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Mechanisms Involved in the Acidosis Enhancement of the Isoproterenol-induced Phosphorylation of Phospholamban in the Intact Heart*

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Previous experiments have shown that acidosis enhances isoproterenol-induced phospholamban (PHL) phosphorylation (Mundiña-Weilenmann, C., Vittone, L., Cingolani, H. E., Orchard, C. H. (1996) *Am. J. Physiol.* 270, C107–C114). In the present experiments, performed in isolated Langendorff perfused rat hearts, phosphorylation site-specific antibodies to PHL combined with the quantitative measurement of ^{32}P incorporation into PHL were used as experimental tools to gain further insight into the mechanism involved in this effect. At all isoproterenol concentrations tested (3–300 nM), phosphorylation of Thr^{17} of PHL was significantly higher at pH_o 6.80 than at pH_o 7.40, without significant changes in Ser^{16} phosphorylation. This increase in Thr^{17} phosphorylation was associated with an enhancement of the isoproterenol-induced relaxant effect. In the absence of isoproterenol, the increase in $[Ca]_o$ at pH_o 6.80 (but not at pH_o 7.40) evoked an increase in PHL phosphorylation that was exclusively due to an increase in Thr^{17} phosphorylation and that was also associated with a significant relaxant effect. This effect and the phosphorylation of Thr^{17} evoked by acidosis were both offset by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62. In the presence of isoproterenol, either the increase in $[Ca]_o$ or the addition of a 1 μM concentration of the phosphatase inhibitor okadaic acid was able to mimic the increase in isoproterenol-induced Thr^{17} phosphorylation produced by acidosis. In contrast, these two interventions have opposite effects on phosphorylation of Ser^{16}. Whereas the increase in $[Ca]_o$ significantly decreased phosphorylation of Ser^{16}, the addition of okadaic acid significantly increased the phosphorylation of this residue. The results are consistent with the hypothesis that the increase in phospholamban phosphorylation produced by acidosis in the presence of isoproterenol is the consequence of two different mechanisms triggered by acidosis: an increase in $[Ca^{2+}]_i$ and an inhibition of phosphatases.

The sarcoplasmic reticulum (SR)[1] Ca^{2+}-ATPase plays a pivotal role in the contraction and relaxation process of myocardial cells (1). Ca^{2+}-ATPase activity is tonically inhibited by the interaction with another SR protein named phospholamban. *In vitro* experiments indicate that phosphorylation of phospholamban occurs by either cAMP-dependent protein kinase (PKA) at Ser^{16} or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) at Thr^{17} (2). Phosphorylation of phospholamban causes dissociation of this protein from the pump, thus increasing ATPase activity and the rate of Ca^{2+} uptake by the SR (3). In the intact beating heart, β-adrenoreceptor stimulation phosphorylates phospholamban at both Ser^{16} and Thr^{17} (4, 5). Experimental evidence from our own laboratory strongly suggested that this dual phosphorylation requires both stimulation of the PKA and CaMKII cascades of phospholamban phosphorylation and simultaneous inhibition of phospholamban phosphatase (5). These two prerequisites appear to be fulfilled by β-adrenoreceptor stimulation, which, as a result of PKA activation, triggers the activation of CaMKII by increasing intracellular Ca^{2+} and produces the inhibition of PP1, the major phosphatase that dephosphorylates phospholamban (6–11). This may be the reason why several attempts to phosphorylate phospholamban by increasing $[Ca^{2+}]_i$ through cAMP-independent mechanisms have failed (12–15). Inhibition of phospholamban phosphatase was required (5).

The effect of β-adrenoreceptor stimulation on phospholamban phosphorylation and myocardial relaxation is dependent on the acid-base status of the myocardium. It has been shown that acidosis enhances isoproterenol-induced phospholamban phosphorylation and myocardial relaxation (16). The mechanism of this action remains unknown. Among several different effects, acidosis increases intracellular calcium levels (17, 18) and inhibits PP1 activity (16) in the rat myocardium. Thus, it seems reasonable to consider that the acidosis enhancement of the isoproterenol-induced increase in phospholamban phosphorylation could be due to a further increase either in Ser^{16} phosphorylation (by inhibition of PP1) and/or in Thr^{17} phosphorylation (by inhibition of PP1 and/or activation of CaMKII).

In this work, immunodetection of site-specific phosphorylated phospholamban was used in combination with the classical isotopic labeling technique of quantification of phospholamban phosphorylation to investigate the contribution of each phosphorylation site of phospholamban to the increase in the isoproterenol-induced phosphorylation of this protein evoked by acidosis. The possible mechanisms involved in this effect were also explored. Simultaneous measurements of mechanical

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; PKA, cAMP-dependent protein kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PSS, physiological salt solution; PP1, protein phosphatase type 1; PHL, phospholamban.

