0026-895X/91/060923-09$03.00/0

MOLECULAR PHARMACOLOGY, 40:923-931

Regulation of Sarcoplasmic Reticulum Protein Phosphorylation by Localized Cyclic GMP-Dependent Protein Kinase in Vascular Smooth Muscle Cells

TRUDY L. CORNWELL, KATHERINE B. PRYZWANSKY, TODD A. WYATT, and THOMAS M. LINCOLN

Department of Pharmacology, College of Medicine, University of South Alabama, Mobile, Alabama 36688 (T.L.C., T.M.L.), and Department of Pathology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 25799 (K.B.P., T.A.W.)

Received May 20, 1991; Accepted August 28, 1991

SUMMARY

The role of cGMP-dependent protein kinase in the regulation of intracellular Ca^{2+} levels in vascular smooth muscle cells was examined by studying the effects of cGMP on the phosphorylation of the Ca^{2+}-ATPase regulatory protein phospholamban. Cultured rat aortic smooth muscle cells incubated with atrial natriuretic peptide II or sodium nitroprusside responded with increased phosphorylation of the 6000-Da subunit of phospholamban. The identity of phospholamban was confirmed using immunoprecipitation methods. Phosphorylation was associated with an increase in the activation of membrane-associated ATPase by Ca^{2+}. These results indicated that at least one site of action of cGMP in smooth muscle cells is the sarcoplasmic reticulum, where phosphorylation of proteins regulating Ca^{2+} fluxes occurs. Studies using confocal laser scanning microscopy to define the cellular distribution of cGMP-dependent protein kinase suggested that the enzyme was localized to the same cellular region(s) as was phospholamban. Phosphorylation of proteins by cGMP in broken cell fractions from rabbit aorta was also performed. Phospholamban and other proteins were phosphorylated in the presence of cGMP but not cAMP, suggesting that only cGMP-dependent protein kinase was associated with smooth muscle membrane fractions containing phospholamban. These results suggest that one mechanism of action of cGMP in the reduction of intracellular Ca^{2+} is the activation of sarcoplasmic reticulum Ca^{2+}-ATPase via phosphorylation of phospholamban. The data also support the concept that compartmentalization of protein kinases with substrates in the intact cell is an important factor involved in protein phosphorylation.

Endogenous regulators of vascular smooth muscle relaxation include agents that elevate cAMP levels (e.g., β-adrenergic catecholamines and eicosanoids) and agents that elevate cGMP levels (e.g., atrial natriuretic peptides and endothelium-derived relaxing factor) (1, 2). In addition, many vasodilator drugs, such as glyceryl trinitrate and NP, appear to produce relaxation through increases in cGMP (3). The mechanisms of cyclic nucleotide-dependent relaxation of smooth muscle are not well understood, although it is now believed that the reduction of $[Ca^{2+}]_i$ is an important component of relaxation (see Ref. 2 for a review). Other mechanisms have been proposed, such as the inactivation of myosin light chain kinase by cAMP-dependent protein phosphorylation (4), but the physiological significance of this effect is not known. Our laboratory has reported that activation of cGMP-dependent protein kinase is both necessary and sufficient to account for the reduction of $[Ca^{2+}]_i$ in vascular smooth muscle cells by agents that increase both cGMP *and* cAMP (5, 6). Therefore, cGMP-dependent protein kinase occupies a pivotal role in the regulation of smooth muscle $[Ca^{2+}]_i$ and vasodilation.

The biochemical events beginning with the activation of cGMP-dependent protein kinase by either cGMP or cAMP, to the eventual reduction in $[Ca^{2+}]_i$, are not defined. Various hypotheses have been advanced to explain the effects of cGMP on $[Ca^{2+}]_i$. These include (i) the inhibition of IP_3 formation via inhibition of phospholipase C activation (7, 8), (ii) the inhibition of G protein coupling to phospholipase C, thus inhibiting IP_3 production (9, 10), (iii) the inhibition of Ca^{2+} release by the sarcoplasmic reticulum (11, 12), and (iv) the activation of Ca^{2+}-ATPase in some cellular compartment that eventually produces a reduction in $[Ca^{2+}]_i$ (13, 14). Each hypothesis has some support, and none are mutually exclusive.

This work was supported by Grants HL 34646 from the National Institutes of Health and PCM 8408902 from the National Science Foundation. T.M.L. is an Established Investigator for the American Heart Association. T.L.C. is a Harriet P. Dustan Fellow for the American Heart Association, Alabama Affiliate.

ABBREVIATIONS: NP, sodium nitroprusside; IP_3, inositol 1,4,5-trisphosphate; ANP, atrial natriuretic peptide II; CLSM, confocal lasar scanning microscopy; BSS, balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PI, phosphatidylinositol; FITC, fluorescein isothiocyanate; G protein, GTP-binding protein; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PBS, phosphate-buffered saline.

In this report, we have examined further the role of cGMP-dependent protein kinase in the regulation of $[Ca^{2+}]_i$, by examining the phosphorylation of the regulatory protein phospholamban. Phosphorylation of phospholamban is known to decrease its interaction with sarcoplasmic reticulum Ca^{2+}-ATPase, thus activating the enzyme (15). Furthermore, we have used immunocytochemistry to study the localization of cGMP-dependent protein kinase in smooth muscle cells, because the locus of the kinase might shed light on its mechanism of action. Our results suggest that a major role of cGMP is, in fact, the regulation of sarcoplasmic reticulum protein phosphorylation.

