cGMP-mediated Ca\(^{2+}\) release from IP\(_3\)-insensitive Ca\(^{2+}\) stores in smooth muscle

KARNAM S. MURTHY AND GABRIEL M. MAKLHOUF
Departments of Medicine and Physiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0711

Murthy, Karnam S., and Gabriel M. Makhlouf. cGMP-mediated Ca\(^{2+}\) release from IP\(_3\)-insensitive Ca\(^{2+}\) stores in smooth muscle. Am. J. Physiol. 274 (Cell Physiol. 43): C1199–C1205, 1998.—Recent studies on the role of nitric oxide (NO) in gastrointestinal smooth muscle have raised the possibility that NO-stimulated cGMP could, in the absence of cGMP-dependent protein kinase (PKG) activity, act as a Ca\(^{2+}\)-mobilizing messenger [K. S. Murthy, K.-M. Zhang, J. -G. Jin, J. T. Grider, and G. M. Makhlouf. Am. J. Physiol. 265 (Gastrointest. Liver Physiol. 28): G660–G671, 1993]. This notion was examined in dispersed gastric smooth muscle cells with 8-bromo-cGMP (8-BrcGMP) and with NO and vasoactive intestinal peptide (VIP), which stimulate endogenous cGMP. In muscle cells treated with cAMP-dependent protein kinase (PKA) and PKG inhibitors (H-89 and KT-5823), 8-BrcGMP (10 μM), NO (1 μM), and VIP (1 μM) stimulated \( ^{45}\)Ca\(^{2+}\) release (21 ± 3 to 30 ± 1% decrease in \(^{45}\)Ca\(^{2+}\) cell content); Ca\(^{2+}\) release stimulated by 8-BrcGMP was concentration dependent with an EC\(_{50}\) of 0.4 ± 0.1 μM and a threshold of 10 nM. 8-BrcGMP and NO increased cytosolic free Ca\(^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) and induced contraction; both responses were abolished after Ca\(^{2+}\) stores were depleted with thapsigargin. With VIP, which normally increases [Ca\(^{2+}\)], by stimulating Ca\(^{2+}\) influx, treatment with PKA and PKG inhibitors caused a further increase in [Ca\(^{2+}\)]; that reverted to control levels in cells pretreated with thapsigargin. Neither Ca\(^{2+}\) release nor contraction induced by cGMP and NO in permeabilized muscle cells was affected by heparin or ruthenium red. Ca\(^{2+}\) release induced by maximally effective concentrations of cGMP and inositol 1,4,5-trisphosphate (IP\(_3\)) was additive, independent of which agent was applied first. We conclude that, in the absence of PKA and PKG activity, cGMP stimulates Ca\(^{2+}\) release from an IP\(_3\)-insensitive store and that its effect is additive to that of IP\(_3\).

cytosolic free calcium; cAMP-dependent protein kinase; cGMP-dependent protein kinase; calcium stores

THREE MAJOR TARGETS for cGMP have been identified: cGMP-dependent protein kinases (types I, II, and II) (22, 38, 42); cGMP-gated cation channels (6, 16); and various cyclic nucleotide phosphodiesterases (PDE), including cGMP-specific PDE5, cGMP-stimulated PDE2, and cGMP-inhibited PDE3 (2). cGMP, acting via cGMP-dependent protein kinase (PKG), has distinct effects on intracellular Ca\(^{2+}\) levels in different cells, increasing cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in hepatocytes (36) and sea urchin eggs (12, 13, 20) and decreasing [Ca\(^{2+}\)]\(_i\) in vascular (8) and visceral smooth muscle (27, 28), cardiac myocytes (23), and cerebellar neurons (3). In hepatocytes, phosphorylation of the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor by PKG potentiates IP\(_3\)-dependent Ca\(^{2+}\) release (36), whereas in sea urchin eggs phosphorylation of ADP ribosyl cyclase stimulating the synthesis of the Ca\(^{2+}\)-mobilizing messenger, cyclic ADP-ribose, which potentiates Ca\(^{2+}\)-induced Ca\(^{2+}\) release from ryadonidine-sensitive stores (12, 13). In smooth muscle, on the other hand, phosphorylation of a different IP\(_3\) receptor isoform by PKG inhibits IP\(_3\)-dependent Ca\(^{2+}\) release (17, 41). In addition, PKG acts on other targets in smooth muscle to attenuate Ca\(^{2+}\). Thus PKG (1) inhibits the activity of phospholipase C-β and generation of IP\(_3\) (28); 2) stimulates the activity of plasmalemmal (36, 44) and sarcoplasmic Ca\(^{2+}\)-ATPase pumps (9, 34, 39), thereby increasing Ca\(^{2+}\) uptake into the stores and Ca\(^{2+}\) efflux from the cells; and 3) inhibits the activity of Ca\(^{2+}\) channels (23) and stimulates the activity of K\(^+\) channels in the plasma membranes, thereby reducing Ca\(^{2+}\) influx into the cells via voltage-sensitive Ca\(^{2+}\) channels (4).

