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Effects of 8-bromo-cGMP on Ca^{2+} levels in vascular smooth muscle cells: Possible regulation of Ca^{2+}-ATPase by cGMP-dependent protein kinase

(phosphorylation/smooth muscle relaxation)

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ABSTRACT **The effects of 8-bromo-cGMP on intracellular calcium concentrations in cultured rat aortic smooth muscle cells were studied. Both angiotensin II and depolarizing concentrations of K^+ stimulated Ca^{2+} accumulation in the cytoplasm. The increase in Ca^{2+} due to angiotensin II was associated with an increase in inositol phosphates, while that due to K^+ was not. Preincubation of cells with 8-bromo-cGMP (100 μM) caused an inhibition of peak Ca^{2+} accumulation to either angiotensin II or K^+. To probe the mechanism of action of cGMP in vascular smooth muscle, the effects of cGMP-dependent protein kinase on Ca^{2+}-ATPase from the cultured cell particulate material were investigated. Ca^{2+}-activated ATPase was stimulated ≈2-fold by exogenous calmodulin and up to 4-fold by low concentrations of purified cGMP-dependent protein kinase. The inclusion of both calmodulin and cGMP-dependent protein kinase resulted in an additive stimulation of Ca^{2+}-ATPase. Stimulation of Ca^{2+}-ATPase activity was observed at all Ca^{2+} concentrations tested (0.01–1.0 μM). cAMP-dependent protein kinase catalytic subunit and protein kinase C were either ineffective or less effective than cGMP-dependent protein kinase in stimulating the Ca^{2+}-ATPase from rat aortic smooth muscle cells. These results suggest a possible mechanism of action for cGMP in mediating decreases in cytosolic Ca^{2+} through activation of a Ca^{2+}-ATPase and the subsequent removal of Ca^{2+} from the cell.**

cGMP is now considered to be an important regulator of vascular smooth muscle tone (1). Several smooth muscle relaxants including nitrogen oxide-containing vasodilators (2–4), endothelial-derived relaxing factors (5, 6), and atrial natriuretic peptides (7, 8) stimulate cGMP production in vascular smooth muscle. In addition, many of these agents have been shown to inhibit Ca^{2+}-stimulated enzymes such as phosphorylase kinase and myosin light chain kinase (9, 10) in aortic smooth muscle, suggesting that one major action of cGMP is to reduce the levels of free intracellular Ca^{2+}. However, direct evidence for a role for cGMP in lowering cell Ca^{2+} is lacking.

Intracellular Ca^{2+} concentrations in eukaryotic cells are several orders of magnitude lower than those found in the extracellular fluid. This is due in part to the low permeability of the plasma membrane to Ca^{2+} and to the presence of efficient Ca^{2+} transporting systems found in the plasma membrane and organelles, such as the endoplasmic reticulum. If cGMP affects Ca^{2+} levels in smooth muscle, then it might act to prevent increases in intracellular Ca^{2+} release or it might affect one or more Ca^{2+}-ATPases present in particulate fractions from smooth muscle cells.

A major problem encountered when studying Ca^{2+}-ATPases from membrane fractions is the presence of a large amount of Mg^{2+}-ATPase associated with plasma membranes. Some of this Mg^{2+}-ATPase appears to be derived from contractile proteins and other filamentous material (unpublished observations). Although whole blood vessels have been used for studying Ca^{2+} fluxes and Ca^{2+}-ATPases, primary cultures of smooth muscle cells may offer some advantages over intact tissues. Ca^{2+} concentrations can be measured directly using fluorometric probes such as fura-2. In addition, the cells respond to a variety of hormones such as angiotensin II (Ang-II) and atriopeptins, making these cultures a valuable model system for studying cyclic nucleotide effects, Ca^{2+} levels, and other events.

EXPERIMENTAL PROCEDURES

Cell Culture. Primary rat aortic smooth muscle cells were prepared by collagenase digestion using the method of Smith and Brock (11) and were used during passages 6–13. Cells were grown in 75-cm^2 plastic tissue culture flasks or 60-mm^2 Petri dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing 100 units of penicillin G per ml, 100 μg of streptomycin per ml, and in some instances 2.5 μg of amphotericin B per ml. All experiments were performed with confluent cultures that had been subcultured (1:3) by brief treatment with 0.05% trypsin.

