Atrial Natriuretic Peptide-dependent Phosphorylation of Smooth Muscle Cell Particulate Fraction Proteins Is Mediated by cGMP-dependent Protein Kinase*

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Boris Sarcevicć, Victoria Brookes, Thomas J. Martin, Bruce E. Kemp, and Phillip J. Robinson§

From the St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

The atrial natriuretic peptide (ANP) stimulates cGMP production and protein phosphorylation in a particulate fraction of cultured rat aortic smooth muscle cells. Three proteins of 225, 132, and 11 kDa were specifically phosphorylated in response to ANP treatment, addition of cGMP (5 nM), or addition of purified cGMP-dependent protein kinase. The cAMP-dependent protein kinase inhibitor had no effect on the cGMP-stimulated phosphorylation of the three proteins but inhibited cAMP-dependent phosphorylation of a 17-kDa protein. These results demonstrate that the particulate cGMP-dependent protein kinase mediates the phosphorylation of the 225-, 132-, and 11-kDa proteins. The 11-kDa protein is phosphorylamban based on the characteristic shift in apparent Mr, from 11,000 to 27,000 on heating at 37 °C rather than boiling prior to electrophoresis. ANP (1 μM) increased the cGMP concentration approximately 4-fold in the particulate fractions, from 4.3 to 17.7 nM, as well as the phosphorylation of the 225-, 132-, and 11-kDa proteins. In contrast, the biologically inactive form of ANP, carboxymethylated ANP (1 μM), did not stimulate phosphorylation of any proteins nor did the unrelated peptide hormone, angiotensin II (1 μM). These results demonstrate the presence of the cGMP-mediated ANP signal transduction pathway in a particulate fraction of smooth muscle cells and the specific phosphorylation of three proteins including phosphorylamban, which may be involved in ANP-dependent relaxation of smooth muscle.

Atrial natriuretic peptide (ANP)* is released by mammalian atria and has diverse biological effects important in regulating blood pressure, electrolyte, and fluid homeostasis (1). These effects include increased salt and water excretion, relaxation of vascular smooth muscle, inhibition of aldosterone, renin, and vasopressin secretion (2), as well as modulation of angiotensin II action (3). Specific ANP receptors have been identified in a range of tissues where the peptide elicits biological responses. These include vascular smooth muscle (4), adrenal, kidney (5), and brain (6). There are a family of receptors with two classes (130 and 66 kDa) in cultured bovine aortic endothelial cells (7). In rat aortic smooth muscle cells, kidney tubular epithelium, and Leydig tumor cells ANP receptors of 140, 120-135, and 66-70 kDa have been reported (8). In general, the ANP receptor types present depend largely on the species and tissue type.

ANP has been shown to stimulate cGMP production in many cell types that contain its receptor (9), but it is not known if increasing the intracellular concentration of cGMP is responsible for all of its actions. The ANP binding activity and guanylate cyclase activity have been reported to co-purify as a 120-130-kDa protein in rat lung (10) and the adrenal gland (11), suggesting that hormone binding and guanylate cyclase activity reside on the same contiguous glycoprotein. This is consistent with the recent report that the complementary DNA clone encoding the membrane form of guanylate cyclase from rat brain consists of a single polypeptide of 1057 amino acids encoding both ANP binding and guanylate cyclase domains (12). These findings suggest that cGMP is the major intracellular messenger for ANP via this form of the receptor and may be responsible for mediating most, if not all, of the intracellular effects of ANP.

The regulatory pathway whereby ANP relaxes vascular smooth muscle is incompletely understood, however, there is considerable evidence that increases in the intracellular concentration of cGMP is associated with smooth muscle relaxation (13, 14). Leitman et al. (9) and others have shown that ANP stimulates cGMP production in aortic smooth muscle cells and early studies by Fiscus et al. (15) indicated that ANP activates cGMP-dependent protein kinase in rat thoracic aorta. Phospholamban from the endoplasmic reticulum of bovine pulmonary artery smooth muscle is one known in vitro substrate of cGMP-dependent protein kinase (16). Other substrates for cGMP-dependent protein kinase of 240, 130, and 85 kDa have been reported in particulate fractions of rabbit aortic smooth muscle (17). Schlichter et al. (18) previously identified a cGMP-dependent protein kinase substrate of 23 kDa in the soluble fraction of rabbit cerebellum and De Jonge (19) has identified a substrate of 86 kDa for this enzyme in intestinal brush-border epithelium. The functions of these proteins are unknown and it is not clear whether there are smooth muscle counterparts.