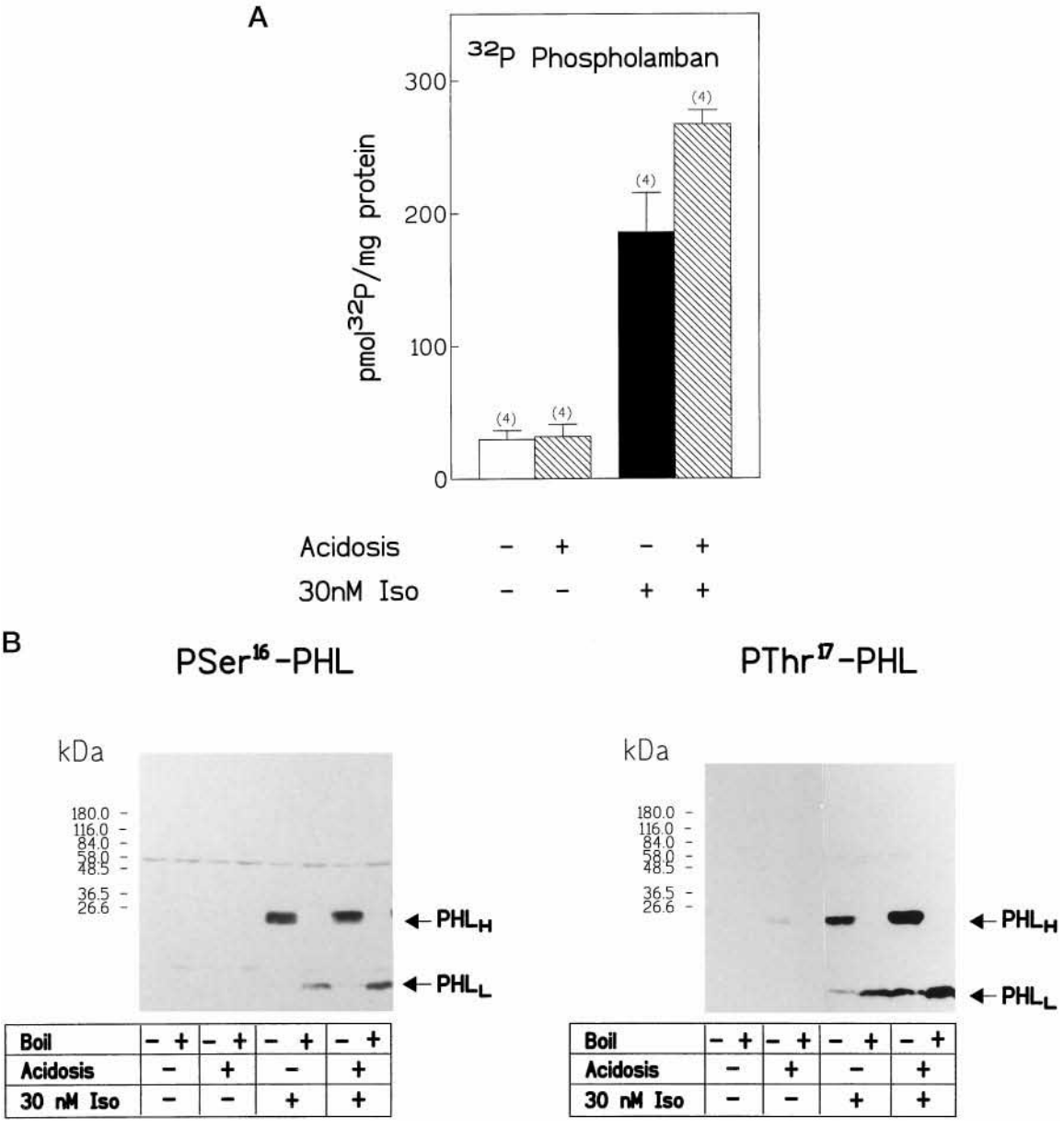

FIG. 1. *A*, overall results of ^{32}P incorporation into PHL. Experiments were performed in SR membrane vesicles isolated from rat hearts perfused with ^{32}P and then without and with 30 nM isoproterenol (*Iso*) at pH_o 7.40 or 6.80 (*Acidosis*). Samples (300 μg) were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The number of experiments in each treatment group is indicated in *parentheses*. The results show that acidosis enhanced isoproterenol-induced PHL phosphorylation. *B*, immunoblots of SR membrane vesicles isolated from hearts perfused under the same conditions described for *A*. 10 μg of SR protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were probed with anti-Ser^{16} PHL phosphopeptide (*$PSer^{16}$-PHL*) and anti-Thr^{17} PHL phosphopeptide (*$PThr^{17}$-PHL*). Antibody binding was visualized using a chemiluminescence detection kit. *PHL_H* and *PHL_L* designate the pentameric and monomeric forms of PHL, respectively.

parameters will provide a clue to the functional consequences of the effects observed.

EXPERIMENTAL PROCEDURES

Heart Perfusions—Experiments were performed in isolated hearts from male Wistar rats (250–350 g of body weight) perfused according to the Langendorff technique as described previously (13). The composition of the physiological salt solution (PSS) was 128.3 mM NaCl, 4.7 mM KCl, 1.35 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 11.1 mM glucose, and 0.04 mM Na_2EDTA; this solution was equilibrated with 95% O_2 and 5% CO_2 to give an extracellular pH (pH_o) of 7.40 ± 0.01 or with 80% O_2 and 20% CO_2 (pH_o 6.80 ± 0.02) in the experiments of hypercapnic acidosis. The mechanical activity of the heart was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer (Namic, Perceptor DT disposable transducer). The balloon was filled with aqueous solution to achieve a left ventricular end diastolic pressure of 8–14 mm Hg. Isovolumic pressure and its first derivative were recorded on a four-channel pen recorder (Gould Model RS 3400) fitted with a transducer amplifier (Gould Model 13-4615-50) and a differentiating amplifier (Gould Model 13-4615-71). Hearts were perfused with PSS at pH_o 7.40 for 10–15 min for stabilization and then for the next 3 min with either PSS (control) or different interventions as described under "Results." To quantify ^{32}P incorporation into phospholamban, hearts were perfused for 60 min by recirculation with PSS containing 10 μCi/ml $^{32}P_i$ after the stabilization period and previously to the interventions assessed. At the end of the experimental period, the ventricles were freeze-clamped, pulverized, and stored at −70 °C until biochemical assay.

Preparation of SR Membrane Vesicles—Membrane vesicles were prepared as described previously (13), except that the pulverized tissue from each heart was homogenized in 6 volumes of a medium containing 30 mM KH_2PO_4 (pH 7.0 at 4 °C), 5 mM Na_2EDTA, 25 mM NaF, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. Samples from ^{32}P-perfused hearts were homogenized in the same me-

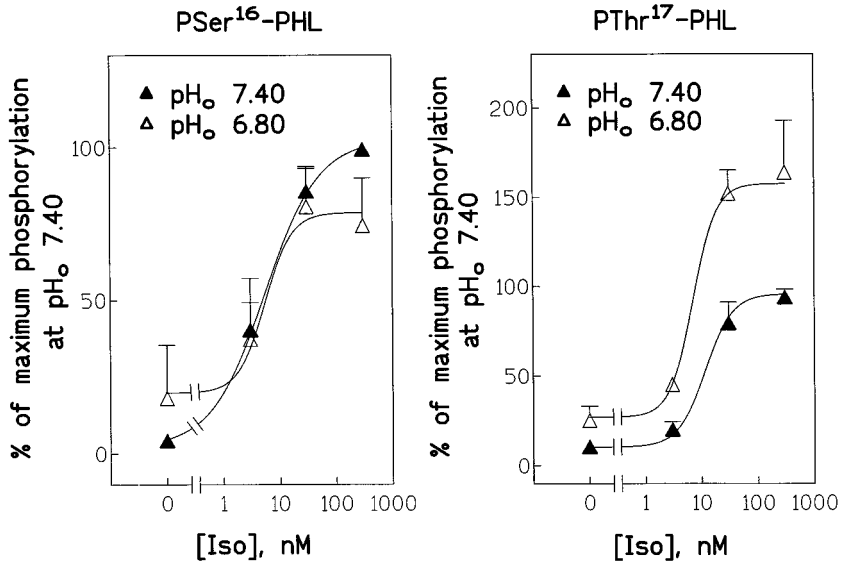

FIG. 2. **Dose-response curves of isoproterenol for phosphorylation of Ser[16] and Thr[17] at pH_o 7.40 and 6.80.** Values are the means ± S.E. obtained after densitometric analysis of the signal of four (anti-Ser[16] PHL phosphopeptide (*PSer[16]-PHL*)) and five (anti-Thr[17] PHL phosphopeptide (*PThr[17]-PHL*)) immunoblots. Results are expressed as a percentage of the maximal isoproterenol (*Iso*; pH_o 7.40)-induced site-specific phosphorylation. At all isoproterenol concentrations, acidosis enhanced the phosphorylation of Thr[17] without affecting the phosphorylation of Ser[16].