Measurement of Intracellular Calcium. Cells were grown to confluency on glass coverslips, which were fitted into a cuvette holder of Perkin-Elmer LS5 recording fluorometer. Cells were loaded with fura-2,AM (acetoxymethyl ester of fura-2; 5 μM) for 20 min followed by a 20-min incubation in medium lacking fura-2. This procedure allowed for the complete hydrolysis of the acetoxymethyl ester of fura-2, yielding the free acid. Recordings were made with an excitatory wavelength of 336 nm and an emission wavelength of 500 nm as described (12). At the end of the experimental treatment, cells were treated with saponin (50 μg/ml) followed by 1 mM $MnCl_2$ to obtain maximal and minimal fluorescence, respectively.

Measurement of Inositol Phosphates in Rat Aortic Vascular Smooth Muscle Cells. Cells were grown in 60-mm^2 Petri dishes for 48 hr in culture medium containing *myo*-[^{3}H]inositol (2 μCi/ml; 1 Ci = 37 GBq) supplemented with 0.7 μM unlabeled *myo*-inositol. For the determination of labeled inositol phosphates, dishes were rinsed once with a balanced salt solution consisting of 120 mM NaCl/5 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/20 mM Hepes, pH 7.4, and treated with buffer containing no additions, 100 nM Ang-II or 40 mM KCl. The procedure was terminated by removing the buffered medium

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Abbreviations: Ang-II, angiotensin II; InsP_3, inositol 1,4,5-trisphosphate; InsP_2, inositol 1,4-bisphosphate.

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by aspiration followed by the addition of 1 ml of ice-cold 0.2 M formic acid. The plates were scraped using a plastic cell scraper and the material was centrifuged at 1000 × *g* for 1 min. The supernatants were applied to Dowex-1 (formate) columns and the inositol phosphates were eluted according to the procedure of Downes and Michell (13). The elution of the various inositol phosphates was determined using radioactive standards.

Preparation of Smooth Muscle Cell Microsomes. Microsomes were prepared as follows: culture medium was removed by aspiration and the cells were rinsed twice with low calcium (0.15 mM) Hank's balanced salt solution. The cells from six to eight flasks were removed, washed once with balanced salt solution, and suspended at 4°C in 2 ml of a buffer containing 50 mM Hepes (pH 7.4), 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 15 mM 2-mercaptoethanol, and 0.25 M sucrose. Homogenization was performed in a 15-ml glass Dounce homogenizer with two strokes and the resulting suspension was transferred to a 12- × 75-mm tube. An additional cell disruption step was then performed using a Polytron homogenizer equipped with a microshear blade. Homogenization was carried out twice for 15 sec each at a setting of 50. The resulting suspension was centrifuged at 1000 × *g* for 5 min to remove debris and intact nuclei, and the supernatant fraction was centrifuged at 100,000 × *g* for 45 min in a Beckman type 50 ultracentrifuge rotor. The pellet was resuspended in 10 mM Tris·HCl (pH 7.2) containing 0.25 M sucrose and 1 mM dithiothreitol.

Ca^{2+}-ATPase Assay. Ca^{2+}-ATPase activity was measured using the charcoal method described (14). Microsomal protein (5–10 μg) was assayed for ATPase activity in 150 μl of a reaction mixture containing (except where indicated) 20 mM imidazole chloride (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 1 μM free calcium (buffered with 0.5 mM EGTA), and 40 μM [γ-^{32}P]ATP. cGMP-dependent protein kinase (cGMP kinase) when present was 0.8 unit (0.02 μM). Reactions were started with the addition of ATP and the tubes were incubated at 37°C for 5 min unless otherwise stated. The assay was terminated by the addition of cold trichloroacetic acid (final concentration, 5%) and protein was coprecipitated with bovine serum albumin (10 mg/ml) in a solution of KH_2PO_4 (13 mg/ml). After centrifugation at 1000 × *g* for 5 min, activated charcoal (50 mg/ml) was added to the supernatant fraction. The suspension was mixed and the charcoal was removed by centrifugation. Aliquots of the supernatant were removed and $^{32}P_i$ was determined by scintillation counting. Blank values (<0.01% of total counts) were subtracted from the experimental values. Total specific ATPase activity is defined as nmol of P_i released per min per mg of protein in the presence of Mg^{2+} and Ca^{2+}; Ca^{2+}-ATPase is defined as total ATPase minus that observed in the presence of Mg^{2+} only.