The aim of the present study was to reconstitute ANP-dependent protein phosphorylation in a particulate fraction from cultured vascular smooth muscle cells in order to identify possible protein substrates whose phosphorylation is regulated by ANP. Three substrates of proteins of 225, 132, and 11 kDa were specifically phosphorylated either directly by cGMP-dependent protein kinase or indirectly by ANP in the...
particulate fraction. The 11-kDa protein was identified as phospholamban on the basis of its electrophoretic properties.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium and tissue culture dishes were obtained from Flow Laboratories. Fetal calf serum, streptomycin, and penicillin were from Commonwealth Serum Laboratories (Melbourne, Australia). Fungizone and glutamine were from Gibco. Collagenase, elastase, leupeptin, cAMP, cGMP, angiotensin II, catalytic subunit of cAMP-dependent protein kinase, and phosphatidylserine were from Sigma. 3-Isobutyl-1-methylxanthine (IBMX) was from Aldrich Chemical Co. [γ-32P]ATP was from Du Pont-New England Nuclear. Human atrial natriuretic peptide (1–28) was a gift from Dr. D. F. Veber of Merck Sharp and Dohme. Porcine brain natriuretic peptide was from Auspep. GTP was from Boehringer Mannheim. Phorbol 1-myristate 2-acetate was from Avanti Polar Lipids. Electrophoretic materials were from Bio-Rad. Calmodulin was purified from sheep heart as described previously (23).

Preparation of particulate fractions was from Bio-Rad. Collagenase, prepared as described previously (23). Chemical Co. [γ-32P]ATP was from Du Pont-New England Nuclear. Fetal calf serum, streptomycin, and penicillin were from Gibco. Collagenase, elastase, leupeptin, cAMP, cGMP, angiotensin II, catalytic subunit of cAMP-dependent protein kinase, and phosphatidylserine were from Sigma. 3-Isobutyl-1-methylxanthine (IBMX) was from Aldrich Chemical Co. [γ-32P]ATP was from Du Pont-New England Nuclear. Human atrial natriuretic peptide (1–28) was a gift from Dr. D. F. Veber of Merck Sharp and Dohme. Porcine brain natriuretic peptide was from Auspep. GTP was from Boehringer Mannheim. Phorbol 1-myristate 2-acetate was from Avanti Polar Lipids. Electrophoretic materials were from Bio-Rad. Calmodulin was purified from sheep heart as described previously (23).

Preparation of Cultured Smooth Muscle Cell Particulate Fractions—Confluent cells from 1 500-cm2 tray were washed twice with 50 ml of warm (37 °C) phosphate-buffered saline buffer (155 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.3) with EDTA added to a final concentration of 50 μM. The cells were harvested by scraping in 40 ml of ice-cold phosphate-buffered saline containing 1 mM EDTA, 1 mM EGTA, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin. The cells were pelleted at 1,000 × g for 5 min, then resuspended in 4 ml of ice-cold lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 100 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized in a 10-ml Wheaton Teflon/glass homogenizer. The homogenate was centrifuged at 1,000 × g for 5 min at 4 °C and the membrane-rich supernatant fraction collected. The pellet was resuspended in a further 4 ml of lysis buffer, homogenized, and centrifuged to obtain a second supernatant fraction which was pooled with the first. The membrane-rich supernatant was centrifuged at 100,000 × g for 30 min at 4 °C. The pellet was resuspended in 1 ml of 20 mM Tris-HCl, 100 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 100 μg/ml leupeptin, pH 7.4, and stored on ice. Protein concentrations were determined using the Amido Black method (25).