Materials and Methods

Intact cell phosphorylation. For the examination of phospholamban phosphorylation in intact cells, rat aortic smooth muscle cells were isolated using the procedure of Smith and Brock (16), with slight modifications. Cells from six rat aortas were placed in six 60-mm tissue culture plates and passaged twice at near-confluency before plating in 35-mm tissue culture plates. Cells were used in early passage, because expression of cGMP-dependent protein kinase is maintained at earlier passages. The monolayers were labeled with 0.25 mCi/ml ^{32}P for 2 hr at 37°, in an air incubator, in a BSS consisting of 10 mM HEPES buffered with Tris to pH 7.4, 140 mM NaCl, 4.5 mM KCl, 1 mM $MgSO_4$, and 1.5 mM $CaCl_2$. After labeling, the cells were rinsed twice with BSS and resuspended in 1 ml of BSS. Incubations with 0.1 μM NP or ANP were performed for the times indicated, at room temperature. At the end of the incubation period, the medium was rapidly aspirated, and 0.5 ml of cell lysis buffer was added, consisting of 10 mM HEPES, pH 7.4, 100 mM NaF, 10 mM EDTA, 2 mM EGTA, 1 μM okadaic acid, 0.1 mM PMSF, 8% sucrose, and 10 μg/ml digitonin. The NaF, EDTA, and EGTA inhibit protein kinase activities, NaF, EDTA, and okadaic acid inhibit phosphatase activities, PMSF and EGTA inhibit proteases, 8% sucrose preserves the integrity of the intracellular organelles, and digitonin permeablizes the plasma membrane. We observed that incubating cells at room temperature and lysing cells as described above were optimal for preserving phospholamban phosphorylation in the intact cell. The cells were "quick frozen" by placing the culture plates on a block of dry ice covered with ethanol, and the plates were stored at −20° until used. For resolving phosphorylated proteins, the cells were subjected to two rounds of freeze-thawing, and the plates were scraped using a plastic cell scraper. The contents were emptied into a 1.5-ml microfuge tube and centrifuged at 12,000 × g for 30 min at 4°. The pellet was then resuspended in 50 μl of electrophoresis "stop mix," consisting of 1% SDS, 2.5 M β-mercaptoethanol, 5% glycerol, and 0.1% bromophenol blue, and was heated for 3 min at 80° in a heat block. The cooled samples were resolved by SDS-PAGE on 8–25% gels, using the Phast gel apparatus (Pharmacia). Gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography. Protein(s) migrating with phospholamban (M_r 6000) were cut from the gels and quantitated by liquid scintillation counting.

Immunoprecipitation of phospholamban. Membrane fractions that had been prepared as described above were prepared for immunoprecipitation as follows: 10 μl of the denatured protein mixture were diluted into 200 μl of ice-cold PBS, and the sample was treated with 10 μl of 2 M neutralized iodoacetic acid for 10 min. This procedure removed mercaptoethanol and diluted the SDS sufficiently for immunoprecipitation. The samples were then mixed with 10 μl of rabbit antiphospholamban overnight at 4°. The next day, 50 μl of a 50% slurry of Protein G-agarose were added, and the samples were mixed at 4° for 3 hr. The mixture was then transfered to a 0.5-ml microfuge tube, centrifuged for 2 min, and washed twice in PBS, and the agarose pellet was resuspended in electrophoresis stop mix. The samples were separated on 8–25% polyacrylamide gels using the Phast system, stained with Coomassie blue, destained, dried, and subjected to autoradiography.

Broken cell phosphorylation. For examination of the effects of cyclic nucleotides on protein phosphorylation *in vitro*, freshly isolated rabbit aortas were used. The vessels were cleaned of adhering fat and connective tissue and minced with scissors; the pieces were suspended at 4° in a homogenizing buffer (1 g/ml of buffer), consisting of 50 mM HEPES, pH 7.2, 8% sucrose, 0.1 M NaCl, 1 mM EDTA, 15 mM β-mercaptoethanol, and 0.1 mM PMSF, and were homogenized with two strokes, using a Teflon pestle, in a 10-ml glass tube. Studies by Ives *et al.* (17) have shown that this homogenization procedure preserves the integrity of cGMP-dependent protein phosphorylation in smooth muscle membranes. The homogenate was centrifuged at 1000 × g for 5 min to remove nuclei and debris, and the supernatant was centrifuged at 100,000 × g for 45 min. The pellet was resuspended in 0.4 ml of homogenization buffer and used as the source of protein for phosphorylation. For extraction of the proteins, the suspended high speed particulate fraction was treated with 1% Triton X-100 for 20 min at 4° and centrifuged at 100,000 × g for 45 min to obtain a particulate extract and a pellet. For phosphorylation, suspended material (20 μl) was preincubated at 4°, for 5 min, with various concentrations of cyclic nucleotides (where indicated) or protein kinases (where indicated). Phosphorylation was initiated with the addition of a phosphorylation mixture (20 μl) consisting of (final concentrations) 25 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP (approximately 500 cpm/pmol), 0.2 mM 3-isobutyl-1-methylxanthine, and 100 μM EGTA. Incubations were carried out at 4° for the times indicated, and reactions were terminated with the addition of 10 μl of an electrophoresis stop mix, consisting of 10% SDS, 2.5 M β-mercaptoethanol, 30% glycerol, and 0.1% bromophenol blue. The contents were immediately heated at 90° for 3 min, and the denatured proteins were resolved on either 8% or 12% SDS-polyacrylamide gels, according to the procedure of Laemmli (18). Molecular weight markers were myosin (200,000), phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (43,000), glyceraldehyde 3-phosphate dehydrogenase (35,000), soybean trypsin inhibitor (24,000), and cytochrome *c* (12,000). Gels were stained with Coomassie blue, destained, dried, and subjected to autoradiography at −80°.

Ca^{2+}-ATPase assay. Cell monolayers in passage 2 or 3 were treated for the times indicated with 0.1 μM NP or 0.1 μM ANP, at room temperature, and the treatments were terminated by addition of the cell lysis buffer described earlier. After two cycles of freeze-thawing to lyse the cells, the samples were collected in 1.5-ml microfuge tubes and centrifuged at 4° for 30 min at 12,000 × g. The particulate material was resuspended in 50 μl of 10 mM HEPES, pH 7.3, 8% sucrose, 1 μM okadaic acid. Ca^{2+}-ATPase was assayed in a final volume of 150 μl of 20 mM HEPES, pH 7.4, 120 mM KCl, 1 mM $MgCl_2$, 0.07 mM [γ-^{32}P] ATP (1 μCi/ml), 0.5 mM EGTA, in the absence or presence of 0.49 mM $CaCl_2$, which yields a final concentration of 1 μM free Ca^{2+}. Reactions were initiated by the addition of 10 μl of membranes, were conducted at 37° for 2 min, and were terminated with the addition of 150 μl of 10% trichloroacetic acid plus 300 μl of a mixture of 20 mg/ml bovine serum albumin and 26 mg/ml KH_2PO_4. The liberated ^{32}P was determined after the addition of 0.5 ml of 100 mg/ml suspended activated charcoal, as described previously (13). The data are expressed as the percentage of stimulation of ATPase activity in the presence of 1 μM $CaCl_2$.

Immunocytochemistry of vascular smooth muscle cells. Rat aortic smooth muscle cells in passage 2 or 3 were adhered to 12-mm glass coverslips, in the presence of Dulbecco's minimal essential medium plus 10% fetal bovine serum, at 37° for 8 hr before treatment. For immunocytochemical localization of cGMP-dependent protein kinase using CLSM, cells were treated as follows. Monolayers were fixed in 1% paraformaldehyde at room temperature for 5 min, followed by 3.7% formaldehyde in PBS for 10 min, −20° methanol for 4 min, and −20° acetone for 1 min. The cells were washed three times with PBS after formaldehyde and after acetone. Cells were incubated for 1 hr at room temperature with rabbit anti-bovine aorta cGMP-dependent protein kinase, washed in PBS, and stained for 30 min with FITC-goat anti-rabbit IgG. Control cells were incubated with preimmune rabbit IgG. The cells were then mounted in polyvinyl alcohol and viewed with

a Zeiss confocal dual lasar scanning microscope. The confocal laser scanning capability allowed optical sectioning of cells. In these experiments, the cells were scanned in depth at 0.5-μm increments. The digital images were recorded using a Polaroid freeze-frame video recorder.

