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Inhibition of type 1 protein phosphatase activity by activation of β-adrenoceptors in ventricular myocardium

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Abstract

The regulation of protein phosphatase (PP) activity by cardiac β-adrenergic receptor stimulation with isoproterenol (ISO) was studied in four groups of guinea pigs consisting of seven animals each. Group 1 received the vehicle solution only intraperitoneally; group 2, 6 μg/kg of ISO; group 3, 60 μg/kg of ISO; and group 4, 600 μg/kg of ISO. Total PP activity (consisting of both type 1 and type 2A PP), activity of each PP subtype, the cAMP-dependent protein kinase activity ratio (−cAMP/+cAMP), the phosphorylation of PP inhibitor 1, and the phosphorylation of phospholamban were measured in ventricular tissue. PP activity was also studied in ventricular cardiomyocytes isolated from guinea pigs treated with and without 1 μM ISO or 1 μM ISO plus 10 μM propranolol, an antagonist of the β-adrenoceptor. PP activity decreased significantly in membrane vesicles, but not in cytosolic fractions, of guinea pigs treated with 60 and 600 μg/kg of ISO compared with untreated animals. The PKA activity ratio, PLB phosphorylation, and PP inhibitor 1 phosphorylation increased in ventricles of guinea pigs treated with 60 and 600 μg/kg of ISO compared with vehicle-treated animals. The decrease in overall PP activity was due primarily to a reduction in type 1 but not type 2A PP activity. In isolated ventricular cardiomyocytes, PP activity was decreased significantly after treatment with 1 μM ISO, and this inhibition was reversed by treatment with 10 μM propranolol. The membrane vesicles of group 1 animals did not release any catalytic subunit of type 1 PP upon phosphorylation by exogenous PKA. These results indicate that activation of cardiac β-adrenoceptors inhibits type 1 PP activity *via* phosphorylation of PP inhibitor 1 in the ventricles. This effect is associated with the well-known effect of ISO on increases in the PKA activity ratio and PLB phosphorylation. Inhibition of type 1 PP activity could be one possible mechanism, in addition to activation of adenylate cyclase, by which ISO mediates enhanced contractility of the heart.

Keywords: Isoproterenol; Ventricles; Phospholamban; Protein phosphatase; Inhibitor 1

1. Introduction

The mechanism by which β-adrenergic agonists, such as norepinephrine or ISO, increase contractility of the heart is thought to be well understood. Several laboratories [1–5] have shown that ISO binds to β-adrenergic receptors located on the plasma membrane, which, subsequently, activates adenylate cyclase to convert ATP into second messenger cAMP. Increased intracellular cAMP levels activate PKA, which, in turn, phosphorylates several target proteins such as the voltage-sensitive Ca^{2+} channels located on the sarcolemma, phospholamban (PLB) in the sarcoplasmic reticulum, and troponin inhibitor in myofilaments. Phosphorylation of these proteins correlates well with the increase in contractility of the heart [1,4]. In addition to this metabolic cascade, a few laboratories, including ours, have suggested that ISO can also increase the contractility of the heart by inhibiting PP. In guinea pig perfused ventricles, ISO inhibited membrane-bound type 1 PP activity and simultaneously applied acetylcholine attenuated ISO-inhibited membrane-bound PP activity [6]. These investigators, however, did not study changes in type 2A PP activity, which is also associated with membrane vesicles [6]. In skeletal muscle, type 1 PP is regulated by the phosphorylation and dephosphorylation of PP inhibitor 1, a 26 kDa trypsin labile thermostable polypeptide [7,8]. While recognized to exist in skeletal muscle, PP

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Abbreviations: ISO, isoproterenol; cAMP, cyclic AMP; PP, protein phosphatase; PKA, cAMP-dependent protein kinase; DTT, dithiothreitol; PLB, phospholamban; MV, membrane vesicles.

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inhibitor 1 was not known to exist in the heart [9]. In studies using isolated perfused guinea pig hearts [10] and isolated cardiomyocytes [11], we showed that PP inhibitor 1 is phosphorylated by ISO [10]. The presence of this protein was also documented in the rat heart [12]. In the present study, we examined whether ISO regulates PP activity in ventricular tissues and isolated ventricular cardiomyocytes. Further, we examined whether inhibition of the membrane-bound PP activity is associated with either the phosphorylation of PP inhibitor 1 or the phosphorylation of the regulatory subunit of type 1 PP bound to the membrane or both.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP was purchased from DuPont, New England Nuclear Research Products. Adenosine deaminase, (−)-isoproterenol-(+)-bitartrate, phosphorylase kinase, and sodium metabisulfite were obtained from the Sigma. All electrophoretic reagents were purchased from Bio-Rad Laboratories. All other chemicals were analytical reagent grade.