The diverse and marked effects of PKG on Ca\(^{2+}\) mobilization in smooth muscle have hampered an assessment of a direct role for cGMP. cGMP can directly activate cationic channels of the outer segment of the retinal rod and in the cilia of olfactory receptor neurons (6, 30) and Ca\(^{2+}\) channels in pancreatic acinar cells and NIH/3T3 cells, leading to sustained Ca\(^{2+}\) influx (1, 31, 43). Our recent studies (25, 27) on the regulatory role of nitric oxide (NO) in gastrointestinal smooth muscle cells have raised the possibility that cGMP could, in the absence of PKG activity, induce Ca\(^{2+}\) mobilization. The transient increase in [Ca\(^{2+}\)]\(_i\), that accompanies stimulation of NO and cGMP formation in muscle cells by vasoactive intestinal peptide (VIP) is enhanced when cAMP-dependent protein kinase (PKA) and PKG activity is blocked, converting the smooth muscle response from relaxation to contraction (29). We have postulated that a product of the cascade that results in activation of PKG could be responsible for the increase in [Ca\(^{2+}\)]\(_i\). In the present study, we have examined the possibility that cGMP can act as a Ca\(^{2+}\)-mobilizing messenger in the absence of protein kinase activity. The effect of cGMP was tested with the potent derivative of cGMP, 8-bromo-cGMP (8-BrcGMP), and with NO and VIP, which stimulate endogenous cGMP formation. The results indicate that cGMP stimulates Ca\(^{2+}\) release from an IP\(_3\)-insensitive store and that its effect is additive to that of IP\(_3\).

METHODS

Dispersion of smooth muscle cells. Muscle cells were isolated from the circular muscle layer of the rabbit stomach by successive enzymatic digestion, filtration, and centrifugation as described previously (25, 29). After digestion, the muscle cells were resuspended in enzyme-free medium consisting of (in mM) 120 NaCl, 4 KCl, 2.6 KH\(_2\)PO\(_4\), 0.6 MgCl\(_2\), 25 HEPES, 14 glucose, and 2.1% Eagle’s essential amino acid mixture. The cells were harvested by filtration through
500-µm Nitex mesh and centrifuged twice at 350 g for 10 min. In some experiments, the cells were permeabilized by incubation with saponin (35 µg/ml) for 10 min as previously described (18, 28). The cells were centrifuged at 350 g for 10 min, washed free of saponin, and resuspended in a medium containing 100 nM Ca\(^{2+}\) and an ATP-regenerating system consisting of 5 mM creatine phosphate and 10 U/ml creatine phosphokinase.

Measurement of [Ca\(^{2+}\)]\(_i\) in dispersed smooth muscle cells. [Ca\(^{2+}\)]\(_i\) was measured in suspensions of muscle cells using the Ca\(^{2+}\) fluorescent dye fura 2, as described previously (24, 29). Muscle cells were suspended in a medium containing (in mM) 10 HEPES, 125 NaCl, 5 KCl, 1 CaCl\(_2\), 0.5 MgSO\(_4\), 5 glucose, 20 taurine, 45 sodium pyruvate, and 5 creatine and were incubated with fura 2-AM (2 µM) for 20 min at 31°C. After centrifugation at 350 g for 20 min, the cells were incubated in fura 2-free medium for immediate measurement of Ca\(^{2+}\)\(_i\). Fluorescence was monitored at 510 nm, with excitation wavelengths alternating between 340 and 380 nm, and the measurements were corrected for autofluorescence of un-loaded cells. An estimate of [Ca\(^{2+}\)]\(_i\) was obtained from observed, maximal, and minimal fluorescence ratios as described previously (24, 29).

