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Phosphorylation of Phospholamban Correlates With Relaxation of Coronary Artery Induced by Nitric Oxide, Adenosine, and Prostacyclin in the Pig

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Abstract The intracellular mechanisms underlying the action of the endogenous vasodilators such as NO/EDRF, adenosine, and prostacyclin acting through cGMP and cAMP, respectively, are not well understood. One important action of cyclic nucleotides in smooth muscle relaxation is to lower the cytosolic Ca^{2+} concentration by enhanced sequestration into the sarcoplasmic reticulum. The present study was undertaken to elucidate the potential role of phosphorylation of phospholamban, the regulator of sarcoplasmic reticulum Ca^{2+} pump, for the control of coronary vascular tone by NO/EDRF, adenosine, and prostacyclin. Phospholamban was identified in pig coronary artery preparations by immunofluorescence microscopy, Western blotting and in vitro phosphorylation. Segments of pig coronary artery, with either intact or denuded endothelium, were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In endothelium-denuded preparations 3-morpholinosydnonimine (SIN-1), 5′-N-ethylcarboxiamidoadenosine (NECA), and iloprost (ILO) caused both relaxation and phospholamban phosphorylation with the potency: SIN-1 > NECA > ILO. The regulatory myosin light chain was significantly dephosphorylated only by SIN-1. In endothelium-intact pig coronary artery, L-NAME caused additional vasoconstriction and a decrease in phospholamban phosphorylation, while phosphorylation of myosin light chain remained unchanged. An inverse relationship between phospholamban phosphorylation and vessel tone was obtained. Our findings demonstrate significant phospholamban phosphorylation during coronary artery relaxation evoked by NO, prostacyclin, and adenosine receptor activation. Because of the close correlation between phosphorylation of phospholamban and vessel relaxation, we propose that phospholamban phosphorylation is an important mechanism by which endogenous vasodilators, especially endothelial NO/EDRF, control coronary vascular smooth muscle tone. J. Cell. Biochem. 70:49–59, 1998.

Key words: coronary artery; NO/EDRF; adenosine; prostacyclin; phospholamban; myosin light chain

Endogenous vasodilators such as endothelial NO/EDRF, adenosine, and prostacyclin elevate either cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP) in vascular smooth muscle cells, and both cyclic nucleotides are thought to be causally involved in mediating smooth muscle relaxation. The intracellular mechnisms underlying cyclic nucleotide-dependent relaxation are only poorly understood. In smooth muscle preparations, in vitro inactivation of myosin light chain kinase by phosphorylation by cAMP-dependent protein kinase was observed [Conti and Adelstein, 1981]. The physiological significance of this effect, however, has not been demonstrated under in vivo conditions. Dephosphorylation of the regulatory myosin light chain is thought to be an important mechanism but does not always correlate with, or is not sufficient to fully explain, smooth muscle relaxation [Gerthoffer et al., 1984; Miller et al., 1983; Katoch et al., 1997]. Relaxation of vascular smooth muscle critically depends on the removal of Ca^{2+} ions from the cytosol. It is now believed that, in smooth muscle cells, the major action of cyclic nucleotides is the reduction of cytosolic Ca^{2+} [Walsh et al., 1995]. This is achieved in part by sequestration into the sarcoplasmic reticulum. The sarcoplasmic reticulum Ca^{2+} uptake was found to be enhanced by cyclic nucleotides [Twort and van Bremen, 1988;

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Itoh et al., 1982] or their related protein kinases [Watras, 1988; Raeymakers et al., 1988,1990]. The molecular mechanism, however, by which cyclic nucleotides control the sarcoplasmic reticulum Ca^{2+} pump in vascular smooth muscle, remained unidentified until the presence of phospholamban was demonstrated [Raeymakers et al., 1986; Ferguson et al.,1988; Eggermont et al., 1990]. Recently, the significance of phospholamban controlled sarcoplasmic reticulum Ca^{2+} sequestration for the contractility of vascular smooth muscle was shown in transgenic mice [Lalli et al., 1997].

In the myocardium, the small pentameric protein phospholamban, intrinsic to membranes of the sarcoplasmic reticulum, is crucial for the relaxant effects of catecholamines [Luo et al., 1994]. In the dephosphorylated state, it inhibits the sarcoplasmic reticulum Ca^{2+}-ATPase, and its phosphorylation relieves this inhibition [Kim et al., 1990]. Phospholamban has been identified in vitro and in vivo as a substrate for cAMP-dependent protein kinase and a Ca^{2+}/calmodulin-dependent protein kinase [Simmerman et al., 1986, Wegener et al., 1989]. Furthermore, it has been shown that cGMP-dependent protein kinase phosphorylates phospholamban at the same site and at about the same rate as cAMP-dependent protein kinase [Raeymakers et al., 1988]. Studies on particulate fractions and intact cells from cultivated vascular smooth muscle myocytes gave evidence that phospholamban is phosphorylated in response to sodium nitroprusside and ANF [Sarcevic et al., 1989; Cornwell et al., 1991]. First efforts to demonstrate phosphorylation of phospholamban in intact vascular smooth muscle did not yield clear results [Huggins et al., 1989]. Previously we provided first evidence that phospholamban is phosphorylated in the intact aorta by endothelium-derived NO/EDRF [Karczewski et al.,1992].

The present study was designed to gain further insight into the possible involvement of phospholamban phosphorylation in the mechanism by which endogenous vasodilators, such as NO/EDRF, adenosine, and prostacyclin, cause vascular smooth muscle relaxation. Our data suggest that phospholamban phosphorylation is an important mechanism involved in the modulation of coronary vascular tone.