dium, except that the phosphate was replaced by 20 mM Tris-HCl (pH 7.0) at 4 °C. Protein was measured by the method of Bradford (19) using bovine serum albumin as a standard. The yield was 1–2 mg of membrane vesicle protein/g of cardiac tissue.

Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis was performed using 10% acrylamide slab gels according to Porzio and Pearson (20) as described previously (13). Samples for electrophoresis were not boiled unless stated. For immunological detection of phospholamban phosphorylation sites, 10 μg of membrane protein were electrophoresed per gel lane. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp.) and probed as described previously (5) according to Drago and Colyer (21) with monoclonal antibody to phospholamban (1:5000) or polyclonal antibodies raised against a phospholamban peptide (residues 9–19) phosphorylated at Ser[16] (1:5000) or at Thr[17] (1:5000) (PhosphoProtein Research, Leeds, United Kingdom). Immunoreactivity was visualized by peroxidase-conjugated antibodies using a peroxidase-based chemiluminescence detection kit (Boehringer, Mannheim, Germany). The signal intensity of the bands on the film was quantified by optical densitometric analysis. To assess ^{32}P incorporation into phospholamban, 300 μg of membrane protein were electrophoresed per gel lane. Gels were run in duplicate to use one of them for autoradiography and the other for liquid scintillation counting. Quantitative results are expressed as picomoles of ^{32}P incorporated into phospholamban/mg of SR protein based on the specific activity of ^{32}P in phosphocreatine (22).

Statistics—All data are expressed as the mean ± S.E. of *n* preparations. Student's *t* test for unpaired observations was used to test for statistical differences. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Acidosis on Phosphorylation of Phospholamban Ser[16] and Thr[17] in the Absence and Presence of β-Adrenoreceptor Stimulation—Fig. 1*A* shows the overall results of ^{32}P incorporation into phospholamban obtained from SR membrane vesicles isolated from hearts perfused with ^{32}P and then in the absence and presence of 30 nM isoproterenol at pH_o 7.40 and 6.80. In agreement with previous findings (16), acidosis did not increase basal phospholamban phosphorylation, but significantly enhanced isoproterenol-induced phospholamban phosphorylation. Fig. 1*B* shows immunoblots of SR membrane vesicles obtained from hearts perfused under the same conditions of Fig. 1*A*. Immunodetection of phosphorylation sites of phospholamban indicated that the acidosis-induced increase in phospholamban phosphorylation was exclusively due to an increase in phosphorylation of Thr[17]. Similar results were obtained when two other different isoproterenol concentrations were explored as shown in Fig. 2. At all isoproterenol concentrations, acidosis significantly increased phosphorylation of Thr[17] of phospholamban without affecting phosphorylation of Ser[16]. In the absence of isoproterenol, acidosis induced a slight and nonsignificant increase in Thr[17] phosphorylation and did not modify Ser[16] phosphorylation. Table I shows the mechanical parameters of this experimental series. Acidosis induced a decrease in the maximal rate of contraction (+Ṗ) in both the absence and presence of isoproterenol. Moreover, acidosis produced an enhancement of the isoproterenol-induced relaxant effect (decrease in $t_{1/2}$), which attained significant levels at 30 nM isoproterenol. This relaxant effect of acidosis may be due at least in part to the significant increase in the phosphorylation of Thr[17] produced by acidosis (see below). In the absence of isoproterenol, the decrease in $t_{1/2}$ produced by acidosis was not associated with any significant increase in phosphorylation of phospholamban and should therefore be attributed to mechanisms unrelated to the phosphorylation of this protein, such as

TABLE I
Effects of different interventions on mechanical parameters in the intact heart

Rat hearts were perfused with the indicated interventions and then freeze-clamped and processed as described under "Experimental Procedures." Results are the means ± S.E. of *n* hearts. Δ indicates differences with respect to the control.

Treatment	*n*	Maximal rate of contraction (+Ṗ)	Half-relaxation time ($t_{1/2}$)
		% of control	Δ*ms*
PSS			
1.35 mM $[Ca]_o$	20	99.18 ± 3.05	−0.2 ± 0.73
1.35 mM $[Ca]_o$-H^+	8	27.49 ± 4.61[a]	−13.75 ± 3.29[a]
Isoproterenol			
3 nM			
1.35 mM $[Ca]_o$	9	137.05 ± 8.13[a]	−12.33 ± 0.99[a]
1.35 mM $[Ca]_o$-H^+	4	72.51 ± 17.81[a,b]	−18.00 ± 4.02[a]
1.35 mM $[Ca]_o$-OA[c]	5	200.63 ± 18.90[a,b]	−20.6 ± 2.16[a,b]
30 nM			
1.35 mM $[Ca]_o$	15	167.43 ± 4.25[a]	−16.33 ± 1.67[a]
1.35 mM $[Ca]_o$-H^+	8	103.5 ± 14.9[b]	−26.13 ± 2.77[a,b]
1.35 mM $[Ca]_o$-OA	6	197.27 ± 20.8[a]	−19.33 ± 2.99[a]
3.85 mM $[Ca]_o$	10	194.45 ± 13.24[a]	−15.7 ± 1.27[a]
300 nM			
1.35 mM $[Ca]_o$	5	155.11 ± 6.29[a]	−17.00 ± 2.23[a]
1.35 mM $[Ca]_o$-H^+	3	119.4 ± 10.55[b]	−23.67 ± 3.18[a]
Calcium			
3.85 mM $[Ca]_o$	13	132.74 ± 10.14[a]	−0.54 ± 0.93
3.85 mM $[Ca]_o$-H^+	9	70.13 ± 5.44[a,b]	−9.55 ± 2.27[a,b]

[a] $p < 0.05$ when compared with the control.
[b] $p < 0.05$ with respect to the same situation in the absence of either acidosis or okadaic acid. The mechanical data correspond to the biochemical data of hearts used in the immunodetection experiments.
[c] OA, okadaic acid.