Protein Phosphorylation—Phosphorylation experiments were conducted on freshly prepared particulate fractions. The standard phosphorylation reaction mixture contained 50 mM Tris-HCl, pH 7.6, 15 mM MgCl2, 1 mM EGTA, 500 μM IBMX, 1 mM GTP, and 1 μM ANP in a final volume of 60 μl and was initiated by the addition of 20 μl of particulate fraction. The samples were incubated for 1 min at 37 °C and then the reaction was terminated by adding 940 μl of ice-cold 1 mM ethylenediaminetetraacetic acid (95% ethanolic acidified with HCl to apparent pH 3). After storage overnight at 4 °C, the ethanolic extracts were dried in a boiling water bath, redissolved in 500 μl of 50 mM sodium acetate buffer, pH 5.0, containing 1 mM theophylline, acetylated, and assayed for cGMP as previously described (27).

Reduction and Carboxymethylation of ANP—ANP (120 μg) was dissolved in 200 ml of 100 mM Tris-HCl, pH 8.1, 1 mM EDTA, 4 mM guanidine HCl. The solution was flushed with nitrogen for 1 min, sealed, and incubated at 50 °C for 30 min. Dithiotreitol was added to a final concentration of 50 mM, the tube flushed briefly with nitrogen and maintained at 50 °C for 4 h. The solution was then cooled to room temperature, and aqueous iodoacetic acid added to a final concentration of 100 mM and left in the dark overnight. The carboxymethylated ANP was purified by reversed-phase high performance liquid chromatography (RP 300 microbore column, 2.1 X 30 mm, Brownlee Labs) using a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid over 30 min at 1 ml/min. Each step of the fractionation was then cooled in an ice water slurry (0 °C) for 3 min. The phosphorylation reaction was then initiated by adding [γ-32P]ATP to a final concentration of 20 μM (12.5 Ci/mmol) and incubating for 1 min at 0 °C. The phosphorylation reactions were terminated by adding 33 μl of SDS buffer and immediately transferred to dry ice. The samples were then boiled for 2 min and 80 μl subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis and Autoradiography—The samples were subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (26) using a linear 7.5–20% acrylamide gradient (140 V overnight at 10 °C). Following electrophoresis the proteins were fixed with 20% trichloroacetic acid, stained with Coomassie Brilliant Blue, dried under vacuum, and exposed to Kodak X-AR, Kodak X-Omat K, or Du Pont Cronex video imaging film.

Particulate Guanylate Cyclase Assay—Guanylate cyclase activity was determined in a buffer containing 30 mM Tris-HCl, pH 7.6, 15 mM MgCl2, 1 mM EGTA, 500 μM IBMX, 1 mM GTP, and 1 μM ANP in a final volume of 60 μl and was initiated by the addition of 20 μl of particulate fraction. The samples were incubated for 1 min at 37 °C and then the reaction was terminated by adding 940 μl of ice-cold 1 mM ethylenediaminetetraacetic acid (95% ethanolic acidified with HCl to apparent pH 3). After storage overnight at 4 °C, the ethanolic extracts were dried in a boiling water bath, redissolved in 500 μl of 50 mM sodium acetate buffer, pH 5.0, containing 1 mM theophylline, acetylated, and assayed for cGMP as previously described (27).

Reagents—Reagents were from Sigma.

Materials and Methods—Materials and Methods.

Fig. 1. Autoradiograph showing serine/threonine protein kinase substrates in smooth muscle cell particulate fractions. Particulate fractions were incubated with [γ-32P]ATP at 37 °C for 1 min in the presence of EGTA (lanes 1 and 4) or with the activators (lanes 2, 3, and 5–7) of the different classes of protein kinases (see “Materials and Methods”) and subjected to SDS-polyacrylamide gel electrophoresis. The autoradiograph shows that the cultured rat aortic smooth muscle cell particulate fraction contains all four major classes of serine/threonine protein kinases as well as endogenous substrates. The position of standard molecular weight marker proteins is shown on the right. This experiment is representative of three separate experiments with the same pattern of protein phosphorylation. PS, phosphatidylserine.