MATERIALS AND METHODS

Experimental Model

Pig coronary arteries were freshly obtained from a local slaughterhouse. Vessels from 5- to 6-month-old animals of both sex were used. The right coronary artery was excised and immediatley rinsed with ice-cold Krebs–Henseleit solution containing NaCl 118 mM, $CaCl_2$ 2.5 mM, KCl 4.7 mM, $MgS0_4$ 1.2 mM, KH_2PO_4 1.2 mM, EDTA 0.5 mM, $NaHCO_3$ 25 mM, and glucose 11 mM, to remove blood and transported to the laboratory. Adherent fat tissue was removed, and the side branches of the coronary artery were ligated with threads of silk. All manipulations were performed at room temperature in Krebs–Henseleit solution gassed with carbogen. Pig coronary artery segments of about 5-cm length were used. Perfusion was performed at 37°C with Krebs–Henseleit solution gassed with carbogen using 2 ml/min flow against a hydrostatic pressure of 60-cm column heights. Each preparation was equilibrated for 25 min. Drugs (see under Protocol) were applied by infusion through a side arm with one-tenth the perfusion speed. Relative changes of the vessel diameter were monitored by the means of paired ultrasonic crystals [Schipke et al., 1984]. In some experiments, the endothelium was removed by perfusion with 0.75% desoxycholic acid for 90 s [Kroll et al., 1987]. The test for functionally intact endothelium was performed with substance P (Sigma, Deisenhofen, Germany). Pig coronary artery segments were precontracted with prostaglandin $F_{2\alpha}$ (PGF_{2_α}reset, Upjohn, Erlangen, Germany) with a maximal effective dose of 50 µM. Vasoconstriction induced by $PGF_{2\alpha}$ was about the same independently whether the endothelium was removed or left intact. At the maximum contraction, the preparation was either freeze-clamped (control group) or infused with different drugs at maximal effective concentrations, as outlined below. In the first set of experiments the endothelium was functionally removed as described above. Groups of precontracted coronary artery segements were infused with the following drugs: 10 µM of the NO-liberating 3-morpholinosydnonimine (SIN-1, Cassella, Frankfurt, Germany), 10 µM of the adenosine receptor agonist 5′-N-ethylcarboxiamidoadenosine (NECA, Serva, Heidelberg, Germany), and 10 µM of the prostacyclin derivative iloprost (Schering, Berlin, Germany). In a second set of experiments, the endothelium was left intact. Here groups of precontracted vessels were perfused with 100 µM of the inhibitor of NO-synthase L-nitroarginine methylester (L-NAME, Serva), 10 µM of the inhinbitor of prostaglandin synthesis indomethacin (Sigma), and 5 µM of the adenosine receptor antagonist 8-phenyltheophylline (Sigma). At the maximum re-

sponse (change in vessel diameter), the coronary artery segments were freeze-clamped. Changes in vessel diameter were expressed relative to the $PGF_{2\alpha}$-precontracted state. Frozen tissue samples were stored at −80°C until analyzed.

Immunohistochemistry of Phospholamban

Cryostat sections of 10-µm thickness from isolated pig coronary artery and from pig heart mounted on glass slides were fixed in phosphate-buffered saline (PBS) buffer (10 mM phosphate buffer, pH 7.4, 150 mM NaCl) containing 15% of a saturated aqueous solution of picric acid and 2% formaldehyde for 10 min and then washed sequentially with aqua bidest, PBS containing 50 mM glycine and PBS with 0.5% bovine serum albumin (BSA) and 0.2% gelatin (PBG). Sections were incubated overnight at 4°C with a mouse MAb against phospholamban diluted 1:500 (Biomol). After three washes with PBG, the slides were incubated with dichlorotriazynyl aminofluorescin (DTAF)-conjugated goat anti-mouse antibodies diluted 1:100 in PBG (Dianova, Hamburg, Germany) for 2 h at room temperature. After washing sections were examined by fluorescence and phase-contrast microscopy with appropriate filters (Zeiss Axioscop, Zeiss, Oberkochen, Germany). The following controls were used: (1) incubation with DTAF conjugates alone, (2) incubation with mouse nonimmune serum, and (3) incubation with the anti-phospholamban antibody that was prereacted with a synthetic peptide corresponding to the amino acids 2–25 of the phospholamban monomer (BioTez, Berlin, Germany).

Immunoblotting of Phospholamban

Microsomal proteins were separated by polyacrylamide gel electrophoresis (PAGE) through a sodium dodecyl sulfate (SDS)-urea polyacrylamide gel [Swank and Munkres, 1971] and electrotransferred to Hybond nitrocellulose membrane (Amersham Buchler). Phospholamban was identified by a monoclonal mouse anti-phospholamban antibody (Biomol, Hamburg, Germany) diluted 1:500. For detection of the antibody binding a second, peroxydase-conjugated, mouse IgG (Sigma), 1:1,000 diluted and the enhanced chemoluminescence assay (ECL-kit, Amersham Buchler, Braunschweig, Germany) was employed. After exposure to x-ray film (ORWO, Wolfen, Germany) the respective bands were quantitated by densitometry (PDI Imaging System, PDI, New York, NY).

Back-Phosphorylation of Phospholamban

To assess changes in phospholamban phosphorylation occurring in the intact vessel preparation, a technique based on the principle of back-phosphorylation was employed [Forn and Greengard, 1978]. This indirect assay technique, as originally described by us [Karczewski et al., 1990], consists of two principal, separate steps. The first is the isolation of subcellular fractions (crude microsomes) from intact tissue under specific conditions that preserve the phosphorylation state of phospholamban. The second step is the in vitro back-phosphorylation (topping-up) of the remaining unphosphorylated sites of phospholamban, using exogenous catalytic subunit of cAMP-dependent protein kinase.