Other Methods. Free Ca^{2+} concentrations were determined using the Ca^{2+}-EGTA buffer system described [log K_a (Ca-EGTA) = 5.98 at pH 7.0] (15). Free Ca^{2+} concentrations were checked independently with a Ca^{2+}-sensitive electrode. cGMP kinase was purified by affinity chromatography (16); the catalytic subunit of the cAMP-dependent protein kinase was purified by the method of Sugden *et al.* (17). Protein was determined by the method of Bradford (18). [γ-^{32}P]ATP was prepared by the method of Walseth and Johnson (19).

Materials. Protein kinase C was generously supplied by Surendra Baliga (Department of Pediatrics, University of South Alabama). Dulbecco's modified Eagle's medium was purchased from GIBCO; fetal bovine serum was from Hyclone, Logan, UT. Fura-2,AM was purchased from Molecular Probes, Junction City, OR. Antibiotics were supplied by Sigma and ^{32}P was purchased from ICN. *myo*-[2-^{3}H]Inositol was purchased from Amersham.

RESULTS AND DISCUSSION

Effects of 8-Br-cGMP on Ca^{2+} Levels in Cultured Rat Aortic Smooth Muscle Cells. Fig. 1 shows the effects of 40 mM KCl on intracellular Ca^{2+} concentrations in fura-2-loaded cells. In Fig. 1*A*, KCl produced an ≈10-fold increase in intracellular Ca^{2+} levels. Preincubation of cells for 1 min with 1.5 mM $LaCl_3$, the inorganic Ca^{2+} antagonist, blocked the increase in Ca^{2+} due to K^+-induced depolarization (Fig. 1*B*). In contrast, cells were still responsive to Ang-II, suggesting that Ang-II acts primarily to mobilize intracellular Ca^{2+}. Because intracellular mobilization is believed to be a result of the accumulation of intracellular inositol phosphates, especially inositol 1,4,5-trisphosphate (InsP_3), the effects of KCl and Ang-II on InsP_3 were determined. As shown in Table 1, Ang-II produced a marked increase in InsP_3 and inositol 1,4-bisphosphate (InsP_2) and had a smaller effect on inositol monophosphate. KCl, on the other hand, had no effect on InsP_2 or InsP_3. Thus, the source of Ca^{2+} mobilized by Ang-II was intracellular, while K^+ appeared to elevate Ca^{2+} through a Ca^{2+} uptake mechanism. The effects of Ang-II on increasing intracellular Ca^{2+} appeared to be receptor linked, since pretreatment of cells for 1 min with the Ang-II receptor antagonist [Sar^1,Leu^8]Ang-II blocked subsequent Ang-II responses (Fig. 2, bottom tracing). Preincubation of fura-2-loaded cells for 15 min with a maximally effective concentration of 8-Br-cGMP (100 μM) decreased Ca^{2+} levels due to either Ang-II (Fig. 2, middle tracing) or KCl (Fig. 3). Thus, the inhibition of Ca^{2+} transients by cGMP occurred regardless of the source of Ca^{2+}—i.e., whether Ca^{2+} entered through the plasma membrane (KCl) or was mobilized from the sarcoplasmic reticulum (Ang-II). This suggested that the mechanism by which cGMP lowered intracellular Ca^{2+} was not related to inhibition of Ca^{2+} uptake or to the inhibition of intracellular Ca^{2+} release. It seemed likely, therefore, that cGMP decreased intracellular Ca^{2+} levels by enhancing its removal from the cytoplasm. This possible mechanism was particularly interesting in light of recent reports suggesting that cGMP enhanced Ca^{2+}-ATPase activity from uterine smooth muscle membranes (20).

Characterization of ATPase Activity of Cultured Rat Aortic Smooth Muscle Cells. High-speed particulate fractions from rat aortic smooth muscle cells contained several ATPase activities representing different enzymes or transport pro-