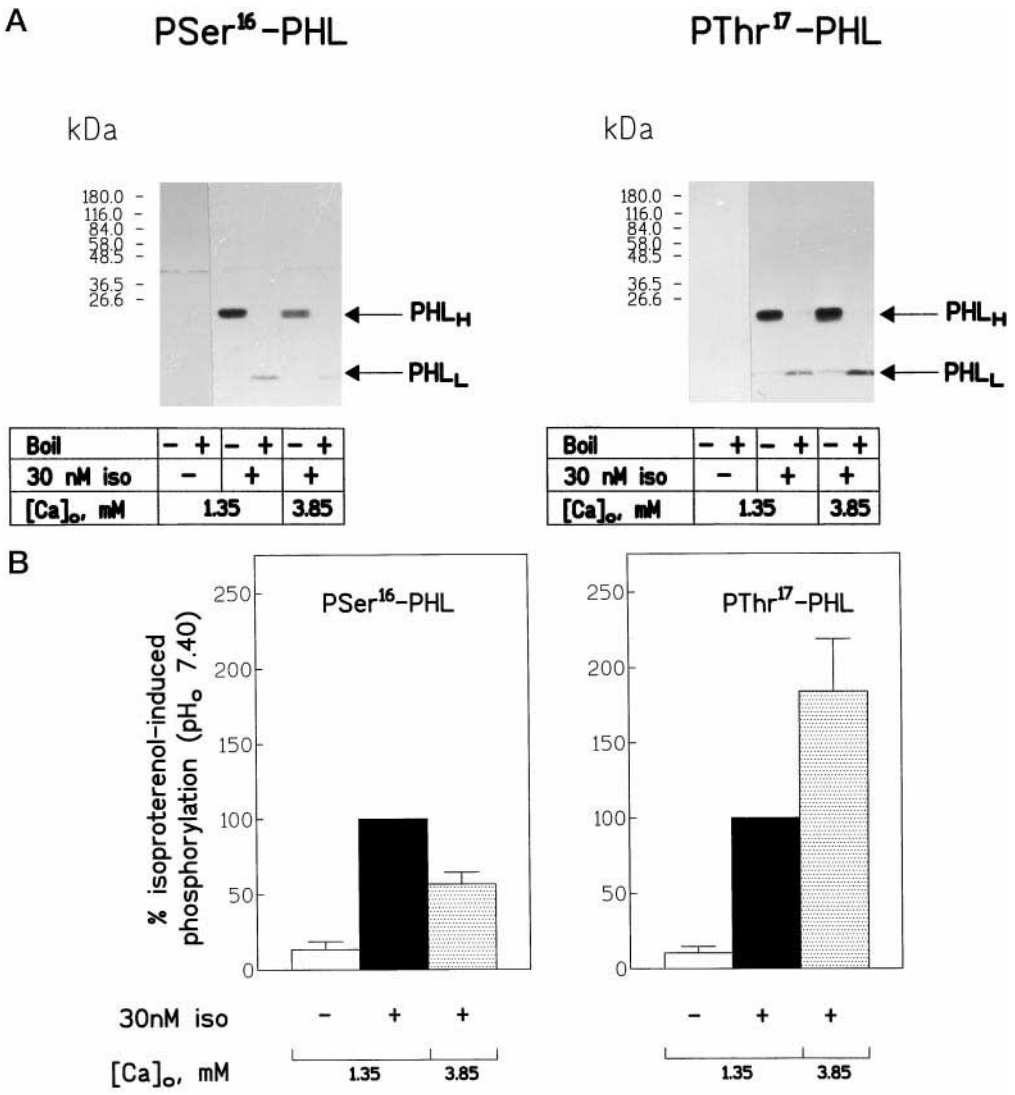

FIG. 3. *A*, immunoblots of SR membrane vesicles isolated from hearts perfused without and with 30 nM isoproterenol (*iso*) at $[Ca]_o$ = 1.35 or 3.85 mM. All experiments were done at pH_o 7.40. 10 μg of SR protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were probed as described for Fig. 1*B*. *B*, means ± S.E. obtained after densitometric analysis of the signal of 8–10 immunoblots. Results are expressed as a percentage of isoproterenol ($[Ca]_o$ = 1.35 mM)-induced site-specific phosphorylation. Increasing $[Ca]_o$ significantly enhanced isoproterenol-induced Thr^{17} phosphorylation. In contrast, increasing $[Ca]_o$ produced a significant decrease in isoproterenol-induced Ser^{16} phosphorylation. *$PSer^{16}$-PHL* and *$PThr^{17}$-PHL*, anti-Ser^{16} and anti-Thr^{17} PHL phosphopeptides, respectively; PHL_H and PHL_L, pentameric and monomeric forms of PHL, respectively.

a decrease in the calcium sensitivity of the myofilaments as suggested by previous results (16).

Does the Increase in $[Ca]_o$ Mimic the Acidosis Enhancement of Isoproterenol-induced Thr^{17} Phosphorylation?—Fig. 3*A* shows immunoblots of SR membrane vesicles obtained from hearts perfused in the absence and presence of isoproterenol at $[Ca]_o$ = 1.35 mM and in the presence of isoproterenol at $[Ca]_o$ = 3.85 mM. Fig. 3*B* shows the overall results of 10 different experiments of this type. Increasing $[Ca]_o$ in the presence of isoproterenol significantly increased phosphorylation of Thr^{17}. Unexpectedly, phosphorylation of Ser^{16} was significantly decreased. The opposite effects of increasing $[Ca]_o$ in the presence of isoproterenol on phosphorylation of phospholamban residues may explain the small effect of this maneuver on total phospholamban phosphorylation. As shown in Fig. 4, increasing $[Ca]_o$ from 1.35 to 3.85 mM evoked a small and nonsignificant increase in the phosphorylation of phospholamban induced by isoproterenol. Table I depicts the mechanical results of this experimental series. The increase in calcium supply to the cell in the presence of 30 nM isoproterenol did not modify the isoproterenol-induced increase in either contractility or relaxation. This result is consistent with the lack of effect of increasing $[Ca]_o$ in the presence of isoproterenol on total phospholamban phosphorylation as quantified by ^{32}P incorporation into phospholamban (Fig. 4).