For simultaneous localization of cGMP-dependent protein kinase and phospholamban in rat aortic smooth muscle cells, the monolayers were fixed and incubated simultaneously with rabbit anti-cGMP-dependent protein kinase and mouse anti-canine heart phospholamban, for 30 min. The cells were then washed with PBS and treated with FITC-goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-sheep anti-mouse IgG, for 30 min. Cells were viewed with a Leitz fluorescence microscope, and images were recorded with a Leitz camera.

Other methods. Cyclic GMP was measured in 5% trichloroacetic acid extracts by radioimmunoassay (19). Bovine lung cGMP-dependent protein kinase was purified by affinity chromatography as described previously (20), and bovine heart catalytic subunit of cAMP-dependent protein kinase was purified by a method modified from that of Sugden *et al.* (21).

Materials. Isotopes were from New England Nuclear-DuPont, Inc., and the various drugs used (NP and ANP) were from Sigma Chemical Co. Media for cell cultures were from GIBCO, and fetal bovine serum from Hyclone, Inc. FITC and TRITC conjugates were from Organon Teknika-Cappel (Malvern, PA). Rabbit anti-bovine cGMP kinase was produced and characterized as described previously (5), and rabbit anti-canine phospholamban and monoclonal antibody to phospholamban were characterized and supplied by Dr. Larry Jones, Indiana University. Okadaic acid was a gift from Dr. Robert W. Dickey, Dauphin Island Sea Laboratory (Dauphin Island, AL).

Results

Phosphorylation of phospholamban in the intact cell. Previous studies suggested that cGMP-dependent protein kinase activated Ca^{2+}-ATPase in smooth muscle cell fractions (13, 14, 22), but purified plasmalemma Ca^{2+}-ATPase was not phosphorylated and activated by cGMP-dependent protein kinase (23, 24). Others have shown that the sarcoplasmic reticulum Ca^{2+}-ATPase regulatory protein phospholamban was phosphorylated using cGMP-dependent protein kinase *in vitro* (25, 26), but studies in the intact cell have been equivocal (27). In order to study more closely the possible phosphorylation of phospholamban by cGMP-dependent protein kinase in the intact smooth muscle cell, careful incubation and cell homogenization conditions were established to optimize the detection of cGMP-stimulated phosphorylation. Cells prelabeled with ^{32}P were treated with NP and ANP to elevate cGMP levels, and proteins were resolved by SDS-PAGE. The proteins having a molecular weight of 6000 were excised from the gel and counted. As shown in Fig. 1, cells treated with NP and ANP demonstrated an increase in phosphorylation of 6-kDa proteins beginning at 30 sec and peaking at 1 min. Although there was some variability in the degree of phosphorylation, particularly at the earlier time points with NP and ANP, it was clear that the level of phosphorylation was greater in the ANP- and NP-treated cells, compared with the control cells.

Several proteins having a molecular weight of approximately 6000 exist in the cell, and some of these could be substrates for cGMP-dependent protein kinase (histones, for example). This might contribute not only to the variability seen in Fig. 1 but also to the background level of phosphorylation. Thus, phospholamban was immunoprecipitated from extracts of ^{32}P-labeled cells, resolved by SDS-PAGE, and subjected to autoradiography. As shown in Fig. 2, the phosphorylation of immu-

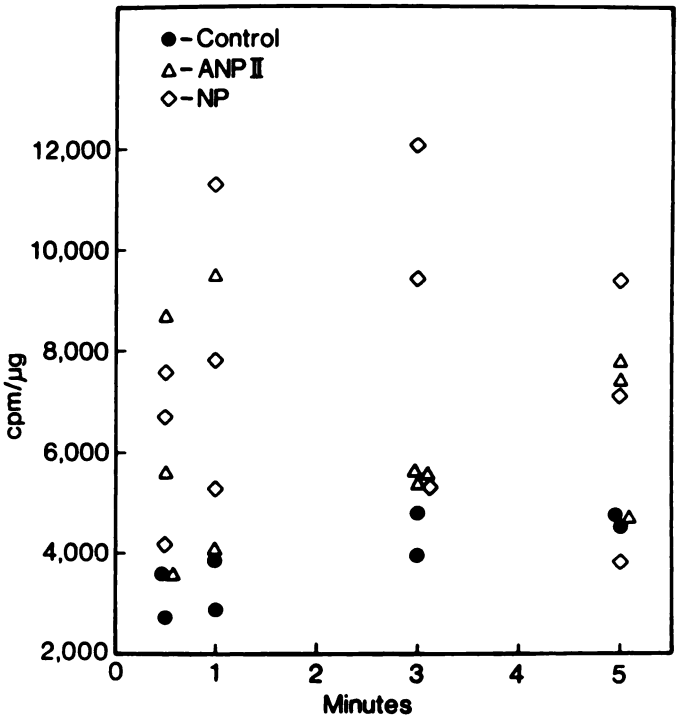

Fig. 1. Effects of ANP and NP on the phosphorylation of phospholamban in cultured rat aortic smooth muscle cells. Cells in passage 3 were grown in 35-mm plastic culture dishes and prelabeled with ^{32}P in phosphate-deficient BSS, as described in Materials and Methods, and were incubated in the absence of drugs (●) or with 0.1 μM ANP (△) or 0.1 μM NP (◇), for the times indicated. Cells were lysed, particulate material containing phospholamban was harvested, and proteins were resolved by 8–25% SDS-PAGE. The molecular weight standards used were myosin (200,000), phosphorylase (94,000), bovine serum albumin (68,000), ovalbumin (43,000), glyceraldehyde-3-phosphate dehydrogenase (35,000), soybean trypsin inhibitor (24,000), cytochrome *c* (12,000), and cardiac phospholamban (6,000). The band corresponding to 6 kDa on autoradiograms was cut out of the gel and counted. The data are the results of three separate experiments and are expressed as the cpm/μg of protein loaded onto the gel.