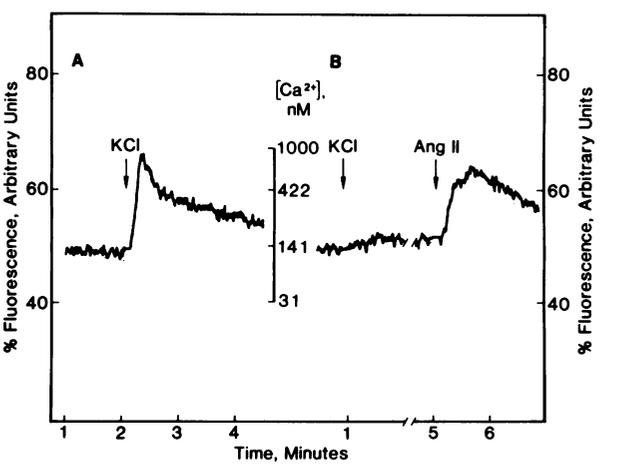

FIG. 1. Effect of KCl and Ang-II on Ca^{2+} concentrations in fura-2-loaded rat aortic smooth muscle cells. Cells were cultured on coverslips as described and incubated with fura-2,AM. At the times indicated, cells were treated with either 40 mM KCl or 10 nM Ang-II. (*A*) KCl was added to control (untreated cells). (*B*) Cells were preincubated in Ca^{2+}-free salt solution containing 1.5 mM $LaCl_3$ for 1 min before the addition of KCl and Ang-II.

Table 1. Effects of Ang-II and KCl on inositol phosphate accumulation in cultured rat aortic smooth muscle cells

	cpm per plate, $\times 10^{-3}$		
	Ins*P*	InsP_2	InsP_3
None	11.02 ± 1.07	0.30 ± 0.02	0.49 ± 0.02
KCl (40 mM)	12.24 ± 0.49	0.29 ± 0.02	0.53 ± 0.02
Ang-II (100 nM)	15.05 ± 0.27	2.43 ± 0.14*	7.67 ± 0.14*

Cells were plated in 60-mm^2 plastic Petri dishes and incubated for 48 hr in 0.7 μM *myo*-[2-^{3}H]inositol. The monolayers were treated with the various compounds for 10 sec and inositol phosphates were measured as described. The data are presented as the mean ± SEM for three separate determinations.
*Statistical significance from untreated cells with $P < 0.01$.

teins, or both. As shown in Table 2, ATPase activity in the absence of exogenous Mg^{2+} and Ca^{2+} was ≈1/10 the maximal ATPase activity observed in the presence of 5 mM Mg^{2+} and 1 μM free Ca^{2+}. The omission of Mg^{2+} from the ATPase assay caused a >50% reduction in ATPase activity, suggesting that most of the ATP hydrolytic activity was dependent on this divalent cation. Ca^{2+}-ATPase was that activity present in membranes after subtracting Mg^{2+}-ATPase activity from Ca^{2+}- plus Mg^{2+}-ATPase activity. It was found that Ca^{2+} alone added to the assay increased basal activity by >3-fold; however, this was not equivalent to the Ca^{2+}-ATPase activity, most likely because Ca^{2+} partially substitutes for nonspecific divalent cation-stimulated ATPases. It was also observed that activity measured in the presence of Ca^{2+} alone and that measured in the presence of Mg^{2+} alone did not equal Ca^{2+}- plus Mg^{2+}-ATPase activity. This could have been due to the presence of a Mg^{2+}-dependent inhibitor of Ca^{2+}-ATPase in these crude fractions (21). When the Ca^{2+}-ATPase was determined as described, it was found that it accounted for ≈10% of the ATP hydrolytic activity. Similar findings have been observed for other tissues (22, 23). Under the assay conditions described here, mitochondrial ATPase did not appear to be a significant factor in that removal of the mitochondria by centrifugation for 20 min at 10,000 × *g* did not affect total ATPase activity (data not shown).

Effects of cGMP Kinase on Ca^{2+}-ATPase Activity. The effect of cGMP kinase on Ca^{2+}-ATPase activity is shown in