Preparation of Microsomal Fractions

All manipulations were performed at 4°C. About 300 mg of frozen pig coronary artery was powdered in a mortar under liquid nitrogen. The powdered tissue was homogenized with a motor-driven Potter glass–glass homogenizer at 600 rpm for 3 times 5 strokes in 5 ml homogenization buffer of pH 7.4 containing 5 mM histidine/HCl, 750 mM KCl, 0.2 mM dithiothreitol, 0.1 mM phenylmethansulfonyl fluoride (PMSF), 50 mM Na_2HPO_4, 25 mM NaF, and 10 mM EDTA. The homogenate was centrifuged at 17,000*g* for 20 min. The supernatant was collected and the pellet re-homogenized as described above. This extraction procedure was routinely repeated twice. Preliminary experiments showed that a third rehomogenization of the pellet did not yield in detectable amounts of microsomal protein. Finally, the supernatants from the three spins were combined, filtered through glass wool, and centrifuged at 150,000*g* for 45 min. The pellet was washed with a buffer containing 10 mM histidine/HCl, 0.2 mM dithiothreitol, 0.1 mM PMSF, 50 mM Na_2HPO_4, 25 mM NaF, and 10 mM EDTA, at pH 7.4, and resuspended in 1.5 vol of 10 mM histidine/HCl, 200 mM sucrose, 50 mM Na_2HPO_4, 25 mM NaF, and 10 mM EDTA, at pH 7.4, frozen in liquid nitrogen, and stored at −80°C until use.

In Vitro Topping-Up Phosphorylation

The reaction mixture contained in a final volume of 100 µl 40 mM histidine–HCl (pH 6.8), 10 mM $MgCl_2$, 15 mM NaF, 1 mM EGTA and 0.1% Triton X-100, 100 µg BSA, 0.5 µM cata-

lytic subunit of cAMP-dependent protein kinase, and 75 µg of microsomal proteins. The reaction was started by addition of [γ-^{32}P] ATP (4,000 cpm/pmol, 25 µM final concentration). Phosphorylation of phospholamban was complete within 3 min and remained at plateau level for at least 8 min. The reaction was linear with respect to increasing amounts of microsomal protein, and maximal phosphate incorporation did not change with increasing concentrations of the catalytic subunit of cAMP-dependent protein kinase. This finding indicates that neither ATP concentration became limiting nor did significant dephosphorylation occur under these conditions. Routinely, incubation was performed for 5 min. The phosphorylation reaction was stopped by the addition of 3 ml of 15% trichloroacetic acid (TCA) containing 50 mM H_3PO_4 and 0.5 mM ATP. Precipitates were sedimented by centrifugation, dissolved in sample buffer and electrophoresed through SDS-urea polyacrylamide gels [Karczewski et al., 1990]. The dried gels and filter paper strips with spots of [γ-^{32}P]ATP standard dilutions were exposed to x-ray films (ORWO). Autoradiograms were scanned on a PDI Imaging system (PDI), and the phosphorylation of phospholamban was calculated from the optical densities of the respective protein bands and the spots of radioactive standards. Back-phosphorylation of phospholamban is expressed as pmoles ^{32}P per mg of crude microsomal protein applied to each gel lane (pmol ^{32}P/mg protein).

Phosphorylation of Myosin Light Chain

Pieces of freeze-clamped vessels were powdered under liquid nitrogen mixed with 10% trichloro acetic acid (TCA). The liquid nitrogen–TCA tissue powder was thawed on ice. The denatured proteins were sedimented by centrifugation (5,000*g* for 10 min). The protein pellet was washed with a mixture of ether/ethanol (8:2 v/v) to remove TCA, overlayed with N_2 gas and stored at −20°C until use. Phosphorylation of myosin light chain was analyzed in these pellets by two-dimensional PAGE [Morano et al., 1993]. Isoelectric focusing in the first dimension was performed in glass capillaries using a pH gradient of 4.5–5.4. The second dimension was SDS slab gel electrophoresis [Laemmli and Favre, 1973]. The gels were silver-stained and the respective spots quantitated by densitometry. Phosphorylation of myosin light chain is expressed as arbitrary units of the optical densities of the phosphorylated form relative to the total amount.

Other Methods

The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart [Peters et al., 1977]. The holoenzyme of cGMP-dependent protein kinase was purified from bovine lung [Walter et al., 1980]. Protein was measured according to [Lowry et al., 1951] with BSA as the standard.

Statistical Analysis

Data are shown as mean ±SEM, where n refers to the number of individual vessel preparations. Statistical comparisons were performed by the Student's t-test after the data had been checked for normal distribution. Values of $P > 0.05$ were considered to indicate statistically significant differences between the experimental groups.

RESULTS

Phospholamban in Pig Coronary Artery

Because expression of phospholamban differs dramatically between various smooth muscle types [Eggermont et al., 1990], our first aim was to identify phospholamban in pig coronary artery. Light micrographs of sections from pig heart (Fig. 1A) and from an isolated coronary artery segment of pig myocardium (Fig. 1B) as phase contrast (Fig. 1A1, B1) and immunofluorescence of the same section areas reacted with a MAb against phospholamban (Fig. 1A2, B2). Intensive staining for phospholamban was observed in pig heart cardiomyocytes and much weaker staining in the artery (Fig. 1A2). This labeling proved to be specific, as the immunofluoresence was completely quenched with a synthetic peptide corresponding to the amino acids 2–25 of the phospholamban monomer (Fig. 1A3). The surrounding connective tissue of the coronary artery is devoid of any labeling. Note, that the signal is only detectable in the smooth muscle layer, whereas there is no reaction in the adventitia of the vessel (Fig. 1B2). As can be seen at higher magnification, fluorescence is clearly localized with smooth muscle cells (Fig. 1B3). After electrophoretic separation of microsomal membrane proteins from coronary artery and cardiac tissue, phospholamban was clearly detected in both preparations by Western blotting (Fig. 2). Preliminary experiments showed