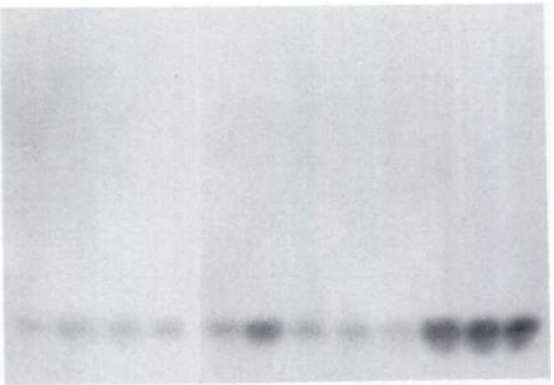

Fig. 2. Immunoprecipitation of phospholamban from cultured rat aortic smooth muscle cells. Cells in passage 3 were prelabeled with ^{32}P and incubated in the absence of drugs (–) or with 0.1 μM ANP II (*A*) or 0.1 μM NP (*N*), for the times indicated. Cells were lysed and phospholamban was immunoprecipitated as described in Materials and Methods. Proteins were resolved by 8–25% SDS-PAGE and subjected to autoradiography.

noprecipitated phospholamban was enhanced by both ANP and NP. Maximum phosphorylation was observed at 1 min with these agents and, with ANP at least, phosphorylation declined to near basal levels after 2–3 min.

Activation of Ca^{2+}-ATPase and elevation of cGMP in the intact cell. The functional consequence of phosphorylation of phospholamban in response to cGMP is not known. However, it might be predicted that, upon phosphorylation of phospholamban, sarcoplasmic reticulum Ca^{2+}-ATPase would become activated. Because this enzyme requires Ca^{2+} for activity, we examined the percentage of activation of ATPase in particulate suspensions of cells treated with ANP. As shown in Fig. 3, cells treated with ANP responded with an increase in intracellular cGMP and an activation of Ca^{2+}-ATPase. Maximal effects for both the activation of the ATPase by Ca^{2+} and the increased cGMP levels were at 1 min, after which a decline in the levels of cGMP and Ca^{2+}-ATPase activity were observed. This time course was similar to that of phosphorylation of phospholamban (Fig. 2). The percentage of activation by Ca^{2+} was modest, due in part to the large background of ATPase activity that was not dependent on Ca^{2+} that was present in the particulate suspensions. These results are consistent with, although perhaps do not prove, the proposal that phosphorylation of phospholamban was associated with an increase in sarcoplasmic reticulum Ca^{2+}-ATPase activity. In the same experiment (data not shown), NP (0.1 μM) also increased Ca^{2+}-ATPase activity (13.4 ± 2.75% at 0.5 min and 9.6 ± 3.09% at 1 min). However, after 1 min in the presence of NP, there was a dramatic decrease in total ATPase activity. The reasons for this finding were not clear, but it was possible that metabolites of NP (e.g., cyanide) might have inhibitory effects on cellular ATPases.

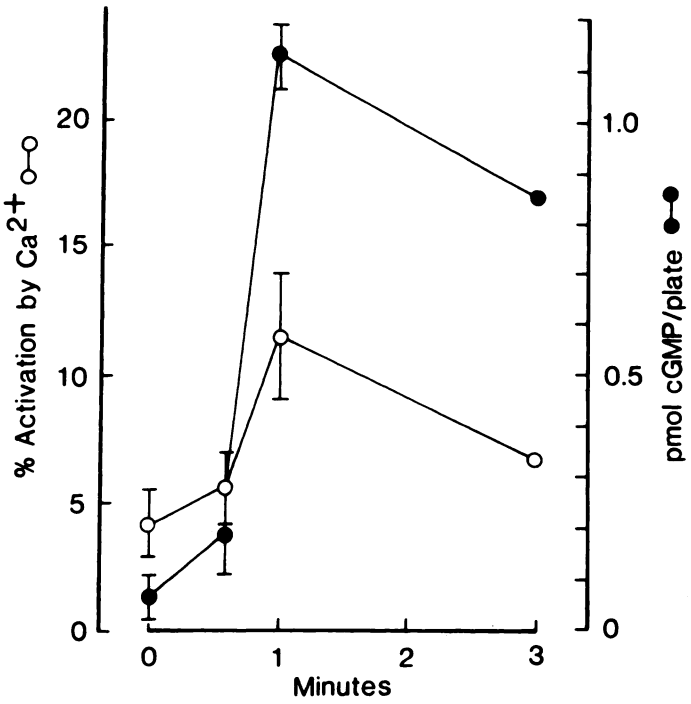

Fig. 3. Effects of ANP on Ca^{2+}-ATPase activity and cGMP levels. Cells in passage 2 or 3 were grown in 35-mm plastic culture dishes and incubated, for the times indicated, with 0.1 μM ANP II. Particulate material containing Ca^{2+}-ATPase was assayed in the absence or presence of 1 μM $CaCl_2$, as described in Materials and Methods. The results are expressed as the percentage of total ATPase activity activated by Ca^{2+}, as the mean ± standard error of three determinations (except for the 3-min time point, which was the average of two determinations). Cyclic GMP was measured in the supernatant fraction of the cells after treatment with 5% trichloroacetic acid.

Localization of cGMP-dependent protein kinase and phospholamban in the intact cell. The results on the phosphorylation of phospholamban and the activation of Ca^{2+}-ATPase suggested that cGMP-dependent protein kinase might regulate Ca^{2+} transport at the sarcoplasmic reticulum. If this is the case, then it should be possible to localize cGMP-dependent protein kinase in the sarcoplasmic reticulum of intact, cultured, smooth muscle cells. We used the technique of CLSM to examine the distribution and localization of cGMP-dependent protein kinase. As shown in Figs. 4 and 5, cells stained with cGMP-dependent protein kinase antibody and visualized using CLSM showed a distribution of cGMP-dependent protein kinase surrounding the nuclear area. The fact that the staining was mainly perinuclear was apparent because different horizontal planes (of 0.5 μm in thickness) demonstrated a "hollow" nuclear area and a dense perinuclear area (Fig. 5d). The punctate staining pattern of cGMP-dependent protein kinase in the cytoplasm could indicate that the enzyme was distributed to vesicles. Previous studies have shown that this region of the cell is known to contain organelles associated with active Ca^{2+} uptake and release and presumably represents smooth muscle sarcoplasmic reticulum (28). Little or no staining for cGMP-dependent protein kinase was associated with the plasma membrane. Some nuclear staining for cGMP-dependent protein kinase was apparent, although the actual nuclear structures containing the kinase could not be ascertained. Nuclear staining is not entirely unexpected, however. Tse *et al.* (29) found that cGMP-dependent protein kinase increased in liver nuclei after partial hepatectomy. Treatment of cells with depolarizing concentrations of KCl to induce contractile activity did not affect the localization of cGMP-dependent protein kinase (compare Fig. 4 with Fig. 5). Cells treated with preimmune serum did not stain (data not shown).