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**ANP-dependent Protein Phosphorylation**

TABLE I

| Protein kinase | M, (kDa) |
|---------------|---------|
| cAMP          | 17,000  |
| cGMP          | 11,000  |
| Protein kinase C | 19,000 |
| Ca²⁺/calmodulin | 11,000 |
|                | 32,000  |
|                | 31,000  |
|                | 21,000  |
|                | 15,000  |
|                | 20,000  |
|                | 225,000 |
|                | 22,000  |
|                | 24,000  |
|                | 25,000  |
|                | 34,000  |
|                | 43,000  |
|                | 53,000  |
|                | 73,000  |

Fig. 2. Temperature-dependent dissociation of a 27-kDa cGMP-dependent protein kinase substrate. Smooth muscle cell particulate fractions were phosphorylated for 1 min under standard reaction conditions as described under "Materials and Methods," in the presence of varying concentrations of cAMP (panel A, lanes 2-6) and cGMP (panel B, lanes 2-8) at 0 °C. The apparent M, of the proteins phosphorylated in response to cGMP is indicated with arrows. The position of standard molecular weight marker proteins is shown on the left. This experiment is representative of three separate experiments with the same pattern of protein phosphorylation.

Fig. 3. Cyclic nucleotide concentration dependence of protein phosphorylation at 0 °C. Smooth muscle cell particulate fractions were phosphorylated for 1 min under standard reaction conditions as described under "Materials and Methods," in the presence of varying concentrations of cAMP (panel A, lanes 2-6) and cGMP (panel B, lanes 2-8) at 0 °C. The apparent M, of the proteins phosphorylated in response to the cyclic nucleotides is indicated with arrows. The position of standard molecular weight marker proteins is shown on the left. This experiment is representative of three separate experiments with the same pattern of protein phosphorylation.

Results

Protein Kinases and Substrates—The presence of endogenous protein kinases and substrates in cultured vascular smooth muscle cell particulate fractions was investigated by adding a variety of exogenous regulators in the presence of [γ-32P]ATP and separating the resultant proteins on SDS-polyacrylamide gels. The particulate fraction contained the cAMP-dependent, cGMP-dependent, calcium/calmodulin-dependent, and calcium/phospholipid-dependent, protein kinases as well as protein substrates for these kinases (Fig. 1). The most prominent phosphoproteins are the calcium/calmodulin-dependent protein kinase substrate proteins (Fig. 1 and Table I). These include substrates of 53, 43, and 34 kDa. Four protein kinase C substrates are phosphorylated with molecular weights 32,000, 31,000, 21,000, and 19,000, in the presence of calcium/phorabol 1-myristate 2-acetate/phosphatidylserine. Both cGMP and cAMP stimulated the phosphorylation of proteins of molecular weights 225,000, 132,000, and 11,000, and in addition, cAMP stimulated the phosphorylation of proteins of molecular weights 17,000, 26,000, 43,000, and 151,000. The 11-kDa protein whose phosphorylation was stimulated by cGMP and cAMP displayed a temperature-dependent mobility shift in polyacrylamide gels. This protein migrated at an apparent 27 kDa if the sample was heated at 37 °C prior to electrophoresis or conversely the protein migrated at an apparent 11 kDa if the sample was boiled prior to electrophoresis (Fig. 2).