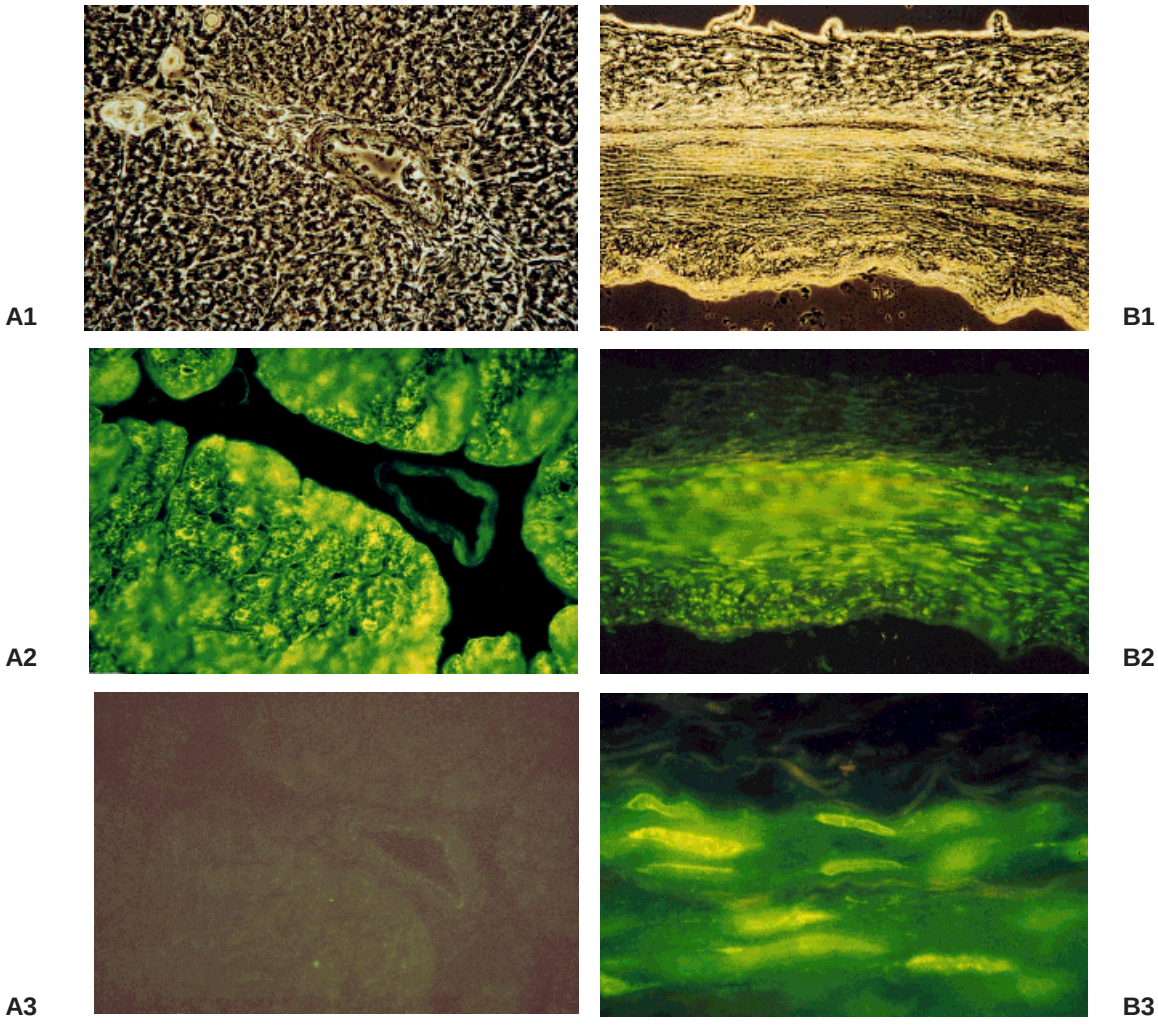

Fig. 1. Light micrographs of sections through pig ventricular myocardium **(A)** and isolated pig coronary artery **(B)**. See under Materials and Methods for experimental details. A1 B1, phase-contrast micrographs; A2 B2, the same section as in A1 B1 treated with a specific monoclonal antibody directed against phospholamban and visualized by a secondary fluorescence-labeled antibody; A3, the same as A1, but with preincubating the section with a 24-amino acid synthetic peptide corresponding to the cytosolic part of phospholamban before reacting it with the monoclonal phospholamban antibody; B3, section of the medial muscle layer taken from B2 at a higher magnification. A1–A3: ×100; B1–B2: ×50; B3: ×240.

no detectable phosphorylation of phospholamban in cardiac as well as vascular smooth muscle microsomes, unless purified cAMP-dependent or cGMP-dependent protein kinase was added (data not shown). Exogenous cAMP-dependent and cGMP-dependent protein kinase phosphorylated phospholamban in cardiac and coronary artery microsomal preparations (Fig. 3). It is important to note that cAMP-dependent protein kinase produced a higher radioactive background as compared with cGMP-dependent protein kinase. However, quantitation revealed the same values of phospholamban phosphorylation for both protein kinases. Furthermore, there was no additivity when both protein kinases were added simultaneously. In this experiment, the average phosphate incorporation for coronary artery and cardiac microsomes was 1.07 ± 0.02 pmol and 74.0 ± 3.8 pmol ^{32}P/mg protein ($n = 3$), respectively. This finding is consistent with the view that both exogenous protein kinases phosphorylate phospholamban at the same site [Raeymakers et al., 1988].