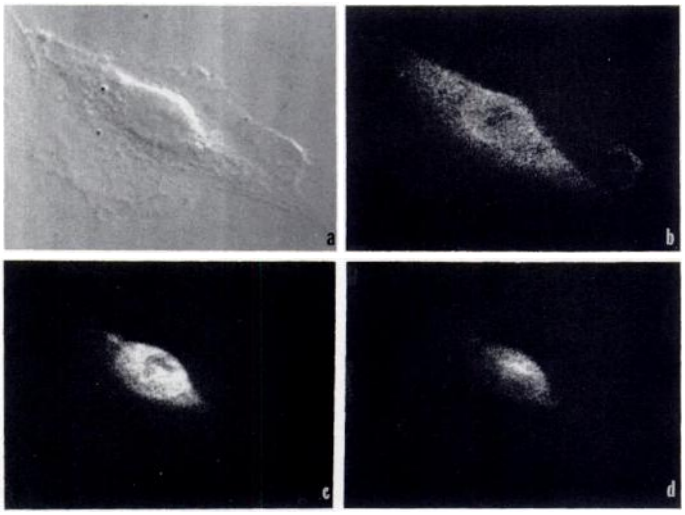

Fig. 4. Localization of cGMP-dependent protein kinase by CLSM in rat aortic smooth muscle cells. Cells in passage 2 were fixed and stained with rabbit anti-cGMP-dependent protein kinase for immunofluorescence, as described in Materials and Methods. The series of images are sections at 1 μm (b), 2 μm (c), and 2.5 μm (d) from the plane of adherence. a, Differential interference contrast micrograph of the cell that is being scanned. Magnification, ×800.

To determine whether cGMP-dependent protein kinase was localized with the sarcoplasmic reticulum, cells were stained, for double-label immunofluorescence microscopy, for cGMP-dependent protein kinase and phospholamban. As shown in Fig. 6, cGMP-dependent protein kinase and phospholamban were found in the same areas of the cell, i.e., in the cytoplasmic area surrounding the nucleus. It is important to note that the micrographs are traditional phase-contrast and epifluorescence microscopy and not confocal laser scanning optics, so that the distribution of kinase and phospholamban may appear to be nuclear. The confocal micrographs shown earlier, however, clearly demonstrated that the cGMP-dependent protein kinase

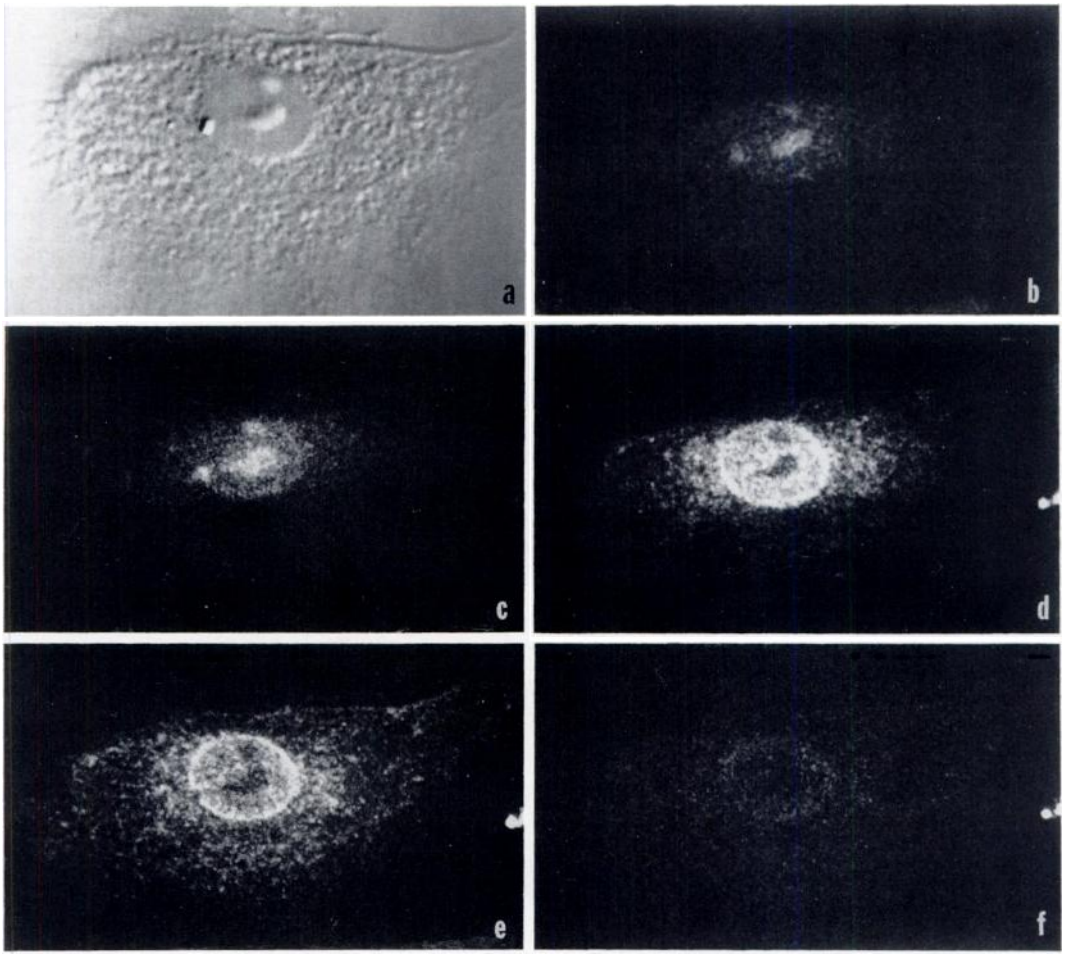

Fig. 5. Localization of cGMP-dependent protein kinase by CLSM in rat aortic smooth muscle cells. Cells in passage 2 were treated with 35 mM KCl for 30 sec before fixing and staining with rabbit anti-cGMP-dependent protein kinase. The series of images are sections at 0.5-μm intervals beginning at the cell surface (a) and continuing toward the plane of adherence (f). a, Differential interference contrast micrograph. Magnification, ×800.