2.2. Methods

2.2.1. Preparation of ventricles

Twenty-eight guinea pigs of either sex, weighing 350–450 g, were divided into four groups, each consisting of seven animals. Each group was injected intraperitoneally with 0.5 mL of vehicle solution (10 mM HEPES buffer, pH 7.0, 100 μM sodium metabisulfite) alone or containing ISO. Group 1 received no ISO; group 2, 6 μg/kg of ISO; group 3, 60 μg/kg of ISO; and group 4, 600 μg/kg of ISO. After 10 min at room temperature, the animals were killed as previously described [11]. Hearts were excised quickly, ventricles were immediately dissected out from the atria, and blood was washed away with a saline solution. The washed ventricles were dried with tissue papers, quickly frozen and pulverized in liquid nitrogen, and stored at −70° for subsequent biochemical analysis.

2.2.2. Isolation and treatment of ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from guinea pig hearts as previously described [13]. A total of eight guinea pigs of either sex, weighing approximately 350–400 g, were used in this experiment. The final preparation of cardiomyocytes was equilibrated with HEPES buffer (1.8 mM $CaCl_2$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 132 mM NaCl, 10 mM HEPES, 10 mM glucose, and 2.5 mM sodium pyruvate, pH 7.4, oxygenated). Approximately 150 mg of freshly isolated cardiomyocytes (or 10 mL of gravity-settled cells) was diluted 5-fold in HEPES buffer and then divided into three groups, each consisting of 8 mL of suspension. One group was incubated with 8 mL of vehicle solution (HEPES buffer, 10 units/mL of adenosine deaminase, 200 μM sodium metabisulfite), another with vehicle solution containing 2 μM ISO, and the third with vehicle solution containing 2 μM ISO plus 20 μM propranolol. Adenosine deaminase was included to avoid interference of the endogenous adenosine produced during the preparation of cardiomyocytes, sodium metabisulfite was included to protect ISO from oxidation, and propranolol was added to antagonize the effect of ISO. After incubation at 37° for 2 min, suspensions were centrifuged at 5000 *g* for 5 min at 4°. The supernatants were discarded, and the pellets were frozen in liquid nitrogen and stored at −70°.

2.2.3. Preparation of membrane and cytosolic fractions

Approximately 300 mg of frozen ventricular tissue or 50 mg of each frozen cardiomyocyte-derived pellet was thawed in 5 mL of medium 1 (10 mM Tris–maleate, pH 6.8, 0.3 M sucrose, 1 mM EDTA, 1 mM EGTA, 2 mM sodium pyrophosphate) containing protease inhibitors (0.8 mM benzamidine, 0.8 mg/L of aprotinin and leupeptin, and 0.4 mg/L of antipain) at room temperature for 5 min with intermittent vortexing. Inclusion of protein kinase inhibitors (EDTA and EGTA) and a PP inhibitor (sodium pyrophosphate) was necessary to protect the state of phosphorylation of proteins in the heart. In our experience, the addition of sodium pyrophosphate has an advantage over that of sodium fluoride in inhibiting PP activity because the effect of the former is reversible in nature, whereas the effect of the latter is irreversible. The thawed samples were homogenized for three 20 s periods using a 10 mm generator (Omni International Inc.) at setting 10. The homogenates were then filtered through glass wool and centrifuged at 100,000 *g* for 30 min at 4°. The resulting supernatants were saved, and the pellets were subsequently washed by resuspension in 10 mL of medium 2 (10 mM Tris–maleate, pH 6.8, 0.3 M sucrose, 0.6 M KCl, 1 mM dithiothreitolm, DTT) followed by centrifugation at 100,000 *g* for 30 min at 4°. The pellets were washed again but this time with 10 mL of medium 2 lacking DTT. The pellet suspensions were centrifuged (100,000 *g* for 30 min at 4°), and then the final pellets were resuspended in 1 mL of medium 3 (10 mM Tris–maleate, pH 6.8, 0.3 M sucrose). These membrane suspensions, referred to as membrane vesicles (MV), were dispensed into tubes in 200 μL aliquots, immediately frozen in liquid nitrogen, and stored at −70° until used. The supernatants saved from the first spin were dialyzed against 2 × 1000 mL of medium 4 (10 mM Tris–maleate, pH 6.8, 0.3 M sucrose, protease inhibitors) for 16 hr at 4°. The dialyzed suspensions were centrifuged at 100,000 *g* for 30 min at 4° to eliminate any precipitate. The clear supernatants, referred to as the cytosolic fraction, were dispensed into 500 μM aliquots, immediately frozen in liquid nitrogen, and stored at −70° until used. The supernatants were dialyzed to remove pyrophosphate.