The decrease in Ser^{16} phosphorylation produced by increasing $[Ca]_o$ in the presence of isoproterenol did not occur during acidosis. This result suggests that other mechanisms triggered by the acidosis may be playing a role. One of these putative mechanisms is the acidosis-induced inhibition of phosphatases, which might be offsetting the effect of an increase in $[Ca^{2+}]_i$ on Ser^{16} phosphorylation. In the next two sections, we will therefore explore the possible role of phosphatases in the acidosis enhancement of isoproterenol-induced phospholamban phosphorylation.

Does the Inhibition of Phosphatases Mimic the Acidosis Enhancement of Isoproterenol-induced Thr^{17} Phosphorylation?—Fig. 5*A* shows immunoblots of SR membrane vesicles obtained from hearts perfused at two different isoproterenol concentrations, 3 and 30 nM (pH_o 7.40), in the absence and presence of 1

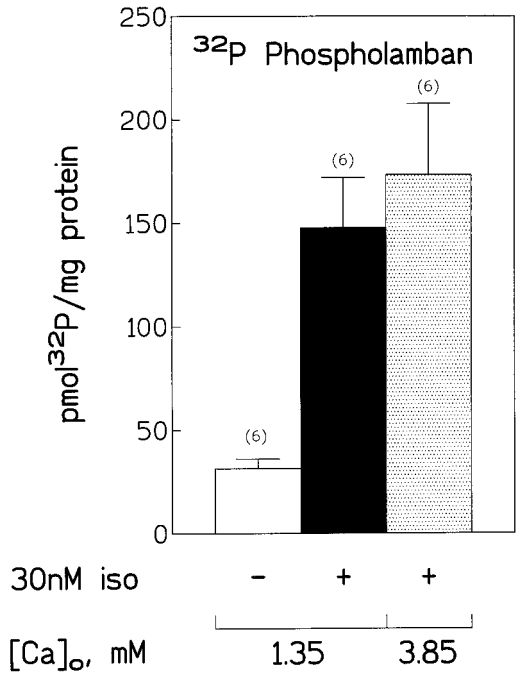

FIG. 4. **Overall results of ^{32}P incorporation into phospholamban under the same conditions described for Fig. 3.** The increase in $[Ca]_o$ failed to significantly enhance phospholamban phosphorylation above the level achieved with isoproterenol at $[Ca]_o$ = 1.35 mM. The number of experiments in each treatment group is indicated in *parentheses*.

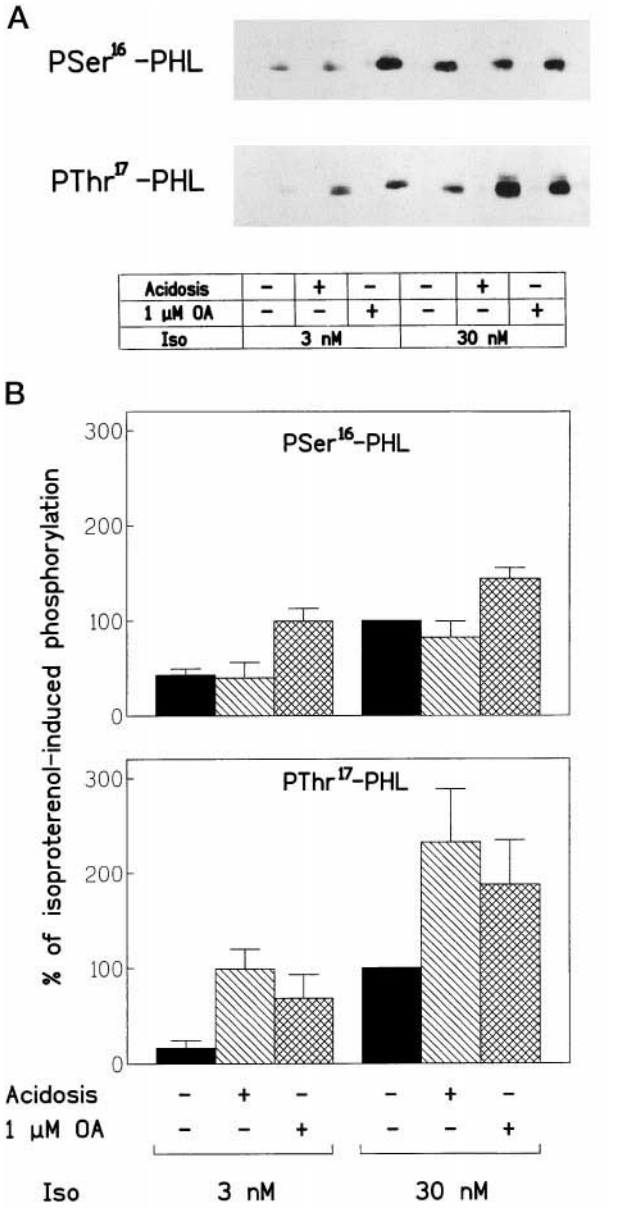

FIG. 5. *A*, immunoblots of SR membrane vesicles isolated from rat hearts perfused at two different isoproterenol (*Iso*) concentrations, 3 and 30 nM, at pH_o 7.40 in the absence and presence of 1 μM okadaic acid (*OA*). To compare the effects of okadaic acid with those of acidosis, SR membranes obtained from hearts perfused with 3 and 30 nM isoproterenol at pH_o 6.80 were run in parallel. Blots were probed as described for Fig. 1*B*. *B*, overall results obtained after densitometric analysis of the signal of three to six immunoblots. Results are expressed as a percentage as described for Fig. 2. Okadaic acid enhanced the isoproterenol-induced increase in phosphorylation of both Ser^{16} or Thr^{17} at the two isoproterenol concentrations tested. This finding revealed that even at the higher isoproterenol concentration, phosphatases are not maximally inhibited and therefore can be inhibited by acidosis. As already shown in Figs. 1*B* and 2, acidosis enhanced only isoproterenol-induced Thr^{17} phosphorylation without affecting Ser^{16} phosphorylation. *$PSer^{16}$-PHL* and *$PThr^{17}$-PHL*, anti-Ser^{16} and anti-Thr^{17} PHL phosphopeptides, respectively.