Measurement of 45Ca\(^{2+}\) efflux in dispersed smooth muscle cells. Net Ca\(^{2+}\) efflux was measured in dispersed muscle cells as described previously (28, 32). The cells were incubated in a medium containing 45Ca\(^{2+}\) (10 µCi/ml) and antimycin (10 µM), and Ca\(^{2+}\) uptake into nonmitochondrial Ca\(^{2+}\) stores was measured at intervals for 60 min when a steady state was attained. VIP, 8-Br-cGMP, or NO was then added, and net Ca\(^{2+}\) efflux was measured after 30 s. Net Ca\(^{2+}\) efflux, reflecting release from nonmitochondrial stores, was expressed as percent decrease in steady-state 45Ca\(^{2+}\) cell content. A similar procedure was followed in permeabilized muscle cells suspended in a medium containing 100 nM Ca\(^{2+}\) and antimycin (10 µM) to prevent mitochondrial Ca\(^{2+}\) uptake. ATP (1.5 mM) in the presence of an ATP-regenerating system (5 mM creatine phosphate and 10 U/ml creatine phosphokinase) was first added to initiate Ca\(^{2+}\) uptake into the nonmitochondrial stores. After 60 min, VIP, 8-Br-cGMP, or NO was added, and net Ca\(^{2+}\) efflux was measured after 30 s. Net Ca\(^{2+}\) efflux was expressed as percent decrease in steady-state 45Ca\(^{2+}\) cell content. Steady-state 45Ca\(^{2+}\) cell content in permeabilized muscle cells (2.63 ± 0.32 nmol/10⁶ cells) was not significantly different from that in intact muscle cells (2.19 ± 0.26 nmol/10⁶ cells).

Assay for NO synthase activity in dispersed smooth muscle cells. NO synthase (NOS) activity was measured in dispersed muscle cells as described previously (15, 25, 29) from the stoichiometric formation of the coproduct, L-citrulline in cells preloaded with L-[\(^{3}H\)]arginine. L-[\(^{3}H\)]arginine (3 µCi/ml) was added to 1 ml of cell suspension for 10 min, and the cells were treated during the last minute with either VIP, 8-Br-cGMP, or NO. The suspension was centrifuged for 1 min at 3,000 g and the pellet was frozen rapidly on dry ice. The samples were stored at −70°C for extraction and separation of L-[\(^{3}H\)]citrulline by flow-through chromatography using Dowex (AG50W-X8) columns. The amount of L-[\(^{3}H\)]citrulline in the sample was measured by liquid scintillation. The results were expressed as counts per minute (cpm) per 10⁶ cells or as percent increase above basal levels measured in separate aliquots of the same cell suspension. Previous studies had shown that this assay yielded results similar to measurement of the end products, NO\(_3\) and NO\(_2\), by HPLC (15, 25).

Measurement of cell contraction. Contraction of dispersed muscle cells was measured by scanning micrometry as described previously (18, 24). Briefly, an aliquot (0.5 ml) of cell suspension containing 10⁶ muscle cells/ml was added to 0.2 ml of HEPES medium containing VIP, NO, or 8-Br-cGMP for 30 s. The reaction was terminated by addition of acrolein to a final concentration of 1%. The length of muscle cells treated with each agent was measured by scanning micrometry and was compared with the length of untreated cells (mean control cell length: 101 ± 5 µm). Contraction was expressed as the decrease in micrometers from mean control cell length.

Materials. 125I-labeled cGMP, L-[\(^{3}H\)]arginine, and 45Ca\(^{2+}\) were obtained from NEN Life Sciences Products; HEPES was from Research Organics (Cleveland, OH); soybean trypsin inhibitor and collagenase (type II) were from Worthington; fura-2 AM was from Molecular Probes; thapsigargin, ruthenium red, 6-anilino-5,8-quinalinedione (LY-83583), and N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinoline-sulfonamide hydrochloride (H-89) were from Calbiochem; (S)-9-methoxy-carbamyl-8-methyl-3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H,-2,7b,11a-trizadibenzo(a,g)cycloocta(cde)-trin-ten-1-one (KT-5823) was from Kamiya Biomedical (Thousand Oaks, CA); low-molecular-weight heparin, N\(^{6}\)-nitro-L-arginine (L-NNA) and all other chemicals were from Sigma Chemical.