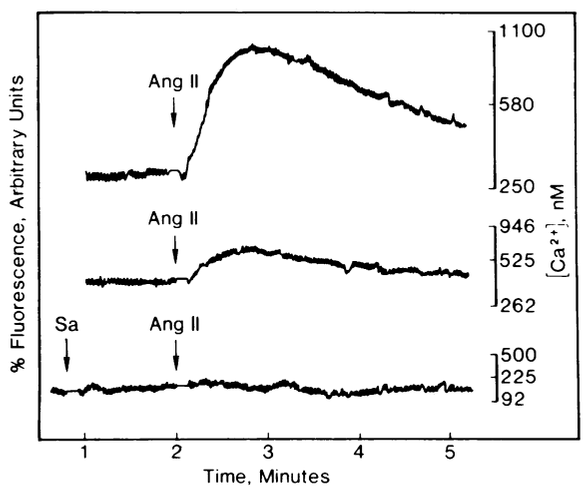

FIG. 2. Effects of 8-Br-cGMP and [Sar^1,Leu^8]Ang-II (Sa) on Ang-II-stimulated rat aortic smooth muscle cells. Cells were cultured on coverslips as described and incubated with fura-2,AM. At the times indicated, Ang-II (10 nM) was added to the cells. Top tracing, no preincubation; middle tracing, cells were preincubated with 100 μM 8-Br-cGMP for 15 min before the addition of Ang-II; bottom tracing, cells were preincubated for 1 min with antagonist (100 nM) before the addition of Ang-II.

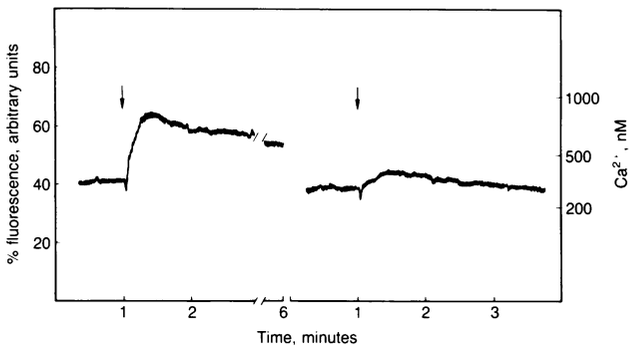

FIG. 3. Effects of 8-Br-cGMP on KCl-stimulated rat aortic smooth muscle cells. Cells were cultured on coverslips as described and incubated with fura-2,AM. At the times indicated (arrows), KCl (40 mM) was added to the cells. Left tracing, no preincubation; right tracing, cells were preincubated with 100 μM 8-Br-cGMP for 15 min before the addition of KCl.

Fig. 4. The rate of ATP hydrolysis in the presence of Ca^{2+} was stimulated up to 4-fold by 0.02 μM cGMP kinase. No ATP hydrolysis was observed with this concentration of the kinase alone (data not shown). cGMP (1.0 μM) was required for maximal stimulation of Ca^{2+}-ATPase when cGMP kinase was added. Heat-denatured cGMP kinase had no effect on Ca^{2+}-ATPase activity, suggesting that one or more possible contaminants in the enzyme preparation were not responsible for the activation (data not shown). The effect of cGMP kinase on ATPase activity assayed in the presence of Mg^{2+} (5 mM) and in the absence of Ca^{2+} (i.e., 0.5 mM EGTA) was also determined. Activity in the absence of kinase was 53.76 ± 3.9 nmol of P_i per min per mg of protein ($n = 3$). This value agrees closely with that shown in Table 2. In the presence of 0.02 μM cGMP kinase, the ATPase activity in the absence of Ca^{2+} was 54.96 ± 4.5 nmol per min per mg of protein ($n = 3$). This value was not significantly different than that shown in Table 2 or reported above. Therefore, the enhanced activity of ATPase in the presence of cGMP kinase was due to an effect on a Ca^{2+}-stimulated ATPase. Workers in several laboratories have reported that the plasma membrane Ca^{2+}-ATPase is stimulated by calmodulin. As shown in Fig. 5, calmodulin stimulated the Ca^{2+}-ATPase 2-fold in the absence of cGMP kinase. Kinase, in the absence of calmodulin, also stimulated Ca^{2+}-ATPase. When cGMP kinase and calmodulin were added together, Ca^{2+}-ATPase activity was increased in an additive fashion. This did not appear to be simply due to the greater affinity of the cGMP kinase-activated Ca^{2+}-ATPase for calmodulin since higher concentrations of calmodulin did not increase the activity of the untreated enzyme (data not shown).