2.2.4. PP activity and subtypes

The activity of PP in MV and cytosolic factions was determined in a total assay volume of 100 μL using ^{32}P-phosphorylase *a* as the substrate, according to a previously described method [6] with some modification. A 30 μL aliquot of each MV or cytosolic fraction was individually incubated with 30 μL of the indicated concentration of $MnCl_2$ at 30° for 30 s. Subsequently, the assay was initiated by adding 40 μL of the assay buffer to each assay tube. The assay buffer consisted of 125 mM Tris–HCl, pH 7.4, 75 mM β-mercaptoethanol, 31.25 mM caffeine, 0.625 mM EDTA, and 2.5 mg/mL of ^{32}P-labeled phosphorylase *a*. The concentrations of the MV or cytosolic fractions were adjusted to allow for the hydrolysis of approximately 20% of the total ^{32}P-phosphorylase *a* added to each reaction. After incubation for 10 min, the reaction was terminated by adding 30 μL of 50% trichloroacetic acid (TCA) and 20 μL of 50 mg BSA/mL to each tube. The assay tubes were centrifuged, and an aliquot (100 μL) of each clear supernatant was assessed for radioactivity, i.e. $^{32}P_i$ released from the hydrolysis of ^{32}P-labeled phosphorylase *a* as previously described [6]. The specific activity of PP was quantified in picomoles of $^{32}P_i$ released per minute per milligram of protein. Inclusion of $MnCl_2$ in the assay restored the membrane- or cytosol-associated PP activity that was previously inhibited by the sodium pyrophosphate in the homogenization buffer.

To determine the activity of type 1 and type 2A PP, the indicated concentration of the purified rabbit skeletal muscle PP inhibitor 2 was incubated with the indicated concentration of the MV or cytosolic fractions for 30 s at 30°. The reaction was initiated by the addition of the assay buffer as already described. Type 1 PP activity was calculated by subtracting the activity measured in the presence of PP inhibitor 2 from the activity found in the absence of inhibitor 2. The PP activity measured in the presence of inhibitor 2 was attributed to type 2A PP.

2.2.5. Phosphorylation of MV

MV isolated from ventricles of the four groups of guinea pigs were phosphorylated by PKA in a total assay volume of 100 μL. The reaction mixture contained 20 μg of MV, 0.1 mM ATP ([γ-^{32}P]ATP = 2 μCi), 10 units of the purified catalytic subunit of PKA, 10 mM Mg $(CH_3COO)_2$, 0.5 mM EDTA, 10 mM NaF, and 30 μM β-mercaptoethanol. After 10 min of incubation at 30°, the reaction was terminated by adding 10 μL of 100% TCA. After cooling in an ice-bath for 10 min, the assay tubes were centrifuged at 14,000 *g* in a table-top centrifuge for 10 min at room temperature. The pellets were washed by resuspension in 500 μL of ice-chilled ether and then recentrifuged at 14,000 *g* for 10 min at room temperature. The resulting pellets were resuspended in 100 μL of SDS-stop solution, and the suspensions were heat-treated (incubated in a boiling water bath for 10 min). Sixty microliters of each heat-treated extract was analyzed by SDS-PAGE. The 12% SDS gels were dried and autoradiographed as described previously [11]. Bands corresponding to PLB were excised, and the radioactivity associated with these bands was assessed and expressed as picomoles of ^{32}P incorporated per minute per milligram of MV.

In a separate experiment, approximately 50 μg of MV isolated from the guinea pigs in group 1 was phosphorylated in a total assay volume of 250 μL by PKA in a manner similar to that already described except that radiolabeled [γ-^{32}P]ATP was not added. Following phosphorylation, samples were centrifuged at 100,000 *g* for 10 min at 4°. The supernatants were saved, and the pellets were resuspended in 100 μL of 50 mM Tris–HCl, pH 7.4. PP activity was determined in the resulting supernatant and membrane fractions by the method already described.

2.2.6. Miscellaneous methods

Phosphorylase *a* was purified from rabbit skeletal muscle as described previously [14]. ^{32}P-Labeled phosphorylase *a* was prepared according to the method given in [15]. Both the determination of protein (with the use of BSA as a standard) and the electrophoresis of the protein samples were performed according to previously described methods [11,13]. The PKA activity ratio (−cAMP/+cAMP) was determined as previously described [13]. The catalytic subunit of PKA was purified from bovine heart [16], and PP inhibitor 2 was purified from rabbit skeletal muscle [17], using methods described in the cited references. Phosphorylated PP inhibitor 1 was determined in ventricular tissue according to the methods described in [6,15].

2.2.7. Data analysis

Statistical comparisons among the four groups of ISO-treated guinea pigs were performed using one-way ANOVA with alpha set at 0.05. If significance was obtained, pairwise comparisons were performed using the Student–Neuman–Keuls test. For this test, a probability of $P < 0.05$ was considered significant. All data are reported as means ± SEM.