In order to determine whether the phosphorylation of the 225-, 132-, and 11-kDa proteins was mediated by cAMP- or cGMP-dependent protein kinase, the following three experiments were undertaken. In the first experiment, the phosphorylation of these three proteins was stimulated by cGMP in a concentration-dependent manner (Fig. 3B). The degree of cGMP-dependent phosphorylation was greater at 0 °C (Fig. 3B) than at 37 °C (Fig. 4B) and the concentration-response curve was shifted to lower concentrations of cGMP at 0 °C. At 0 °C the minimum concentration of cGMP required to stimulate the phosphorylation of these proteins was 5 nM, as shown in Fig. 3B, whereas at 37 °C the minimum concentra-
FIG. 4. Cyclic nucleotide concentration dependence of protein phosphorylation at 37 °C. Smooth muscle cell particlate fractions were phosphorylated for 1 min under standard reaction conditions as described under "Materials and Methods," in the presence of varying concentrations of cAMP (panel A, lanes 2-6) and cGMP (panel B, lanes 2-8) at 37 °C. The apparent M, of the proteins phosphorylated in response to the cyclic nucleotides is indicated with arrows. The position of standard molecular weight marker proteins is shown on the left. This experiment is representative of three separate experiments with the same pattern of protein phosphorylation.

stimulated phosphorylation of the 17-kDa protein appeared higher at 37 °C than at 0 °C and the minimal concentration of cAMP required to stimulate phosphorylation of this protein was similar at both temperatures (Figs. 3A and 4A).

The cAMP-dependent protein kinase inhibitor peptide (PKI5-22) was used to distinguish between the actions of cAMP- and cGMP-dependent protein kinases. PKI5-22 specifically inhibited the cAMP-dependent phosphorylation of the 17-kDa protein in a concentration-dependent manner, with 100% inhibition at 0.5 μM PKI5-22, indicating that this protein was a substrate of CAMP-dependent protein kinase (Fig. 5). However, the CAMP- and cGMP-stimulated phosphorylation of the three proteins (225,000, 132,000 and 11,000) was not inhibited by PKI5-22, even at concentrations as high as 1 μM, indicating that their phosphorylation was not catalyzed by cAMP-dependent protein kinase. These results indicate that both exogenous cAMP and cGMP may activate the cGMP-dependent protein kinase and phosphorylate the three proteins.

Addition of purified protein kinases was also employed to verify the endogenous substrate preferences of the two protein kinases. When exogenous cGMP-dependent protein kinase was added to the particulate fractions, the 225-, 132-, and 11-kDa proteins were specifically phosphorylated, whereas exogenous catalytic subunit of CAMP-dependent protein kinase only phosphorylated the 17- and 225-kDa proteins at the concentrations used (Fig. 6).

On the basis of the criteria of 1) cyclic nucleotide dependence, 2) insensitivity to the cAMP protein kinase inhibitor, and 3) capacity to act as substrates for exogenous cGMP-dependent protein kinase and not catalytic subunit of CAMP-dependent protein kinase, the phosphoproteins of 132 and 11 kDa are therefore preferred substrates for the cGMP-dependent protein kinase only phosphorylated the 17- and 225-kDa proteins at the concentrations used.

ANP-dependent cGMP Production and Protein Phosphorylation—It was of interest to test whether the three cGMP-dependent protein kinase substrates could be part of a reconstituted ANP-signal transduction pathway. The effect
FIG. 6. Effect of exogenous catalytic subunit of cAMP-dependent protein kinase and exogenous cGMP-dependent protein kinase. Smooth muscle cell particulate fractions were phosphorylated in the presence of exogenous catalytic subunit of cAMP-dependent protein kinase at varying concentrations (left panel, one-tenth = 0.43 µg/ml) and exogenous cGMP-dependent protein kinase at varying concentrations (right panel, one-tenth = 6 µg/ml). The latter was used in combination with PKIβ-22 (500 nM). For comparison of endogenous phosphorylation by cAMP- and cGMP-dependent protein kinases, cAMP (lane 3, panel A; lane 2, panel B) and cGMP (lane 2, panel A; lane 3, panel B) were added to a final concentration of 1.0 µM. The position of standard molecular weight marker proteins is shown on the left.