Effects of cGMP Kinase on Ca^{2+} Activation of the Ca^{2+}-ATPase. Ca^{2+}-ATPase was activated by free Ca^{2+} concentrations from 0.01 to 1.0 μM in the presence of calmodulin with half-maximal stimulation at ≈0.1 μM (Fig. 6). cGMP

Table 2. ATPase activity in rat aortic smooth muscle cells

Assay conditions	P_i, nmol per min per mg of protein
Basal ($-Mg^{2+}$, $-Ca^{2+}$)	8.52 ± 0.26
Ca^{2+} only (1 μM)	28.52 ± 1.58
Mg^{2+} only (5 mM) + EGTA (0.5 mM)	54.91 ± 1.13
Ca^{2+} + Mg^{2+}	61.01 ± 1.13
Ca^{2+} + ATPase [(Ca^{2+} + Mg^{2+}) − Mg^{2+}]	6.10 ± 0.60

Rat aortic smooth muscle microsomes were prepared as described in the text. Free Ca^{2+} concentrations were calculated as described in ref. 15. Activity was determined in the absence of exogenous calmodulin. Results are expressed as the mean ± SEM of three separate determinations.

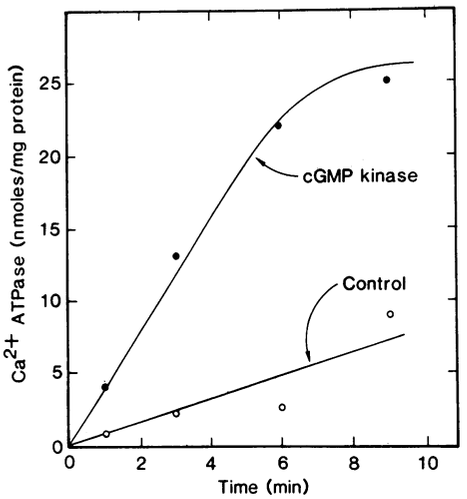

FIG. 4. Effect of cGMP kinase on Ca^{2+}-ATPase from rat aortic smooth muscle cell microsomes. cGMP kinase (final concentration, 0.02 μM) plus 1 μM cGMP (●) or buffer alone (○) was added to the reaction mixture containing 5–10 μg of protein. Calmodulin was not present in this experiment. Each point represents the average of two separate experiments.

kinase increased ATPase activity at all concentrations of Ca^{2+} with maximal effects observed at 1.0 μM free Ca^{2+}. These results suggest that the effect of cGMP kinase was to increase primarily the maximal velocity of the enzyme in the presence of Ca^{2+}. These results also indicate that the effects of cGMP kinase are most apparent within the physiological range of intracellular Ca^{2+} concentrations (i.e., 0.1–1 μM). cGMP kinase increased the V_{max} for ATP hydrolysis from 8.0 to 18.1 nmol per min per mg of protein but had little or no effect on the apparent K_m of the enzyme for ATP (data not shown).

Effects of Various Protein Kinases on Ca^{2+}-ATPase Activity. Studies in several laboratories have demonstrated that protein kinases, especially cyclic nucleotide-dependent protein kinases, have overlapping substrate specificity *in vitro* (24). In general, the cGMP kinase is a poor catalyst relative to the cAMP kinase when various protein substrates are used (24, 25). However, selective protein substrates for the cGMP

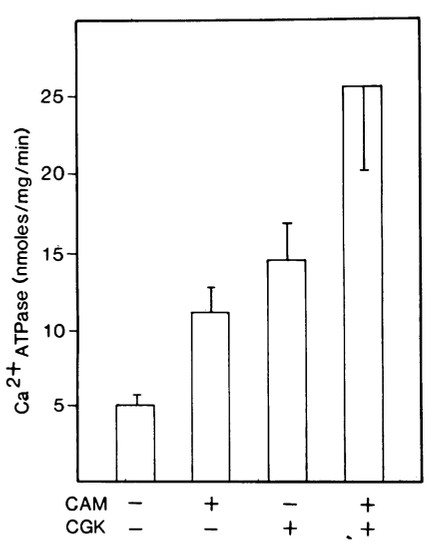

FIG. 5. Effect of cGMP kinase (CGK) and calmodulin (CAM) on Ca^{2+}-ATPase from rat aortic cell microsomes. Additions include calmodulin (final concentration, 0.1 μM) or cGMP kinase (final concentration, 0.02 μM) plus 1 μM cGMP, or both. Results are expressed as the mean ± SEM for three separate experiments.