3. Results

3.1. Effect of PP inhibitor 2 on PP activity

The proportion of PP activity attributed to type 1 and type 2A PP was examined in MV and in cytosolic fractions, using PP inhibitor 2. The results are shown in Fig. 1. PP inhibitor 2 inhibited PP activity of both MV and cytosolic fractions in a concentration-dependent manner. The maximal inhibition by PP inhibitor 2 was approximately 78% in the MV and 32% in the cytosolic fraction. This suggested that PP activity attributable to type 1 PP was 78% in the MV and 32% in the cytosol, whereas the type 2A PP activity was 22% in the MV and 68% in the cytosol.

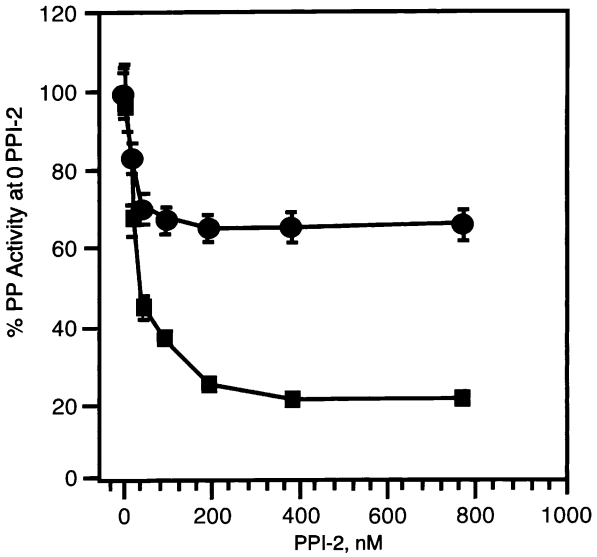

Fig. 1. Effect of PP inhibitor 2 on PP activity in MV or cytosol fractions. Membrane vesicles (■) or cytosol (●) was preincubated with various concentrations of protein phosphatase inhibitor 2 (PPI 2), and then PP activity was determined. Values are means ± SEM from three different experiments.

3.2. Effect of ISO on cardiac PP activity

The effects of increasing concentrations of ISO, injected into guinea pigs, on PP activity in MV and cytosolic fractions are shown in Fig. 2. PP activity assayed in ventricular tissue obtained from the guinea pigs of group 1 (in the absence of ISO) was 3012 ± 270 units/mg protein in the cytosolic fraction and 350 ± 25 units/mg protein in the MV fractions. PP activity in the cytosolic fraction did not change with increasing concentrations of ISO compared to the control (Fig. 2, top). However, PP activity in the MV decreased significantly at an ISO concentration of 60 μg/kg and decreased further at 600 μg/kg compared to the control (Fig. 2, bottom). The values of PP activity at 60 and 600 μg/kg were 300 ± 20 and 150 ± 10 units/mg protein, respectively.

To determine the subtype of PP whose activity was inhibited by ISO, PP activity was measured in MV fractions obtained from the group 1 guinea pigs that did not receive ISO and compared with those that received the highest dose of ISO (group 4, 600 μg/kg). The results are shown in Fig. 3. In the MV fraction without ISO, PP activity attributable to type 1 PP was 300 ± 20 units/mg protein with only 60 ± 3 units/mg protein attributable to type 2A. In the MV fractions from guinea pigs receiving the highest dose of ISO, PP activity attributable to type 1 PP was 98 ± 5 units/mg protein and approximately 60 ± 4 units/mg protein was attributable to type 2A. Thus, the highest dose of ISO resulted in inhibition of type 1 PP activity but not of type 2A PP activity. To determine whether changes in PP activity are associated exclusively with cardiomyocytes, cardiomyocytes were isolated from

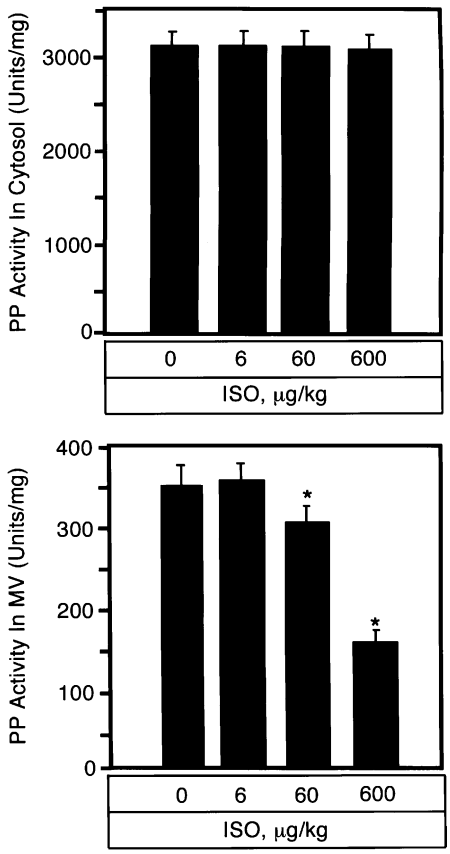

Fig. 2. Effect of ISO on PP activity associated with cytosol (top) and membrane vesicles (bottom) isolated from guinea pig ventricles. Guinea pigs were injected intraperitoneally with vehicle solution containing no (0) ISO or with 6, 60, and 600 μg/kg of ISO, (*) $P < 0.05$ vs. 0 ISO. Values are means ± SEM from three different experiments.