Organ Bath Experiments

SIN-1 caused a rapid and nearly complete relaxation within about 200 s after the onset of dilation in endothelium-denuded preparation. From data for endothelium-denuded preparations summarized in Figure 4A, it is evident

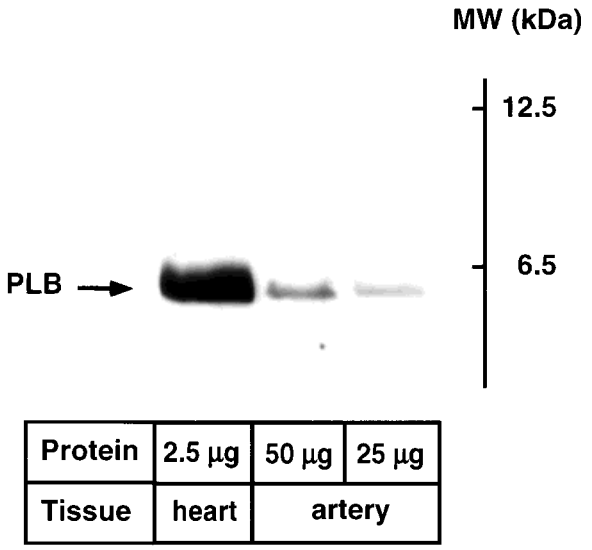

Fig. 2. Western blot analysis of phospholamban in crude microsomal fractions from rabbit heart and pig coronary artery. Microsomal protein (µg as indicated) was separated by SDS-PAGE, electrotransferred onto nitrocellulose, and reacted with a specific monoclonal antibody against phospholamban as described under Methods section. The immunoreaction was visualized by the enhanced chemoluminescence kit. Before application to the gel, the samples were boiled for 5 min, so that phospholamban migrates exclusively as the 5.5-kDa monomer.

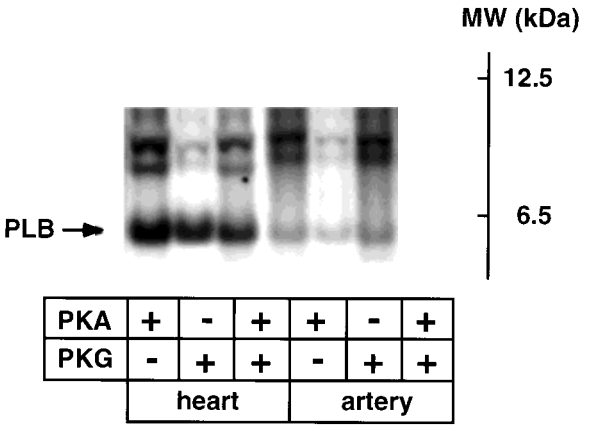

Fig. 3. Autoradiogram of ^{32}P incorporation into phospholamban in microsomal fractions from rabbit heart and pig coronary artery by equimolar concentrations (0.5 µM) of exogenous cyclic AMP-dependent protein kinase, exogenous cyclic GMP-dependent protein kinase and by simultaneous application of both. Either 20 µg of cardiac microsomes or 100 µg of microsomal protein from pig coronary artery preparations was applied to gel lanes. The samples were boiled before electrophoresis, which converts phospholamban completely to the monomeric form migrating at about 5.5 kDa. For details, see Materials and Methods.

that SIN-1 relaxed the vessel by 98 ± 1 % (n = 5) and that the effect of NECA with 95 ± 3% (n = 5) was comparable to that evoked by SIN-1. Iloprost was found to be less efficient in relaxing pig coronary arteries. Only a partial relaxation of $PGF_{2\alpha}$-precontracted vessels of 60 ± 7 % (n = 4) was observed.

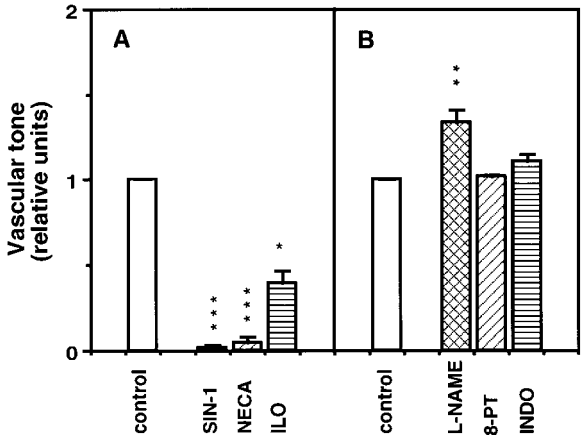

Fig. 4. Bar graphs showing the effect of various agonists and antagonists on the tone of 50 µM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted pig coronary artery (pig coronary artery) segments. See Materials and Methods for experimental details. The changes in vessel diameter are given relative to the $PGF_{2\alpha}$ -precontracted state of the same preparation (set to 1) and referred to as control. **A:** Relaxation of endothelium-denuded, precontracted pig coronary artery segments by 10 µM 3-morpholinosydnonimine (SIN-1), 10 µM 5′-N-ethylcarboxiamidoadenosine (NECA), and 10 µM iloprost (ILO). **B:** Effect of inhibitors of endogenous vasodilators 100 µM L-nitroarginine-methylester (L-NAME), 10 µM indomethacin (INDO), and 5 µM 8-phenyltheophylline (8-PT) on the tone of endothelium-intact, precontracted pig coronary artery preparations. Data for drug-treated groups of vessel segments are presented as mean values +SEM. The number of individual pig coronary artery preparations was 4–5 in each experimental group. Statistically significant differences to controls are depicted as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

In pig coronary artery preparations with intact endothelium, L-NAME significantly ($P < 0.01$) increased vascular tone by 34 ± 7 % (n = 5) (Fig. 4B). Indomethacin also slightly increased vascular tone, which, however, did not reach level of significance ($P = 0.051$), whereas 8-phenyltheophylline had no effect on vascular tone.