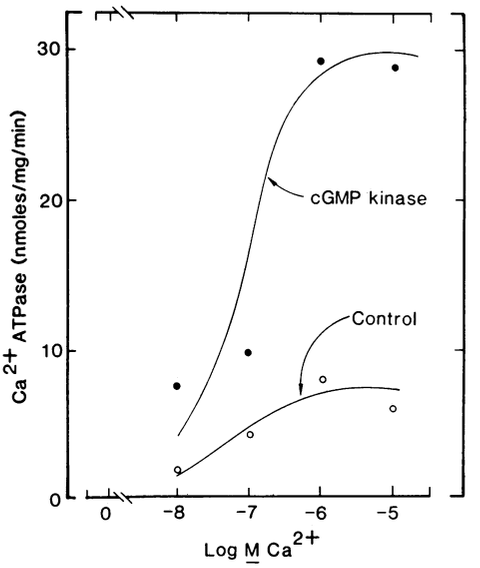

FIG. 6. Effect of cGMP-dependent protein kinase on Ca^{2+}-stimulated Ca^{2+}-ATPase from rat aortic cell microsomes. All Ca^{2+} concentrations were calculated as described. Each assay mixture contained calmodulin (0.1 μg) with or without cGMP-dependent protein kinase (final concentration, 0.02 μM). Each point represents the average of two separate experiments.

kinase have not been characterized. Thus, it was of interest to determine the selectivity of Ca^{2+}-ATPase activation using different protein kinases. As shown in Table 3, 0.02 μM cGMP kinase (i.e., 0.8 unit) was the best activator of Ca^{2+}-ATPase when compared with the cAMP kinase catalytic subunit (0.2 μM) and protein kinase C (2.5 units). It was interesting to note that even a 10-fold greater concentration of cAMP kinase did not activate Ca^{2+}-ATPase activity when compared with the cGMP-dependent enzyme.

Because Ca^{2+}-sensitive processes such as contraction, myosin phosphorylation, and phosphorylase *a* formation are inhibited by increases in cGMP, we have suggested that cGMP lowers intracellular Ca^{2+} concentrations (9, 10). Furthermore, it has been demonstrated that cGMP is capable of inhibiting contraction (26), Ca^{2+}-sensitive enzyme activation (9, 10), and Ca^{2+} accumulation (Figs. 2 and 3) caused by depolarization or by agonist-induced intracellular mobilization of Ca^{2+}, suggesting that the mechanism of action of cGMP may involve Ca^{2+} removal from the cytoplasm. It has also been found that the effect of cGMP on cytoplasmic Ca^{2+} is smaller when the accumulation of Ca^{2+} is greater, suggesting that the Ca^{2+}-removal step regulated by cGMP might be of limited capacity such as a relatively slow transporting system with high affinity for Ca^{2+} (26). The present results

Table 3. Effects of cGMP-dependent and cAMP-dependent protein kinases and protein kinase C on Ca^{2+}-ATPase activity in rat aortic smooth muscle microsomes

Kinase	Ca^{2+}-ATPase, nmol per min per mg of protein
None	6.09
Protein kinase C (2.5 units)	12.60
cAMP-dependent protein kinase (0.2 μM)	6.08
cGMP-dependent protein kinase (0.02 μM)	19.98

Microsomes were prepared as described. Protein kinases were added to reaction mixtures and the reactions were initiated immediately by the addition of [γ-^{32}P]ATP. One unit of lymphocyte protein kinase C is equivalent to that amount of enzyme required to transfer 1 nmol of phosphate from ATP to histone substrate in 1 min. Results represent the average of two separate experiments.

suggest that activation of Ca^{2+}-ATPase through phosphorylation of a specific substrate protein may contribute to the mechanism of action for cGMP-dependent relaxation of vascular smooth muscle. Because the source and location of the Ca^{2+}-ATPase from cultured aortic cells was not ascertained, it is not known which Ca^{2+}-ATPase is affected. Furthermore, it is not known that a specific Ca^{2+}-ATPase is itself a substrate for cGMP kinase. Detergent-solubilized Ca^{2+}-ATPase and cGMP-catalyzed phosphorylation of a protein of M_r 135,000 cochromatographed through several steps (unpublished observations). However, it is conceivable that alterations in ion fluxes produced by cGMP (27) may influence Ca^{2+}-ATPase activity. Further work will be required to dissect the Ca^{2+}-transporting systems from smooth muscle membranes to determine the precise mechanism of action of cGMP. The potential regulation of Ca^{2+}-ATPase activity suggests a role for cGMP in vascular smooth muscle, and perhaps other tissues, in regulating Ca^{2+} transport from cells, thus contributing to the low intracellular concentration of Ca^{2+} with respect to the extracellular fluid.

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