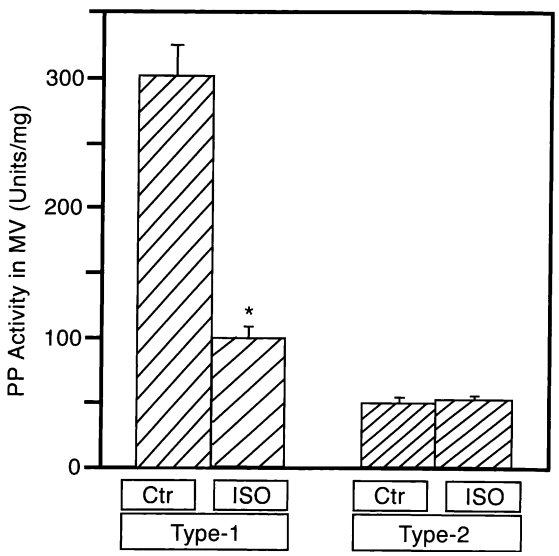

Fig. 3. Effect of ISO on the distribution of type 1 and type 2 PP activity in membrane vesicles isolated from guinea pig ventricles. Guinea pigs were injected with vehicle solution containing no (Ctr) ISO or with 600 μg/kg of ISO, and PP activity was determined in membrane vesicles in the presence and absence of PP inhibitor 2, (*) $P < 0.05$ vs. Ctr, type 1 PP. Values are means ± SEM from three different experiments.

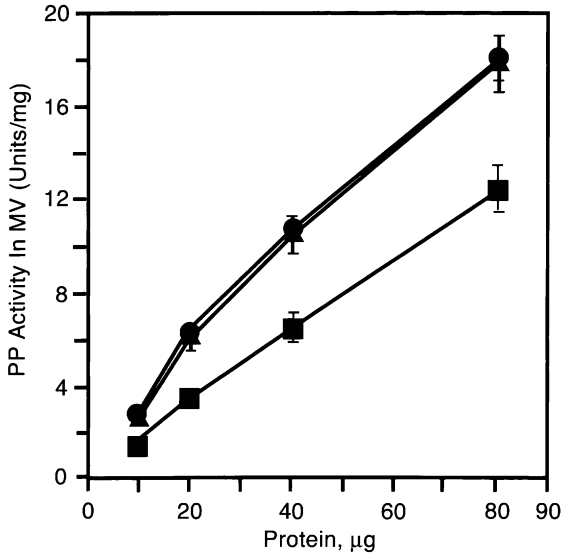

Fig. 4. Protein–response curve of PP activity associated with membrane vesicles isolated from ventricular cardiomyocytes of guinea pigs. Isolated guinea pig ventricular cardiomyocytes were divided into three portions, and then each portion was treated with vehicle alone (●), 1 μM isoproterenol (■), or 1 μM isoproterenol + 10 μM propranolol (▲). After treatment, MV were isolated from cardiomyocytes, and PP activity was determined in various concentrations of MV. Values are means ± SEM from three different experiments.

ventricles of guinea pig hearts and treated with ISO, with ISO and propranolol, and also without either compound. Treatment of cardiomyocytes with ISO inhibited membrane-associated PP activity, but this inhibition was overcome by treatment of cells with propranolol (Fig. 4).

3.3. Effect of ISO on the PKA activity ratio (−cAMP/+cAMP) and the phosphorylation of PLB

To ensure that ISO-injected intraperitoneally into guinea pigs reached the heart, the PKA activity ratio was determined in a 14,000 *g* supernatant prepared from 25 mg of frozen ventricles of the four groups of guinea pigs described earlier. The results are shown in Fig. 5. The PKA activity ratio was 0.24 ± 0.01 in the absence of ISO, remained unchanged in the presence of the lowest dose of ISO (6 μg/kg), and increased significantly to 0.33 ± 0.02 and 0.35 ± 0.02 in the presence of 60 and 600 μg/kg of ISO, respectively (Fig. 5A). Since it is well known that ISO activates PKA and thereby phosphorylates PLB, the phosphorylation of PLB was determined in MV isolated from ventricles of all four groups of animals. The results on PLB phosphorylation in the MV from two guinea pigs of group 1 (0 ISO) and two guinea pigs of group 4 (600 μg/kg of ISO) are shown in Fig. 5B. PLB was identified on the gel as a monomeric protein of M_r 5.5 kDa; several other proteins of high molecular masses also appeared to be phosphorylated (Fig. 5B). This indicates that the effect of ISO on PLB phosphorylation is preferential rather than selective. PLB appeared to be phosphorylated less in guinea pigs from