Back-Phosphorylation of Phospholamban

The in vitro back-phosphorylation of phospholamban in microsomal membrane preparations from endothelium-denuded, $PGF_{2\alpha}$-contracted pig coronary artery segments was found to be highly reproducible for all groups of experiments performed (1.32 ± 0.05 pmol ^{32}P/mg protein, n = 13). In vitro back-phosphorylation of phospholamban showed a reduced incorporation of ^{32}P in preparations from vessels relaxed with SIN-1 compared with precontracted controls (Fig. 5A). The quantitative analysis of the

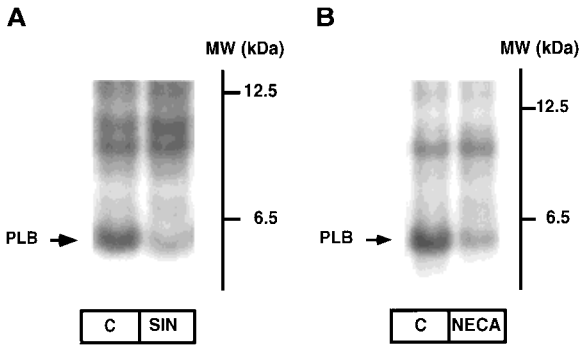

Fig. 5. Autoradiograph of back-phosphorylation of microsomal fractions prepared from 50 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-precontracted pig coronary artery segments freeze-clamped at the maximum of contraction as controls (C) or at the maximum of relaxation induced by subsequent treatment with 10 μM 3-morpholinosydnonimine (SIN-1) **(A)** or 10 μM 5′-N-ethylcarboxiamidoadenosine (NECA) **(B).** See Materials and Methods for details.

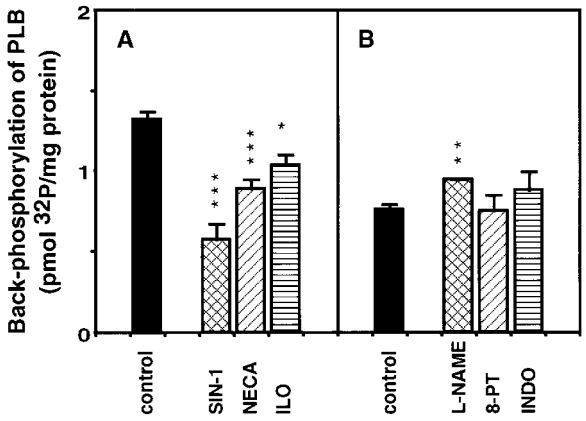

Fig. 6. Bar graph showing the quantitated data for back-phosphorylation of microsomal fractions from pig coronary arteries precontracted with 50 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Untreated controls were freeze-clamped at the maximum of tension development. **A:** Endothelium-denuded vessel preparations were relaxed by 10 μM 3-morpholinosydnonimine (SIN-1), 10 μM 5′-N-ethylcarboxiamidoadenosine (NECA), or 10 μM iloprost (ILO). **B:** Intact endothelium preparations were perfused with 100 μM L-nitroarginine-methylester (L-NAME), 10 μM indomethacin (INDO), or 5 μM 8-phenyltheophylline (8-PT). See Materials and Methods for details of the experimental procedure. Data are presented as mean values +SEM and were obtained from 12–13 individual vessel prepaprations for controls and from 4–5 individual artery segments for experimental groups treated with drugs. Statistically significant differences to controls are depicted as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

in vitro topping-up phosphorylation yielded a significant difference in ^{32}P incorporation in phospholamban between these two experimental groups (1.32 ± 0.05 pmol ^{32}P/mg protein for $PGF_{2\alpha}$ controls versus 0.58 ± 0.09 for SIN-1-treated vessels, n = 4, $P < 0.001$) (Fig. 6A). From these differences in ^{32}P incorporation observed in vitro the increase in phospholamban phosphorylation that occured in the intact vessel in response to SIN-1 was calculated to be 0.74 ± 0.09 pmol P/mg protein. For the NECA-treated tissues the autoradiograph of back-phosphorylated crude microsomes clearly shows reduced ^{32}P incorporation (Fig. 5B). Quantitation of ^{32}P incorporation obtained by back-phosphorylation resulted in a significant difference ($P < 0.001$) between the $PGF_{2\alpha}$ controls and the NECA group (Fig. 6A). The calculated increase in phospholamban phosphorylation in the intact vessel induced by NECA (0.41 ± 0.06 pmol P/mg protein) was significantly lower than that for SIN-1 ($P < 0.05$). In microsomal preparations from tissue specimens treated with iloprost, back-phosphorylation of phospholamban was measured to be significantly ($P < 0.05$) reduced as well, compared with controls (Fig. 6A).

Comparing membrane fractions derived from endothelium-denuded and endothelium-intact $PGF_{2\alpha}$-precontracted pig coronary artery, there was a striking difference in phospholamban phosphorylation (Fig. 6). In membranes from endothelium-intact vessels in vitro back-phosphorylation of phospholamban was 0.76 ± 0.03 pmol ^{32}P/mg protein (n = 12), which compares with 1.32 ± 0.05 pmol ^{32}P/mg protein (n = 13) in endothelium-denuded preparations ($P < 0.001$) . This difference suggests a strong tonic influence of the unstimulated endothelium on phospholamban phosphorylation. In preparations from L-NAME-treated artery segments, the in vitro ^{32}P incorporation into phospholamban was found significantly increased compared with controls (Fig. 6B). The difference in ^{32}P incorporation in vitro between L-NAME-treated preparations and controls was 0.19 ± 0.03 pmol P/mg protein (n = 5; $P < 0.01$) and indicates a decrease in the phosphorylation state of phospholamban in the intact, L-NAME-treated pig coronary artery. Indomethacin showed a tendency to increase the in vitro back-phosphorylation of phospholamban (not significant, $P = 0.145$) (Fig. 6B), and 8-phenyltheophylline was without influence on the phospholamban phosphorylation (Fig. 6B).