μM okadaic acid, a PP1 inhibitor. To compare the effects of okadaic acid with those of acidosis, SR membranes obtained from hearts perfused at 3 and 30 nM isoproterenol at pH_o 6.80 were run in parallel. As already shown in Fig. 2, acidosis enhanced only isoproterenol-induced Thr^{17} phosphorylation without affecting Ser^{16} phosphorylation. In contrast, okadaic acid enhanced the isoproterenol-induced phosphorylation of both Ser^{16} and Thr^{17} at the two isoproterenol concentrations. Fig. 5*B* shows the mean values obtained by optic densitometric analysis of the different experiments of this series. These findings revealed that the sole inhibition of phosphatases by okadaic acid has a different effect on phosphorylation of Ser^{16} than that produced by acidosis. They also showed that even at the higher isoproterenol concentration used, which produced the maximum phosphorylation of phospholamban at pH_o 7.40 (5), phosphatases are not maximally inhibited. Evidence for an enhancement of the isoproterenol-induced increase in phospholamban phosphorylation produced by okadaic acid has been previously reported in isolated myocytes (10). The present results further showed that this increase in phospholamban phosphorylation is due to the simultaneous increase in the phosphorylation of both Thr^{17} and Ser^{16} of phospholamban.

Table I shows the effects of okadaic acid in the presence of isoproterenol on the mechanical parameters of contraction and relaxation. At both isoproterenol concentrations, okadaic acid produced a further decrease in the isoproterenol-induced decrease of $t_{½}$. This reduction attained significant levels at 3 nM isoproterenol. Okadaic acid also significantly increased the positive inotropic effect of this isoproterenol concentration without affecting the effect of 30 nM isoproterenol on contractility.

Does Acidosis Increase Phospholamban Phosphorylation in the Absence of Isoproterenol?—The rationale behind this group of experiments was to study the effects of stimulating the CaMKII pathway of phospholamban phosphorylation in the absence of the inhibition of phosphatases produced by cAMP-dependent mechanisms (7–11). Previous experiments have shown that in the absence of isoproterenol, the increase in $[Ca]_o$ did not phosphorylate phospholamban unless the phosphatases were inhibited. This was so even when the increase in contractility (and therefore cytosolic calcium) was similar to that evoked by isoproterenol or more prolonged as during tetani (5, 12–15). Thus, any increase in phospholamban phosphorylation produced by increasing $[Ca]_o$ at low pH_o would strongly suggest a significant role of the acidosis-induced inhibition of phos-

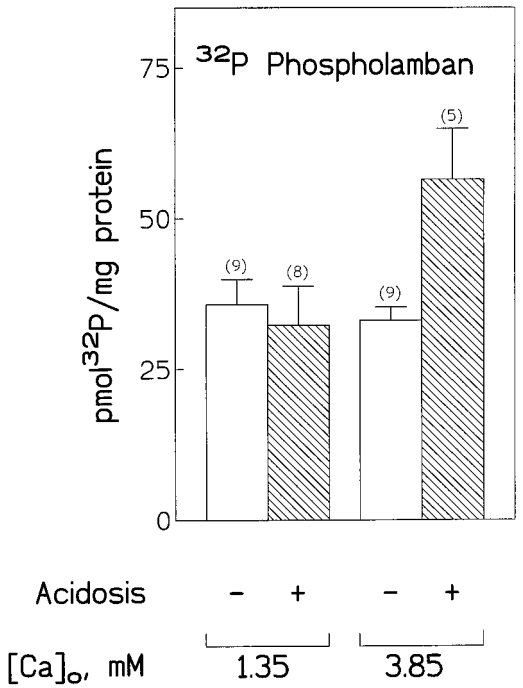

FIG. 6. **Overall results of ^{32}P incorporation into PHL in SR membrane vesicles isolated from rat hearts perfused with ^{32}P and then with Ringer's solution containing 1.35 mM $[Ca]_o$ at pH_o 7.40 or 6.80 or 3.85 mM $[Ca]_o$ at pH_o 7.40 or 6.80.** Acidosis failed to phosphorylate PHL at $[Ca]_o$ = 1.35 mM, but increased PHL phosphorylation at high $[Ca]_o$.

phatases in this effect. Fig. 6 shows the overall results of the experiments performed in SR membrane vesicles from hearts perfused with ^{32}P and then at low and high $[Ca]_o$ at pH_o 7.40 and 6.80. The increase in $[Ca]_o$ significantly increased phosphorylation of phospholamban only under acidotic conditions. Fig. 7*A* shows immunoblots of SR membrane vesicles isolated from hearts perfused as described for Fig. 6, except that ^{32}P perfusion was omitted. Immunological detection of the two phosphorylation sites of phospholamban showed that the increase in phospholamban phosphorylation observed in Fig. 6 was exclusively due to the phosphorylation of Thr^{17}. The overall results of this series are shown in Fig. 7*B*. Table I shows the mechanical results of this experimental series. Acidosis produced a significant decrease in $+\dot{P}$ and $t_{½}$ at $[Ca]_o$ = 1.35 mM that was not associated with any significant increase in phospholamban phosphorylation. At $[Ca]_o$ = 3.85 mM, acidosis produced a decrease in $+\dot{P}$ similar to that produced at low $[Ca]_o$ and a significant decrease in $t_{½}$ that occurred in association with the increase in Thr^{17} phosphorylation.

Contribution of Acidosis-induced Thr^{17} Phosphorylation to the Relaxant Effect of Acidosis—As shown in Table I, the acidosis-induced relaxant effect at high $[Ca]_o$ in the absence of isoproterenol was not greater than at $[Ca]_o$ = 1.35 mM, as would be expected from the significant increase in Thr^{17} phosphorylation observed at high $[Ca]_o$. Similarly, the decrease in $t_{½}$ induced by acidosis in the presence of isoproterenol was not higher than that produced by acidosis in the absence of the β-agonist. To further explore this point, additional experiments were performed in which the effect of acidosis on Thr^{17} phosphorylation and $t_{½}$ was studied at high $[Ca]_o$ in the presence and absence of a 10 μM concentration of the CaMKII inhibitor KN-62. In these experiments, acidosis produced an increase in Thr^{17} phosphorylation (expressed as percent of the phosphorylation of Thr^{17} at 30 nM isoproterenol, run in parallel) from 24.6 ± 8.3 to 82.4 ± 19.8%, which returned to control levels (28.6 ± 10.9%) in the presence of 10 μM KN-62 (n = 5). These changes in Thr^{17} phosphorylation were paralleled by changes in $t_{½}$. The corresponding $t_{½}$ values were 65.3 ± 4.1, 55.3 ± 4.4, and 66.0 ± 4.9 ms at high $[Ca]_o$, high $[Ca]_o$ + acidosis, and high $[Ca]_o$ + acidosis + KN-62, respectively. These results indicated that the increase in Thr^{17} phosphorylation evoked by acidosis was closely associated with a relaxant effect. It is possible that at high $[Ca^{2+}]_i$ levels, as should occur at high $[Ca]_o$ and in the presence of isoproterenol, the tension developed by the contractile proteins may be close to or at the "plateau" of the $[Ca^{2+}]_i$-tension curve. Under these conditions, the acidosis-induced decrease in myofilament calcium sensitivity and the resultant relaxant effect might be minimized. This effect would be further minimized in the presence of isoproterenol by the decrease in myofilament calcium sensitivity produced by the β-agonist.