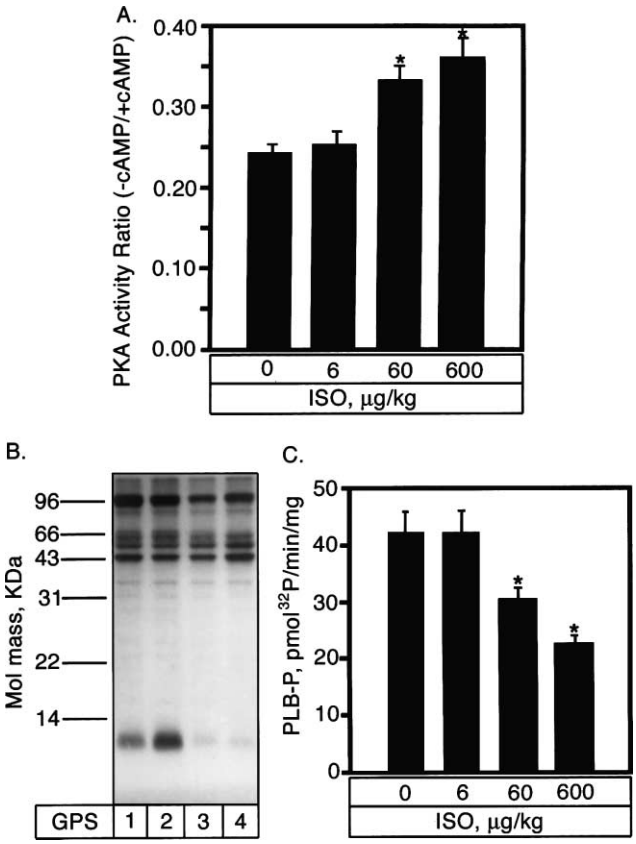

Fig. 5. (A) Effect of ISO on the PKA activity ratio (−cAMP/+cAMP). The PKA activity ratio was determined in ventricles obtained from guinea pigs injected with vehicle solution containing no (0) ISO or the indicated concentrations of ISO. Values are means ± SEM from three different experiments. (B) Autoradiograph showing the phosphorylation of PLB by exogenous PKA in membrane vesicles isolated from ventricles of guinea pigs treated with and without ISO. Membrane vesicles, isolated from each animal separately, were injected with vehicle solution containing no (lanes 1 and 2) ISO or 600 μg/kg of ISO (lanes 3 and 4). Subsequently, membrane vesicles were phosphorylated by PKA in the presence of [γ-^{32}P]ATP and then were electrophoresed and autoradiographed. (C) Analysis of PLB phosphorylation at different concentrations of ISO. The PLB band was excised from the gel containing MV from seven animals in each of the four groups, and then radioactivity was assessed. Values are means ± SEM from three different experiments, (∗) $P < 0.05$ vs. 0 ISO.

group 4 than from group 1. When the phosphorylation of PLB was analyzed in all four groups of animals, PLB phosphorylation was found to be decreased approximately 29% in group 3 guinea pigs and approximately 49% in group 4 animals compared with those of group 1 (Fig. 5C), suggesting that PLB in group 3 and group 4 animals exists in the phosphorylated form. There was no difference in the extent of phosphorylation of PLB between group 1 and group 2 animals, suggesting that the amount of ISO was not enough in the group 2 guinea pigs to let PLB be phosphorylated (Fig. 5C).

3.4. Phosphorylation of PP inhibitor 1

The extent of phosphorylation of PP inhibitor 1 was determined in ventricular tissue obtained from all four

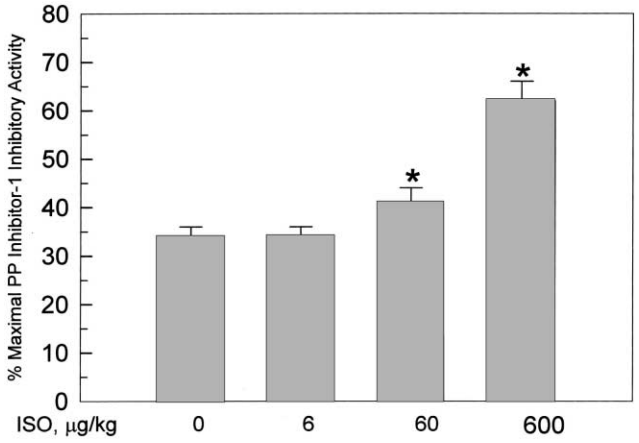

Fig. 6. Effect of ISO on the phosphorylation of PP inhibitor 1 in guinea pig ventricles. The phosphorylation state of PP inhibitor 1 was determined in ventricles isolated from guinea pigs injected with vehicle solution containing no (0) ISO, or 6, 60, or 600 μg/kg of ISO. An increase in the percentage of maximal inhibitory activity indicates an increase in the phosphorylation state of PP inhibitor 1. Values are means ± SEM from three different experiments, (∗) $P < 0.05$ vs. 0 ISO.