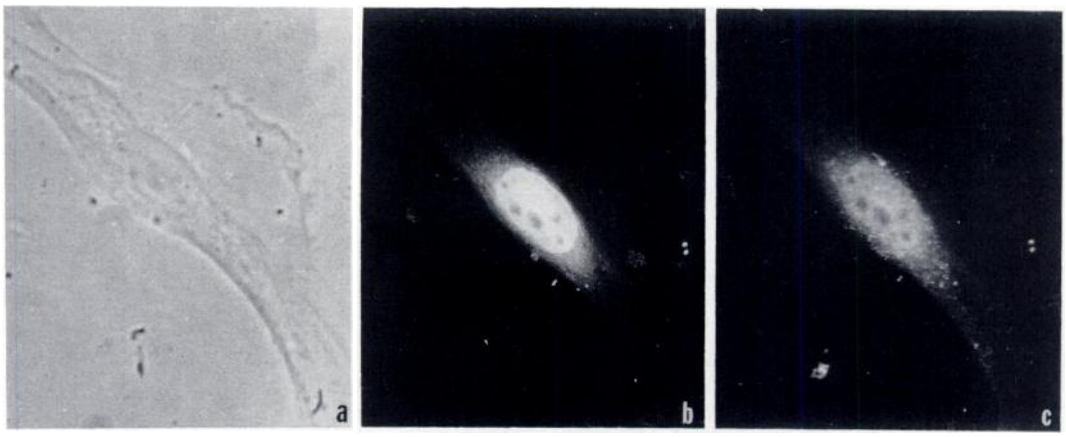

Fig. 6. Localization of cGMP-dependent protein kinase and phospholamban using double-label immunofluorescence microscopy in rat aortic smooth muscle cells. Cells in passage 4 were fixed and stained with rabbit anti-cGMP-dependent protein kinase (b) and mouse monoclonal antiphospholamban (c). a, Phase contrast micrograph. The perinuclear staining of both kinase and phospholamban is apparent. Magnification, ×570.

was also localized to perinuclear areas. Again, no staining of the plasma membrane was observed for either antibody. The morphological data reported here complement and support the biochemical data shown earlier, suggesting that cGMP-dependent protein kinase regulated Ca^{2+}-ATPase in an intracellular compartment in smooth muscle that contains the sarcoplasmic reticulum protein phospholamban.

Phosphorylation of rabbit aortic membrane proteins *in vitro*. Early studies had shown that cAMP and cAMP-dependent protein kinase activation promoted the phosphorylation of phospholamban in intact cardiac muscle (27, 30). Cyclic AMP elevations also caused reductions of $[Ca^{2+}]_i$ in vascular smooth muscle cells, but this effect is now thought to be mediated by activation of cGMP-dependent protein kinase (6, 31). If the cGMP-dependent protein kinase is the enzyme that catalyzes the phosphorylation of phospholamban in smooth muscle cells, then it should be possible to demonstrate this in isolated membrane fractions from smooth muscle tissue. In fact, Sarcevic *et al.* (26) reported that cGMP and cGMP-dependent protein kinase selectively catalyzed the phosphorylation of phospholamban in isolated membranes from rat aortic smooth muscle cells. We considered it important to reproduce these findings. As shown in Figs. 7 and 8, the addition of cGMP to isolated particulate fractions from rabbit aorta promoted the phosphorylation of at least three proteins. These proteins had estimated molecular weights of 140,000, 75,000, and 6,000. At 15 sec and at 4°, however, no protein was phosphorylated in the presence of cAMP, even at 1 μM. When phosphorylation was carried out for 30 sec at 4° (Fig. 8), greater amounts of radioactive phosphate were incorporated into these proteins by cGMP, making it difficult to resolve proteins above 50 kDa. However, at 30 sec, the phosphorylation of the 6-kDa protein was conspicuously increased by cGMP, from 100 to 500 nM, but not by cAMP. This protein was identified as phospholamban, using polyclonal antibodies to canine phospholamban (data not shown). These results confirmed those of Sarcevic *et al.* (26), who demonstrated that phospholamban was phosphorylated selectively by cGMP in cultured rat aortic smooth muscle cell fractions. Because submicromolar concentrations of cAMP did not lead to phosphorylation of phospholamban (or any other protein) in this experiment, we concluded that these fractions did not contain cAMP-dependent protein kinase, but only cGMP-dependent protein kinase. Hence, upon addition of cyclic nucleotide, only cGMP promoted phosphorylation.

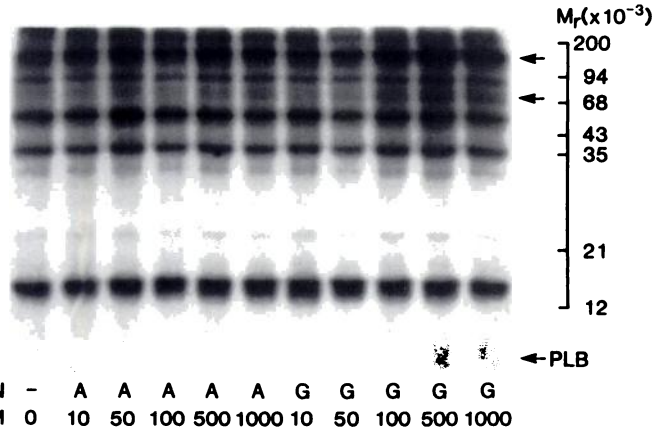

Fig. 7. Phosphorylation of rabbit aortic membrane proteins *in vitro* by different concentrations of cAMP and cGMP. Rabbit aortic membranes were prepared by the homogenization technique of Ives *et al.* (17), as outlined in Materials and Methods. Proteins were phosphorylated using [γ-^{32}P]ATP, in the presence of cAMP (*A*) or cGMP (*G*), for 15 sec at 4° and were resolved by 12% SDS-PAGE. *Arrows*, proteins phosphorylated selectively by cGMP. *PLB*, position of phospholamban in this gel system.

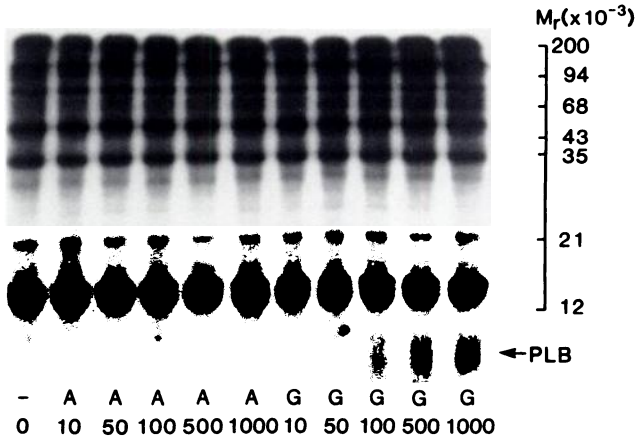

Fig. 8. Phosphorylation of rabbit aortic membrane proteins *in vitro* by different concentrations of cAMP and cGMP. Phosphorylation was performed as described in the legend to Fig. 7, except that the phosphorylation assays were conducted for 30 sec at 4° instead of 15 sec. Note the selective phosphorylation of phospholamban (*PLB*) by cGMP.