DISCUSSION

Phosphorylation site-specific antibodies have proven to be highly specific in the discrimination between the two sites of phosphorylation of phospholamban since no cross-reactivity with the other site of phosphorylation was observed (5). The combination of this technique with the quantification of ^{32}P incorporation into phospholamban along with simultaneous measurements of mechanical parameters constitute invaluable tools to characterize the underlying mechanisms of phospholamban phosphorylation and their regulation.

Isoproterenol-induced phospholamban phosphorylation has been shown to be dependent upon the acid-base status of the myocardium, with acidosis enhancing the increase in phospholamban phosphorylation produced by the β-adrenergic agonist (Ref. 16 and our results in Fig. 1*A*). The relevant findings of this study were that the increase in the isoproterenol-induced phospholamban phosphorylation produced by acidosis was exclusively due to an increase in phosphorylation of Thr^{17} of phospholamban (Figs. 1*B* and 2) and that this increase would contribute to the enhancement of the relaxant effect of isoproterenol evoked by acidosis (Table I). The results also provided evidence that an activation of CaMKII and an inhibition of phosphatases may have both played a significant role in the observed increase in Thr^{17} phosphorylation produced by acidosis in the presence of isoproterenol.

Phosphorylation of phospholamban depends on a basic mechanism that is common to any phosphorylation process, *i.e.* the relative activities of kinases and phosphatases that phosphorylate and dephosphorylate the protein, respectively. β-Adrenoreceptor stimulation increases phospholamban phosphorylation by increasing the phosphorylation of both Ser^{16} and Thr^{17} (4, 5). Different types of evidence further indicate that this dual phosphorylation occurs because isoproterenol not only increases PKA activity, but as a consequence of PKA activation, also produces a simultaneous activation of CaMKII (by increasing $[Ca^{2+}]_i$) and inhibition of PP1, the major phosphatase that dephosphorylates phospholamban (6–11). In this context and in the search for the mechanisms underlying the enhancement of the isoproterenol-induced phospholamban phosphorylation produced by acidosis, two possibilities should be necessarily explored: 1) acidosis produces a further increase in CaMKII activity, and/or 2) acidosis evokes a further inhibition of PP1. Both possibilities have some experimental support (16–18). First, it has been shown in several species that hypercapnic acidosis increases $[Ca^{2+}]_i$ more in the presence than in the absence of isoproterenol (17, 18). This increase in $[Ca^{2+}]_i$ may add to the increase in $[Ca^{2+}]_i$ produced by isoproterenol to further activate CaMKII. Second, it has also been shown that acidosis inhibits PP1. A decrease in pH_o from 7.40 to 6.80 produced by increasing the CO_2 of the gas mixture from 5 to