Phosphorylation of Myosin Light Chain

In endothelium-denuded, $PGF_{2\alpha}$-precontracted pig coronary artery segments 37.6 ± 3.7 % (n = 12) of the 20-kDa myosin light chain was found to be phosphorylated. The data shown in

Table I show that SIN-1 and NECA reduced myosin light chain phosphorylation. These changes were statistically significant only for the SIN-1-treated preparations ($P < 0.01$). Iloprost-induced relaxation was not accompanied by a decrease in phosphorylation.

As with measurements of phospholamban phosphorylation, the phosphorylation state of myosin light chain was also found to be significantly influenced by the presence of intact endothelium. In $PGF_{2\alpha}$-precontracted artery segments with intact endothelium, 53.8 ± 3.2% (n = 0) of MLCs were phosphorylated as compared to 37.6 ± 3.7% in endothelium-denuded vessels ($P < 0.01$). L-NAME and indomethacin were without significant effect on myosin light chain phosphorylation.

Phosphorylation of Phospholamban and Vascular Tone

Phosphorylation of phospholamban was calculated from the data presented and plotted against the respective changes in diameter of vessel segments (Fig. 7). A close inversely linear relationship was found for alterations of phospholamban phosphorylation and vessel diameter in endothelium-denuded and endothelium-intact pig coronary artery. Near-maximal relaxation was achieved with both SIN-1 and NECA. Note, however, that SIN-1 elicited a significantly higher phosphorylation of phospholamban, which might be due to a supermaximal effect of the cGMP-mediated phosphorylation of phospholamban on vessel relaxation.

TABLE I. Phosphorylation of the Regulatory 20-kDa Myosin Light Chain (MLC) in Isolated Intact Perfused $PGF_{2\alpha}$-Precontracted Pig Coronary[a] Artery Segments

Preparation	Intervention	MLC-phosphorylation relative to total MLC
Endothelium (−)	Control	0.38 ± 0.04 (12)
	SIN-1	0.20 ± 0.05* (4)
	NECA	0.28 ± 0.02 (5)
	ILO	0.48 ± 0.04 (5)
Endothelium (+)	Control	0.54 ± 0.03 (10)
	L-NAME	0.51 ± 0.04 (5)
	INDO	0.48 ± 0.03 (5)

[a]See Materials and Methods for experimental details. Data are expressed as mean ±SEM. Numbers in parentheses give the numbers of individual artery segments tested in each group.
*$P < 0.05$ compared with control group.

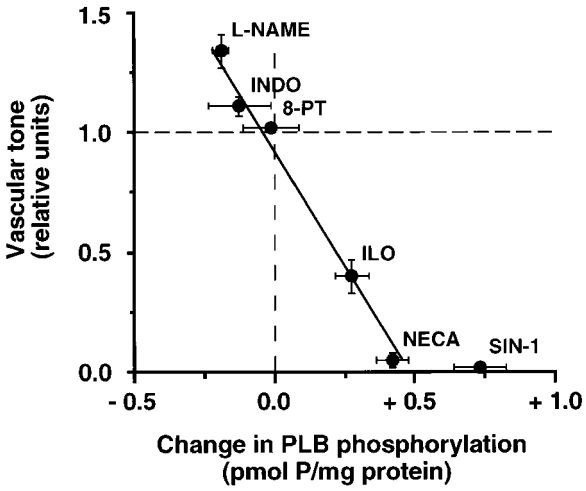

Fig. 7. Relationship between changes in phosphorylation of phospholamban versus changes in vascular tone in response to various agonists and antagonists. Changes in phosphorylation of phospholamban were calculated from data shown in Figures 4 and 6 as differences between the treated groups and their respective controls. Positive values represent an increase in phosphorylation. Negative values express dephosphorylation. The values are shown as means ±SEM. SIN-1, 3-morpholinosydnonimine; NECA, 5′-N-ethylcarboxiamidoadenosine; ILO, iloprost; L-NAME, L-nitroarginine-methylester; INDO, indomethacin; and 8-PT, 8-phenyltheophylline.

DISCUSSION

This study demonstrates phosphorylation of phospholamban in intact pig coronary artery in response to vasodilatory substances such as NO/EDRF, adenosine, and prostacyclin acting through cGMP- or cAMP-dependent mechanisms. Our findings extend studies on smooth muscle cell particulate fractions [Sarcevic et al., 1989], cultivated aortic smooth muscle cells [Cornwell et al., 1991] and results we have previously obtained on intact rat aorta [Karczewski et al., 1992].

The present data confirm that the tissue concentration of phospholamban in vascular smooth muscle is much lower than in the myocardium. However, comparing highly purified vesicles of cardiac sarcoplasmic reticulum and sarcoplasmic reticulum from bovine pulmonary artery Raeymaekers et al. [1990] reported a ratio of 7.1 for phospholamban and of 8.6 for the Ca^{2+}-transport ATPase. Thus, in smooth muscle the stoichiometry of phospholamban and its target for regulation, the sarcoplasmic reticulum Ca^{2+}-ATPase, is similar as it has been

estimated for cardiac muscle [Colyer and Wang, 1991; Movsesian et al., 1992–1993]. Furthermore, vascular smooth muscle expresses the SERCA2 enzyme, the same isoform of the Ca^{2+}-ATPase as cardiac muscle [Eggermont et al., 1990], known to be regulated by phospholamban. Recently, studies on phospholamban-deficient transgenic mice clearly demonstrated that phospholamban modulates both KCl-induced and receptor-mediated contractility in aortic smooth muscle [Lalli et al., 1997]. Collectively, these data suggest that phospholamban is a potent modulator of sarcoplasmic reticulum Ca^{2+} pump also in vascular smooth muscle.