In order to provide additional support for this conclusion, we assayed cAMP-dependent protein kinase activity in rabbit aortic membrane fractions and compared this activity with that in bovine cardiac sarcoplasmic reticulum, a subcellular fraction known to contain cAMP-dependent protein kinase. Cardiac fractions contained 1.19 nmol/min/mg of protein of cAMP-dependent protein kinase activity, using histone (F2b) as the substrate, and the activity was stimulated >10-fold by 2 μM cAMP. In contrast, rabbit aortic fractions contained <0.18 nmol/min/mg of protein of cAMP-dependent protein kinase activity, and histone phosphorylation was stimulated <25% by cAMP. Thus, no measurable cAMP-dependent protein kinase activity was associated with the rabbit microsomal fraction.

To demonstrate that proteins that can be phosphorylated using both cAMP- and cGMP-dependent protein kinases do exist in the aortic membranes, rabbit aortic membrane fractions were prepared as described above and phosphorylated using exogenous kinases. As shown in Fig. 9, *lanes 1–3*, both the catalytic subunit of cAMP-dependent protein kinase and cGMP-dependent protein kinase catalyzed the phosphorylation of a number of proteins. Furthermore, more proteins were phosphorylated by the exogenous addition of the protein kinases than by the addition of only the cyclic nucleotide. These results suggested that protein substrates for both kinases exist in the aortic membrane fraction and that those proteins in Figs. 7 and 8 whose phosphorylation was stimulated by cGMP alone probably represented the "best" substrates for cGMP-dependent protein kinase, because their phosphorylation was enhanced with what were most likely only small amounts of endogenous cGMP-dependent protein kinase. The lack of specificity of phosphorylation shown in Fig. 9, *lanes 1–3*, is in contrast to the selectivity for cGMP-stimulated phosphorylation shown in Figs. 7 and 8 and further supports the suggestion that the specificity for cGMP-catalyzed protein phosphorylation was due to the presence of endogenous cGMP-dependent protein kinase localized with the substrates, rather than to the presence of "specific" protein substrates for cGMP-dependent

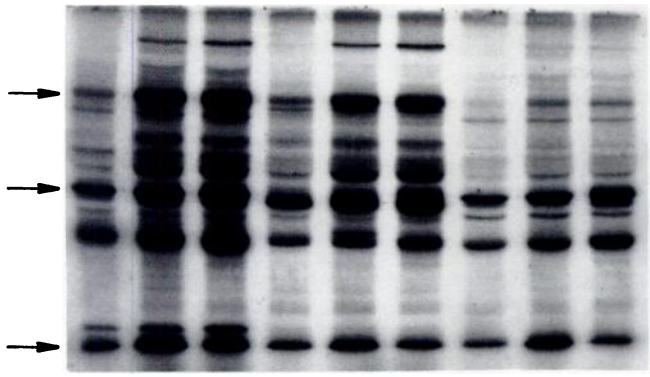

Fig. 9. Phosphorylation of rabbit aortic membrane proteins *in vitro* by exogenous cAMP- and cGMP-dependent protein kinase. Rabbit aortic membranes (*M*) were prepared as described in Materials and Methods and extracted with Triton X-100 (1% final concentration), to yield a Triton-soluble protein fraction (*T-S*) and an Triton-insoluble protein fraction (*T-P*). Phosphorylations were performed for 60 sec at 4°, using 10 nM purified catalytic subunit of cAMP-dependent protein kinase (*A*), 10 nM purified cGMP-dependent protein kinase (*G*), or no exogenous kinase (*O*). Proteins were resolved by 8% SDS-PAGE and subjected to autoradiography. *Arrows*, positions of 140-kDa, 65-kDa, and 17-kDa proteins.

protein kinase. However, it was possible that solubilized membrane proteins could be better substrates for one kinase or the other; thus, Triton X-100-extracted membrane proteins were phosphorylated using exogenous catalytic subunit and cGMP-dependent protein kinase. As shown in Fig. 9, *lanes 4–6*, these extracted soluble proteins were, in general, effectively phosphorylated using both protein kinases. There were some exceptions; for example, one protein, of approximately 43 kDa, was selectively phosphorylated using the catalytic subunit of cAMP-dependent protein kinase, whereas another, of approximately 50 kDa, was selectively phosphorylated using cGMP-dependent protein kinase. Few unique substrates for the kinases remained behind with the cytoskeleton pellet in this experiment (Fig. 9, *lanes 7–9*). We believe that this was an important observation, because it suggested that the major substrates for cGMP-dependent protein kinase in smooth muscle particulate fractions were membrane proteins, not those present in the cytoskeleton. We conclude, therefore, that proteins capable of serving as substrates for both cyclic nucleotide-dependent protein kinases were present in aortic membranes; however, under homogenization conditions designed to retain the endogenous kinases that were associated with these membranes in the intact cell, only cGMP-dependent protein kinase was involved in the phosphorylation of these proteins.

The identity of all the proteins phosphorylated in Fig. 9 is not known. The 250-kDa protein that was extracted by Triton X-100 and, therefore, was not associated with the cytoskeleton has not been unequivocally identified; however, its localization to membranes, its molecular weight, and its capacity to be phosphorylated using the cAMP-dependent protein kinase suggests that it could be the IP_3 receptor protein (32). If so, then this would be the first demonstration that cGMP-dependent protein kinase catalyzes the phosphorylation of the IP_3 receptor *in vitro*. Phospholamban migrated at the dye front in Fig. 9.

Discussion

The results in this study provide, in part at least, a plausible mechanism of action for cGMP in the lowering of $[Ca^{2+}]_i$ in vascular smooth muscle cells. These data suggest that cGMP-dependent protein kinase regulates Ca^{2+}-ATPase activity in smooth muscle sarcoplasmic reticulum via phosphorylation of phospholamban. Phospholamban is known to interact with the Ca^{2+}-ATPase in the sarcoplasmic reticulum membrane and to inhibit its activity. When phosphorylated, phospholamban dissociates from the ATPase, resulting in activation of the enzyme. Presumably this leads to the reduction of $[Ca^{2+}]_i$ and, subsequently, tone in isolated arterial smooth muscle cells and tissue in response to elevations in cGMP levels. Most of the information concerning the regulation of Ca^{2+} flux in the sarcoplasmic reticulum has come from studies in the isolated mammalian heart, where cAMP-dependent protein kinase regulates the phosphorylation of phospholamban. Until now, however, no studies have demonstrated that cGMP-dependent protein kinase catalyzes the phosphorylation of phospholamban in the intact cell. Thus, our results also suggest that cGMP-dependent protein kinase performs a similar regulatory function in vascular smooth muscle.