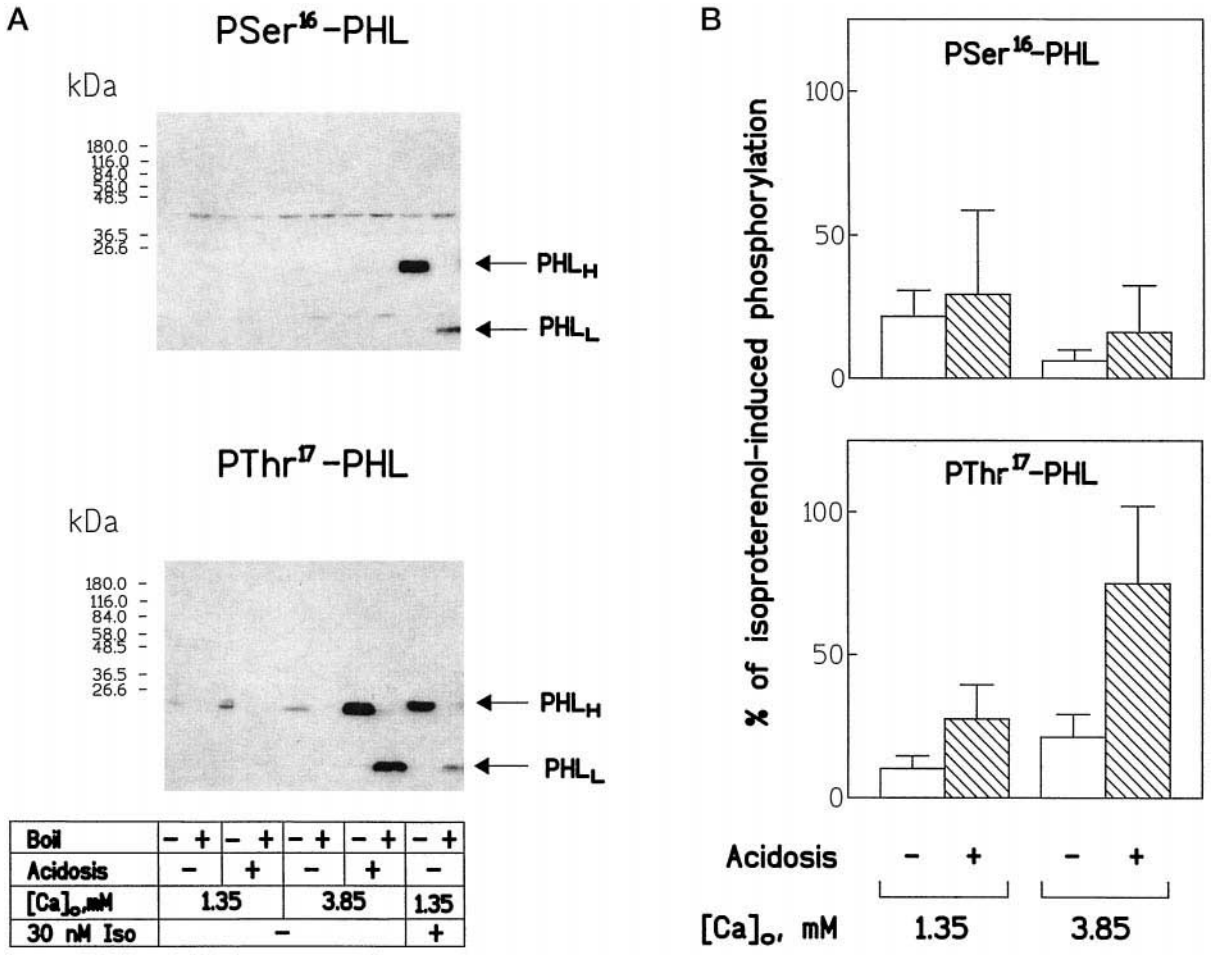

FIG. 7. *A*, immunoblots of SR membrane vesicles isolated from rat hearts perfused as described for Fig. 6. Site-specific phosphorylation produced by 30 nM isoproterenol (*Iso*) is shown for comparison. *B*, overall results obtained after densitometric analysis of the signal of three immunoblots. At $[Ca]_o$ = 1.35 mM, acidosis failed to significantly increase either Ser^{16} or Thr^{17} phosphorylation. At $[Ca]_o$ = 3.85 mM, acidosis did not affect Ser^{16} phosphorylation, but produced an evident enhancement of Thr^{17} phosphorylation, which would account for the increase in total PHL phosphorylation observed in Fig. 6. *$PSer^{16}$-PHL* and *$PThr^{17}$-PHL*, anti-Ser^{16} and anti-Thr^{17} PHL phosphopeptides, respectively; PHL_H and PHL_L, pentameric and monomeric forms of PHL, respectively.

20% (external bicarbonate of 18.5 mM) has been reported to produce a decrease in pH_i from 7.14 to ~6.70 in isolated cat and guinea pig myocytes (23). Similar results were obtained by us in experiments performed in rat isolated myocytes under the same conditions described in the present experiments. In these experiments, the pH_i decreased from 7.18 ± 0.06 to 6.73 ± 0.04.[2] In this pH_i range, we have previously shown in *in vitro* experiments that PP1 was inhibited by acidosis by ~30% (16).

Previous results have shown that the increase in $[Ca]_o$ produced an increase in Thr^{17} phosphorylation only when the phosphatases were inhibited as in the presence of okadaic acid (5). In the present experiments, acidosis produced effects similar to those observed with okadaic acid: the activation of CaMKII by increasing $[Ca]_o$ in the absence of isoproterenol evoked an increase in Thr^{17} phosphorylation only under acidotic conditions (Fig. 7). This finding indicates that the acidosis-induced inhibition of phosphatases played a significant role in the increase in Thr^{17} phosphorylation observed. Moreover, the increase in Thr^{17} phosphorylation was closely paralleled by a relaxant effect. The fact that, at $[Ca]_o$ = 1.35 mM, acidosis did not produce any significant change in either Ser^{16} or Thr^{17} means that for the level of phosphatase inhibition produced by acidosis, the activity of kinases was too low to increase the hosphorylation of the corresponding residues.

The acidosis enhancement of isoproterenol-induced Thr^{17} phosphorylation could be mimicked by either increasing $[Ca]_o$ (Fig. 3) or adding okadaic acid (Fig. 5). These findings indicated that the CaMKII cascade can be activated above the level achieved by stimulation with isoproterenol and that even at the higher isoproterenol concentration used, phosphatases are not maximally inhibited by cAMP and therefore can be further inhibited by acidosis. However, neither the increase in $[Ca]_o$ nor the presence of okadaic acid mimicked the lack of effect of acidosis on Ser^{16}. Whereas okadaic acid significantly enhanced the isoproterenol-induced phosphorylation of Ser^{16}, the increase in $[Ca]_o$ significantly decreased it. The results obtained on phosphorylation of Ser^{16} under the different experimental situations are therefore consistent with the hypothesis that acidosis was acting by two different mechanisms, *i.e.* the increase in $[Ca^{2+}]_i$ and the inhibition of phosphatases, both of which contribute to the increase in Thr^{17} phosphorylation, but have opposite effects on Ser^{16} phosphorylation.

The decrease in Ser^{16} phosphorylation when $[Ca]_o$ was increased in the presence of isoproterenol (Fig. 3) was an unexpected finding that deserves some additional comments. 1) The result is in line with the fact that the increase in $[Ca]_o$ in the presence of the β-agonist failed to significantly increase total

[2] L. Vittone, C. Mundiña-Weilenmann, M. Said, and A. Mattiazzi, unpublished results.

phospholamban phosphorylation (Fig. 4) and myocardial relaxation (Table I) above the levels attained by β-adrenoreceptor stimulation. 2) As discussed above, the result is also consistent with the idea that both the activation of CaMKII and the inhibition of phosphatases have contributed to the enhancement of the isoproterenol-induced Thr^{17} phosphorylation produced by acidosis. The cause for this decrease in Ser^{16} phosphorylation is not apparent to us. The inhibition of type V adenylyl cyclase, the major adenylyl cyclase isoform present in adult ventricle (24), by submicromolar concentrations of calcium has been previously described (25). However, this possibility is not supported by previous experiments in our laboratory showing that the increase in $[Ca]_o$ did not affect intracellular cAMP levels (14). Another possible clue to explain the above findings can be found in the mechanisms regulating PP1 activity. SR-associated PP1 could be inhibited by direct PKA-dependent phosphorylation of the PP1 regulatory subunit and PKA-dependent phosphorylation of inhibitor-1, which in the phosphorylated state is a potent inhibitor of the catalytic subunit of PP1 (8). In turn, the PP1 regulatory subunit and inhibitor-1 are dephosphorylated by two other phosphatases, PP2A and PP2B (8). Since PP2B is activated by calcium and calmodulin, the increase in $[Ca^{2+}]_i$ is a potential mechanism by which PP1 can be activated (8). As a consequence, the inhibitory effect of PKA on PP1 would be attenuated. This phosphatase regulatory cascade might explain the decrease in the phosphorylation of Ser^{16} when $[Ca^{2+}]_i$ was increased.

This decrease may not occur in acidosis if it is overrided by acidosis-induced phosphatase inhibition. The decrease in isoproterenol-induced Ser^{16} phosphorylation by increasing $[Ca]_o$ (Fig. 3) may therefore be the expression of a mechanism by which the increase in $[Ca^{2+}]_i$, acting through a protein phosphatase cascade, attenuates the signals that act via cAMP (8, 26).

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