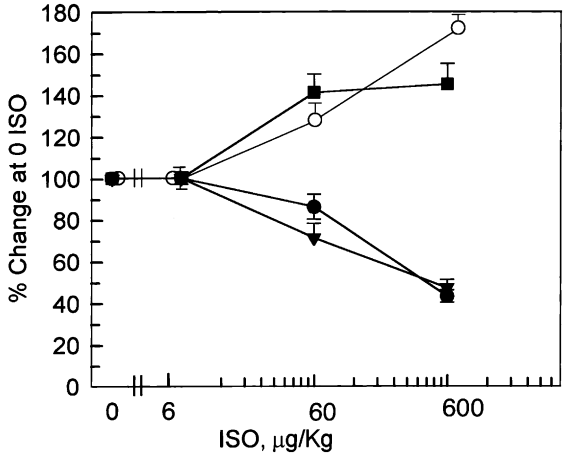

Fig. 7. Relationship among inhibition of PP activity, cAMP-independent protein kinase activity, back-phosphorylation of PLB, and PP inhibitor 1 at different concentrations of ISO. A decrease in the back-phosphorylation of PLB in response to ISO suggests that ISO increases PLB phosphorylation. Values are means ± SEM from three different experiments, (●) inhibition of PP activity, (■) cAMP-independent protein kinase activity, (▲) back-phosphorylation of PLB, and (○) phosphorylation of PP inhibitor 1.

groups of animals and expressed as percentage of maximal inhibitory activity of the PP inhibitor 1. In group 1 animals (0 ISO), the extent of PP inhibitor 1 phosphorylation was found to be 34 ± 2%. This value remained the same in group 2 animals (6 μg/kg of ISO), and increased to 41 ± 3 and 62 ± 4% in group 3 (60 μg/kg of ISO) and group 4 (600 μg/kg of ISO) animals, respectively (Fig. 6).

3.5. PP activity in phosphorylated MV

MV isolated from ventricles of group 1 animals were phosphorylated by PKA and then ''airfuged'' to separate out the cytosol and membrane fractions, as described in Section 2. PP activity was not detected in the supernatant, whereas the enzyme activity in the phosphorylated and unphosphorylated membrane fractions was similar (349 ± 16 pmol ^{32}P/min mg protein).

3.6. Relationship of biochemical parameters

The data calculated from Figs. 2, 5, and 6 are depicted in Fig. 7 for showing the relationship of the effect of different concentrations of ISO on the phosphorylation of PLB and PP inhibitor 1, the inhibition of PP activity, and the increase in cAMP-independent protein kinase (PKA) activity. In group 1 (0 ISO) and group 2 animals (6 μg/kg of ISO), ISO did not produce any effects on these biochemical parameters. In contrast, ISO in group 3 (60 μg/kg) and group 4 (600 μg/kg) animals increased cAMP-independent PKA activity by 41 and 45%, and PP inhibitor 1 phosphorylation by 20 and 76%, but reduced MV-bound PP activity by 15 and 56%, respectively, compared with group 1 animals. In addition, back-phosphorylation of PLB in MV was decreased by 29 and 53% in group 3 and group 4 animals, respectively, suggesting that PLB existed in a prephosphorylated form in the animals that received ISO (Fig. 7).

4. Discussion

The main findings of this study are: (a) ISO inhibits PP activity through the activation of the β-adrenergic receptor pathway as evidenced by stimulation of PKA and an increase in the phosphorylation of PLB; (b) activation of the β-adrenergic receptor pathway with ISO mediates a reduction in PP activity in the MV but not in the cytosol; (c) the reduction of MV-associated PP activity is limited to type 1 and not type 2A PP, and (d) ISO-induced inhibition of type 1 PP activity is mediated through the phosphorylation of the PP inhibitor 1 and is not due to dissociation of the catalytic subunit of type 1 PP (PP1c) from its regulatory subunit which is bound to the MV. The findings of the study also provide evidence that activation of the β-adrenergic receptor signal transduction system by ISO mediates inhibition of PP activity at the ventricular cardiomyocyte level.