Regulation of phospholamban phosphorylation and subsequent activation of Ca^{2+}-ATPase may not be the only mechanism of action of cGMP for the reduction of $[Ca^{2+}]_i$. Other investigators have suggested that cGMP may regulate signal transduction in smooth muscle and other cell types. Takai *et al.* (7) were first to propose that cGMP might inhibit PI turnover in platelets. Rapoport (8) provided the first evidence that cGMP inhibits PI hydrolysis and inositol phosphate production in rat aorta. Collins *et al.* (12) and Godfraind (33), on the other hand, provided evidence that cGMP inhibits Ca^{2+} influx in smooth muscle cells, consistent with the findings of Richards *et al.* (34) and Ousterhout and Sperelakis (35) that 8-Br-cGMP inhibits action potential formation in smooth muscle. Subsequently, two recent and provocative reports indicate that cGMP-dependent protein kinase leads to the phosphorylation of G proteins that regulate PI turnover or ion channel activity, or both (9, 10). One may question whether this is the sole mechanism of action of cGMP-dependent protein kinase, however, because no G protein studied to date appears to be a substrate for the kinase *in vitro* (36). It will be critical to demonstrate that phosphorylation of G proteins occurs in the intact cell in response to cGMP, as we have done here for phospholamban, before this proposed mechanism can be accepted. Moreover, our laboratory has demonstrated that cGMP elevations lower $[Ca^{2+}]_i$ in depolarized rat aortic smooth muscle cells and tissues (5, 13, 37, 38). Because depolarization does not increase inositol phosphate production in rat aortic smooth muscle cells (13, 36), inhibition of phospholipase C via G protein phosphorylation (or by any other mechanism, for that matter) cannot totally explain the effects of cGMP on $[Ca^{2+}]_i$. Perhaps most significantly, however, the studies reported here on the localization of cGMP-dependent protein kinase in smooth muscle cells, using immunocytochemical techniques, indicate that areas in the vicinity of the sarcoplasmic reticulum and nucleus contain significant amounts of the enzyme, in comparison with areas outside of this zone. The plasma membrane appears to be devoid of cGMP-dependent protein kinase. However, the presence of cGMP-dependent protein kinase in plasma membrane cannot be totally ruled out, because the fixation procedure used in our protocol may not have preserved membrane-bound antigens. On the other hand, if cGMP-dependent protein kinase catalyzed phosphorylation of plasma

membrane-associated proteins in smooth muscle, such as K^+-channels, G proteins, phospholipases, and Ca^{2+}-calmodulin-activated Ca^{2+}-ATPase, then one would expect to find some of the kinase located here, as it is with the sarcoplasmic reticulum. Based upon these findings, we conclude that the major site of action of cGMP-dependent protein kinase in aortic smooth muscle is the sarcoplasmic reticulum and perhaps nucleus.

The phosphorylation of phospholamban by cAMP-dependent protein kinase in the intact heart (27, 30) and by cGMP-dependent protein kinase in intact smooth muscle cells apparently leads to the same effect (i.e., Ca^{2+}-ATPase activation). The difference physiologically may be that, whereas the cAMP-dependent protein kinase is known to regulate cardiac muscle Ca^{2+} levels by catalyzing phosphorylation of proteins in both the plasma membrane (i.e., the voltage-gated Ca^{2+} channel) and the sarcoplasmic reticulum, cGMP-dependent protein kinase may regulate smooth muscle Ca^{2+} levels by catalyzing phosphorylation of proteins only in the sarcoplasmic reticulum. This would certainly include phospholamban but may include other proteins as well. The IP_3 receptor (M_r 250,000) is known to be a substrate for cAMP-dependent protein kinase *in vitro*, and its phosphorylation has been reported to reduce gating activity (39). It has not been demonstrated that the IP_3 receptor is a substrate for cGMP-dependent protein kinase, but the results in Fig. 9 suggest that a membrane protein of 250 kDa is phosphorylated by both kinases. If the IP_3 receptor is a substrate for cGMP-dependent protein kinase in the intact cell, then this could explain the results of Meisheri *et al.* (11) that atrial peptides inhibit Ca^{2+} release in the intact rabbit aorta. This would also provide an explanation for the greater capacity of cGMP to lower Ca^{2+} in agonist-stimulated (i.e., IP_3-mediated) cells, compared with depolarized cells (37, 38). Conceivably, the inhibition of Ca^{2+} release coupled with the stimulation of Ca^{2+} uptake in the sarcoplasmic reticulum would maintain the low $[Ca^{2+}]_i$ needed for relaxation.

One question raised by these studies is why cGMP-dependent protein kinase catalyzes phosphorylation of phospholamban in smooth muscle cells, especially because phospholamban is a substrate for both kinases *in vitro*. The data on broken cell phosphorylation shown in Figs. 7 and 8 may provide some insight into how protein kinases function in the cell to "identify" their substrates. Under the homogenization conditions used in this study, care was taken to preserve the integrity of the membrane fractions. We hypothesize that cGMP-dependent protein kinase, but not cAMP-dependent protein kinase, remains bound to the membrane fractions containing phospholamban via a high affinity binding interaction. This would explain why only cGMP induces phosphorylation of membrane proteins *in vitro*, including phospholamban. The hypothesis is supported by the immunocytochemical evidence, where, in the intact cell, cGMP-dependent protein kinase is bound to areas corresponding to the sarcoplasmic reticulum. Thus, we suggest that cGMP-dependent protein kinase recognizes a specific binding protein (or proteins) in the sarcoplasmic reticulum membrane, which serves to anchor cGMP-dependent protein kinase in close proximity to its substrates. In this way, phosphorylation would be rapid and efficient in response to cGMP elevations. This is attractive from the physiological point of view, because cGMP-dependent protein kinase is not an abundant protein in cells, and its capacity to catalyze phosphorylation of a protein would be enhanced if it were colocalized with its substrate. In the human neutrophil, for example, the colocalization of cGMP-dependent protein kinase with its substrate in that cell, vimentin, is required for cGMP-stimulated vimentin phosphorylation (40). The difference between the neutrophil and the smooth muscle cell is that cGMP-dependent protein kinase levels are so low in the neutrophil that the enzyme must be transiently localized to the intermediate filaments that contain vimentin. In smooth muscle, however, cGMP-dependent protein kinase appears to be a "resident" protein of the sarcoplasmic reticulum, so that it is poised to catalyze phosphorylation of phospholamban and perhaps other sarcoplasmic reticulum proteins upon the elevation of cGMP or cAMP in the cell.

Acknowledgments

The authors thank Donna Valentine for expert technical assistance. We are indebted to Dr. Larry R. Jones, Krannert Institute of Cardiology, Indiana University, for the antibodies to phospholamban. We thank Dr. Robert Bagnell for assistance with the CLSM studies.

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Send reprint requests to: Thomas M. Lincoln, Department of Pathology, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35233.