The largest increase in phospholamban phosphorylation in our study was detected after perfusion of pig coronary artery segments with the NO-liberating compound SIN-1. About 60% of the total capacity of phospholamban to incorporate phosphate was phosphorylated in the intact vessel in response to SIN-1. This value compares well with the phosphorylation of phospholamban in isolated rat hearts challenged with 10^{-7} M isoproterenol, which was the maximal effective dose with regard to stimulation of contractility [Karczewski et al., 1990]. Thus, in the intact vessel the extent of phospholamban phosphorylation elicited by the vasodilators is comparable to that in the catecholamine-stimulated myocardium. The presented data strongly suggest a role for the cyclic nucleotide-dependent phosphorylation of phospholamban in modulating the activity of the smooth muscle sarcoplasmic reticulum Ca^{2+} pump.

The most pronounced effect on pig coronary artery tone, as well as on phosphorylation of phospholamban, was elicited either by activation of the NO-pathway with SIN-1 or inhibiting endothelial NO/EDRF formation with L-NAME. Inhibition of endothelial NO/EDRF elicited an additional vasoconstriction and at the same time significantly reduced the phosphorylation state of phospholamban. It therefore appears that the unstimulated endothelium exerts a tonic influence on phospholamban phosphorylation by basal release of NO/EDRF [Kelm et al., 1988; Kelm and Schrader, 1988]. This is in line with earlier observations that tissue levels of cGMP [Rapoport and Murad, 1984] and cGMP-dependent protein kinase activities[Fiscus et al., 1983–1984] are higher in rat aorta with intact endothelium compared with endothelium-denuded preparations.

Adenosine and prostaglandins are known to be potent vasodilators that are assumed to act through the formation of cGMP and cAMP in vascular smooth muscle cells [Kukovetz et al., 1978; Hardman, 1984; Kurtz, 1987]. Here we demonstrate for the first time that the relaxation of pig coronary artery by both the adenosine receptor agonist NECA and the stable prostacyclin derivative iloprost is accompanied by significant phosphorylation of phospholamban. Our data do not permit definition of which kinase was responsible for the phosphorylation of the cyclic nucleotide-specific site of phospholamban. For cGMP-dependent protein kinase a central role in mediating vasodilation has been proposed [Lincoln and Cornwell, 1991]. Direct evidence for cross-activation of cGMP-dependent protein kinase by agents that elevate cAMP [Jiang et al., 1992] and its association with sarcoplasmic reticulum membranes has been observed [Cornwell et al., 1991]. It appears very likely that phosphorylation of phospholamban in response to adenosine and prostacyclin stimulation was mediated by cGMP-dependent protein kinase.

We have observed that phospholamban phosphorylation is highly sensitive to direct stimulation of pig coronary artery smooth muscle adenosine receptors with luminally applied NECA. In the intact vessel the endothelium constitutes an effective metabolic barrier, and luminal adenosine is rapidly taken up and metabolized [Kroll et al., 1987]. This barrier proved incomplete, and at physiological coronary concentrations of adenosine smooth muscle adenosine has been calculated to be 15–20% of the luminal concentration [Kroll et al., 1989]. Thus, in vivo adenosine either stimulates coronary artery smooth muscle receptors directly and/or acts on the endothelium, causing the release of NO/EDRF [Li et al., 1995]. Both effects, as was demonstrated in this study, lead to phospholamban phosphorylation. Enhanced Ca^{2+} sequestration into the sarcoplasmic reticulum through phosphorylation of phospholamban is therefore likely to play a role in mediating the vasodilatory action of adenosine.

The presence of functionally intact unstimulated endothelium caused a highly significant phosphorylation of phospholamban as compared with 76% phosphorylation induced by SIN-1 in endothelium-denuded preparations. Assuming that L-NAME, 8-phenyltheophylline, and indomethacin exerted maximal effects, the

findings demonstrate that mainly endothelial NO and prostacyclin production can account for the major part of this phospholamban phosphorylation observed with endothelium intact vessels.

Phosphorylation of phospholamban was shown to closely correlate with changes of pig coronary artery tone. No such correlation was obtained for the phosphorylation of myosin light chain. Although it is generally accepted that phosphorylation of myosin light chain is essential to initiate smooth muscle contraction [Hathaway et al., 1991], contradictory results on changes of its phosphorylation state have been reported for relaxation through either cGMP or cAMP-mediated mechanisms [Gerthoffer et al., 1984; Miller et al., 1983; Draznin et al., 1986]. Studies on thromboxane contracted coronary artery gave evidence that a major part of cAMP-induced relaxation was not due to desensitization of contractile proteins to calcium [Yamagishi et al., 1994]. In our experiments, only the activation of the cGMP pathway with SIN-1 elicited significant dephosphorylation of myosin light chain. Interestingly, evidence for a cGMP-dependent increase in smooth muscle myosin light chain phosphatase activity has been recently reported by Wu et al. [1996]. Our data support the notion that dephosphorylation of myosin light chain seems not be the essential event in the molecular mechanism of vasodilators elevating cyclic nucleotides, and are in line with the view, that their major mechanism is to lower cytosolic Ca^{2+}. Apparently, dephosphorylation of myosin light chain does not always occur when cytosolic Ca^{2+} is reduced [Katoch et al., 1997]

In summary, our results demonstrate the phosphorylation of phospholamban in the intact pig coronary artery in response to the potent vasodilators NO, adenosine, and prostacyclin. A strong correlation was obtained between the phosphorylation state of phospholamban and changes in the vessel diameter. Our data are in line with a dominant role of endothelial NO/EDRF for the control of coronary artery tone. It is proposed that enhanced Ca^{2+} sequestration into the sarcoplasmic reticulum by phosphorylation of phospholamban is a major component in the mechanism of vascular smooth muscle relaxation evoked by endothelial NO/EDRF, adenosine, and prostacyclin.

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