It is well known that ISO increases the contractility of the heart *via* activation of adenylate cyclase, increases in cAMP levels, activation of PKA and Ca^{2+}-calmodulin-dependent protein kinase, and phosphorylation of PLB and Ca^{2+}-ATPase [2,14,18]. Phosphorylated PLB, in turn, allows the Ca^{2+}-pump of the sarcoplasmic reticulum to transport more Ca^{2+} from the cytosolic compartment to the lumen of the sarcoplasmic reticulum by increasing the affinity of the pump for Ca^{2+}, whereas phosphorylated Ca^{2+}-ATPase exhibits increased pump activity without any changes in the affinity of the pump for Ca^{2+} [18]. The buildup of Ca^{2+} within the sarcoplasmic reticulum is released with the next heartbeat, resulting in an increase

in the contractility of the heart [1–5]. The findings of the present study support an alternative mechanism by which ISO mediates increased cardiac contractility, namely through ISO-mediated inhibition of PP activity. Our data also provide a mechanism for inhibition of PP activity. This mechanism is due to phosphorylation of PP inhibitor 1. In isolated perfused ventricles of guinea pigs, ISO was shown to inhibit membrane-bound type 1 PP activity *via* phosphorylation of PP inhibitor 1 [6]. In that study, the effect of ISO on MV-bound type 2A PP activity was not examined. Our present results suggest that the effect of ISO is to inhibit type 1 but not type 2A PP activity. Our results also suggest that ISO-induced inhibition of MV-bound type 1 PP activity is associated with the phosphorylation of PP inhibitor 1 by PKA. Earlier, there was no evidence for the existence of PP inhibitor 1 in the heart; in fact on the contrary, some studies showed its absence in the heart [9]. In 1988, Iyer *et al*. [12] reported the presence of a PP inhibitor 1-like protein in the rat heart, which was also phosphorylated by ISO in ventricular slices. In 1991, using an antibody to inhibitor 1, we demonstrated conclusively the presence of PP inhibitor 1 and its hormonal regulation in the intact heart [10]. Subsequently, we showed the same to be true in ventricular cardiomyocytes isolated from guinea pigs [11].

In addition to the phosphorylation of inhibitor 1, an alternate mechanism has also been proposed [19]. In this mechanism, the holoenzyme type 1 PP is composed of the regulatory subunit and the catalytic subunit. The catalytic subunit is bound to the regulatory subunit, which is bound to the membrane. It is suggested that phosphorylation of the regulatory subunit by PKA releases the catalytic subunit of the enzyme into the cytosol where the active enzyme becomes inactive upon interaction with PP inhibitor 1 and inhibitor 2 proteins [19,20]. In the present study, we phosphorylated MV by PKA and determined PP activity in the supernatant and the phosphorylated MV. No PP activity was detected in the supernatant, and the activity associated with phosphorylated MV by PKA was very similar to that of unphosphorylated MV. Thus, these results clearly suggest that ISO inhibits type 1 PP activity through phosphorylation of PP inhibitor 1.

Results of the present study clearly indicate an ISO-mediated reduction of PP activity. This effect was associated with an increased PKA activity ratio and increased PLB phosphorylation. These findings prompted us to ask what effect, if any, does inhibition of PP activity have on cardiac contractility. Previous studies in guinea pig papillary muscles showed that PP inhibitors such as okadaic acid, sodium fluoride, cantharidin, and flosequinoxan increase the positive inotropic state by increasing the phosphorylation of PLB, troponin inhibitor, and myosin light chain [21–24]. These effects of increased positive inotropic response were observed without increases in cAMP levels. Indirect evidence for the involvement of β-adrenergic receptors in modulating PP activity also comes from studies performed in rat ventricular cardiomyocytes in which the activation of β_1 and β_2 elicited qualitatively different cellular responses [25,26]. Activation of the β_1-receptor was shown to increase cAMP levels, Ca^{2+}-transients, and contractility of the heart, whereas activation of the β_2-receptor caused an increase in Ca^{2+}-transients and contractility, but these effects were not associated with an increased level of cAMP [25]. In the same study, activation of the β_1-receptor led to phosphorylation of PLB, but activation of β_2 failed to phosphorylate PLB, suggesting another signal transduction pathway(s) to produce changes in cytosolic Ca^{2+} and contraction [25]. The effect of the cardiac β_2-adrenergic receptor on contractility of rat cardiomyocytes can be explained potentially through modulation of PP.

In summary, ISO administered to guinea pigs results in the inhibition of cardiac PP activity. This effect is associated with the well-known effect of ISO on increases in the PKA activity ratio and the phosphorylation of PLB and PP inhibitor 1. Inhibition of PP activity could be one possible mechanism, in addition to activation of adenylate cyclase, by which ISO mediates enhanced contractility of the heart.

Acknowledgments

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