing the Ca^{2+} dissociation rate [21]. Little is known about regulation of the phosphatases which dephosphorylate cTnI [54].

Adenosine may inhibit the phosphorylation of other proteins and substrates in the heart. For example, inhibition of the phosphorylation of phospholamban would be expected to cause a decreased heart rate and contractility. It was shown previously that adenosine inhibited phosphatidylinositol kinase and myosin light chain kinase in aortic smooth muscle [14], and we have found that adenosine also inhibited phosphatidylinositol kinase in rat heart (unpublished results).

The concentration of adenosine required for inhibition of phosphorylation of cTnI is within the physiological range for intracellular adenosine. Adenosine level are approximately 10 and 100 μM for rat hearts perfused for 10 min with aerobic and anaerobic buffer, respectively [55]. Schrader and Gerlach [56] observed a similar rise in adenosine levels in perfused guinea pig hearts after 6 min of ischemia. It may be possible to develop adenosine analogs which inhibit cTnI phosphorylation at even lower concentrations than are required for inhibition by adenosine or 5′-chloro-5′-deoxyadenosine, as was the case for inhibition of cAMP phosphodiesterase by theophylline analogs [57]. Our results suggest that one mechanism of action of adenosine in heart may be its effect on the phosphorylation of the contractile regulatory protein cTnI.

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