TABLE II

ANP stimulation of guanylate cyclase activity in the particulate fraction of smooth muscle cells

| Variable | cGMP (nM) |
|----------|-----------|
| Control  | 3.04 ± 0.03 |
| GTP (1 mM) | 4.30 ± 1.11 |
| ANP (1 µM) + GTP (1 mM) | 17.72 ± 0.76* |

*p < 0.001 (Student's t test).

of ANP on particulate guanylate cyclase activity was determined by incubating the particulate fraction with 1 µM ANP for 1 min at 37 °C. Addition of 1 mM GTP alone to the particulate fraction increased the basal concentration of cGMP from 3.0 to 4.3 nM. Addition of 1 mM GTP and 1 µM ANP to the particulate fraction further increased the concentration of cGMP approximately 4-fold to 17.7 nM as shown in Table II. Since ANP-dependent production of cGMP was favored at 37 °C and the most sensitive dose-response for cGMP-dependent protein phosphorylation was at 0 °C, the assay of ANP-dependent protein phosphorylation was performed in two parts at these temperatures. Addition of GTP alone had a small stimulatory effect on the phosphorylation of the 225–, 132–, and 11-kDa proteins due to the basal levels of cGMP produced, however, when 1 µM ANP and 1 mM GTP were added, the phosphorylation of these proteins was increased in a manner similar to the increase observed on addition of cGMP (Figs. 7 and 8). The same result was obtained in the presence of the cAMP-dependent protein kinase inhibitor PKIβ-22 (1 µM). These results demonstrate that ANP-dependent protein phosphorylation is mediated through cGMP-dependent protein kinase. Since the biological activity of ANP requires a disulfide cross-bridge within the peptide (28), we reduced and carboxymethylated ANP to produce a biologically inactive form. Carboxymethylated ANP did not stimulate the phosphorylation of any proteins nor did...
the unrelated peptide hormone, angiotensin II (not shown). However, the closely related brain natriuretic peptide produced results indistinguishable from ANP (not shown).

DISCUSSION

The biochemical mechanism whereby ANP relaxes vascular smooth muscle is incompletely understood. Various laboratories have shown that ANP binds to specific membrane-bound receptors on smooth muscle cells, one of which activates the membrane-bound guanylate cyclase, hence increasing the intracellular concentration of cGMP (9). Observations that agents such as nitrovasodilators (29) and ANP (15) which activate the membrane-bound guanylate cyclase, hence increasing the intracellular concentration of cGMP in a time- and dose-dependent manner correlate with smooth muscle relaxation, and that 8-bromo-cGMP, an analogue of cGMP produces a dose-dependent decrease in smooth muscle tension (30), suggest that relaxation in vascular smooth muscle is mediated by cGMP. It has recently been shown that ANP decreases the agonist-induced increase in intracellular calcium concentrations in cultured rat aortic smooth muscle cells (31). This effect appears to be mediated by cGMP-dependent protein kinase as cells repetitively passaged lose their responsiveness to cGMP concurrently with the loss of this enzyme. Therefore, many if not all, of the effects of cGMP appear to be mediated by cGMP-dependent protein kinase (32).

In the present studies, three distinct substrate proteins of cGMP-dependent protein kinase of 225, 132, and 11 kDa have been identified in the particulate fraction of cultured rat aortic smooth muscle cells. Low concentrations of exogenous cGMP (5 nM) specifically stimulated the phosphorylation of these three proteins in the particulate fraction. Although phosphorylation of these proteins can also be stimulated by exogenous cAMP, three criteria were used to rule out the possibility that these were also substrates for cAMP-dependent protein kinase under the present assay conditions and that this stimulatory effect is due to activation of cGMP-dependent protein kinase. First, higher concentrations of cAMP (100 nM) than cGMP (5 nM) were required and second, PKI_5-22 did not inhibit the phosphorylation of these proteins, clearly demonstrating that their phosphorylation is not mediated by cAMP-dependent protein kinase. Furthermore, exogenous cGMP-dependent protein kinase phosphorylated all three proteins while the exogenous catalytic subunit of cAMP-dependent protein kinase did not phosphorylate the 132- and 11-kDa proteins. Previously, Fiscus et al. (15) demonstrated that ANP activates cGMP-dependent protein kinase activity ratios in rat thoracic aortic strips, but substrate protein phosphorylation was not examined. ANP stimulation of protein phosphorylation has previously been reported in the placental membrane fraction where ANP stimulated the phosphorylation of a 130-kDa protein (33). The phosphorylation of this protein was stimulated by addition of exogenous cGMP and not cAMP, suggesting that phosphorylation was mediated by cGMP-dependent protein kinase. Our results are in general agreement with and significantly extend a preliminary communication by Silver et al. (34). They have reported that ANP increased the phosphorylation of 3 proteins of 259, 131, and 108 kDa in the particulate fraction of rabbit aortic smooth muscle when phosphorylated in the presence of Triton X-100. Only the 108-kDa protein was also found to be phosphorylated in the presence of cGMP. However, it was not determined whether these substrates were associated with smooth muscle cells or endothelial cells.

ANP stimulated guanylate cyclase activity and increased cGMP from a basal level of 4.3 to 17.7 nM in the particulate fraction of cultured rat aortic smooth muscle cells. The concentration of cGMP produced was sufficient to activate cGMP-dependent protein kinase, which subsequently phosphorylated its substrate proteins. A crucial variable in studying the level of cGMP-dependent phosphorylation of these three cGMP-dependent protein kinase substrate proteins was the temperature. The threshold concentrations of cGMP required at 0 °C were much lower than at 37 °C to achieve the same stimulation of phosphorylation. The reason for the temperature sensitivity may be due to endogenous phosphatase activity at 37 °C as reported recently (35). Carboxymethylated ANP (1 μM) had no effect on the phosphorylation state of any of the cGMP-dependent protein kinase substrates nor did angiotensin II (1 μM). These results indicate that stimulation of guanylate cyclase activity and the subsequent activation of cGMP-dependent protein kinase is specific for biologically active ANP.

Substrate proteins of cGMP-dependent protein kinase of 240, 130, and 85-kDa, termed GS₉, GS₁, and GS₂ respectively, have previously been reported in the particulate fraction of rabbit aortic smooth muscle (17). The 225- and 132-kDa proteins reported in our work may be rat homologues of GS₉ and GS₁. A rabbit 85-kDa substrate (GS₂) thought to be a proteolytic fragment of GS, was absent from the particulate fraction of cultured rat aortic smooth muscle cells. Another difference is the presence in rat particulate fractions of an 11-kDa cGMP-dependent protein kinase substrate protein which has not been reported in the rabbit particulate fractions. This protein behaved like phospholamban, migrating at an apparent 27 kDa when heated at 37 °C prior to electrophoresis, or 11 kDa when boiled prior to electrophoresis (16, 36, 37). Phospholamban has recently been shown to be present in smooth muscle and has also been shown to be an in vitro substrate of cGMP-dependent protein kinase (16). Phospholamban is believed to be important in modulating intracellular calcium transport in cardiac muscle (38, 39). Raeymaekers et al. (16) have shown that phosphorylation of phospholamban by cGMP-dependent protein kinase concomitantly increased the affinity of the calcium pump for calcium in dog ventricular sarcoplasmic reticulum preparations. Therefore one of the ways ANP may mediate its relaxant effects on smooth muscle is by decreasing the free intracellular calcium concentration...
resulting from cGMP-dependent phosphorylation of phospholamban. This is consistent with the report by Cornwell and Lincoln (31) that ANP and 8-bromo-cGMP decrease intracellular calcium as measured by fura-2 in cultured rat aortic smooth muscle cells. On the other hand Paglin et al. (40) reported that ANP did not alter the free intracellular calcium measured with aequorin.

In conclusion we have reconstituted ANP-mediated protein phosphorylation in a cultured vascular smooth muscle cell particulate fraction. ANP stimulates production of cGMP, activation of cGMP-dependent protein kinase, and phosphorylation of three distinct substrate proteins of 225-, 132-, and the 11-kDa phospholamban. These results are consistent with the concept that relaxation of smooth muscle by ANP results in part from cGMP-mediated phosphorylation of phospholamban.

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