RESULTS

Ca\(^{2+}\) release and contraction induced by cGMP in dispersed smooth muscle cells. The ability of 8-Br-cGMP to stimulate Ca\(^{2+}\) release from sarcoplasmic stores was examined in muscle cells loaded with 45Ca\(^{2+}\) and treated for 10 min with the PKA inhibitor H-89 (1 µM) and the PKG inhibitor KT-5823 (1 µM). These concentrations were previously shown to be both selective and maximally effective in inhibiting PKA and PKG, respectively, based on direct measurement of PKA and PKG activity in these smooth muscle cells (27). In other tissues, H-89 at a concentration of 1 µM was shown also to inhibit PKG (7, 37). Both protein kinase inhibitors were used so as to preclude effects that might result from cross-activation of PKA. The use of both PKA and PKG inhibitors was especially important in experiments with VIP, which stimulates both cAMP and cGMP activity (Fig. 1).

In the presence of both protein kinase inhibitors, 8-Br-cGMP caused a concentration-dependent increase in Ca\(^{2+}\) release (Fig. 1). Maximal release (30 ± 1% decrease in steady-state 45Ca\(^{2+}\) cell content; P < 0.001, n = 4) was similar to that elicited by a maximally effective concentration of IP\(_3\) (1 µM) in permeabilized muscle cells (32 ± 3% decrease in steady-state 45Ca\(^{2+}\) cell content; P < 0.001, n = 4) (18, 24, 27). The EC\(_{50}\) was 0.4 ± 0.1 µM with a threshold concentration of ~10 nM. No Ca\(^{2+}\) release was observed in the absence of PKA and PKG inhibitors (Fig. 1).

Ca\(^{2+}\) release in the presence of PKA and PKG inhibitors was observed also with NO (21 ± 3% decrease in 45Ca\(^{2+}\) cell content; P < 0.01, n = 4) and VIP (23 ± 1%, P < 0.001, n = 4), which were used at maximally effective concentrations (Fig. 2) (15, 29). The EC\(_{50}\) was 0.4 ± 0.1 µM with a threshold concentration of ~10 nM. No Ca\(^{2+}\) release was observed in the absence of PKA and PKG inhibitors (Fig. 1).

The effects of NO and VIP on Ca\(^{2+}\) release were attributed to stimulation of endogenous cGMP. The increase in cGMP induced by NO (0.52 ± 0.05 pmol/10⁶ cells above a basal level of 0.45 ± 0.04 pmol/10⁶ cells; P < 0.01, n = 4) was not affected by the presence of PKA and PKG inhibitors (0.49 ± 0.03 pmol/10⁶ cells) or L-NNA (0.51 ± 0.05 pmol/10⁶ cells).
respectively, above a basal level of 56
the increase was caused by Ca$^{2+}$, 0.01, affected by treatment of the cells with L-NNA (100 µM), and Ca$^{2+}$ uptake into nonmitochondrial Ca$^{2+}$ stores was measured at intervals for 60 min when a steady state was attained. 8-BrcGMP was then added, and net Ca$^{2+}$ efflux was measured after 30 s in presence or absence of cAMP-dependent protein kinase (PKA) inhibitor, H-89 (1 µM), and cGMP-dependent protein kinase (PKG) inhibitor, KT-5823 (1 µM). Net Ca$^{2+}$ efflux, reflecting Ca$^{2+}$ release from nonmitochondrial stores, was expressed as percent decrease in steady-state 24Ca$^{2+}$ cell content. Steady-state 24Ca$^{2+}$ cell content in these experiments was 2.29 ± 0.28 nmol/10$^6$ cells. Values are means ± SE of 4 experiments.

Coincidentally with stimulation of Ca$^{2+}$ release, all three agents (8-BrcGMP, NO, and VIP) induced muscle cell contraction (Fig. 2). The contraction induced by 8-BrcGMP (18 ± 2 µm decrease in cell length; P < 0.01, n = 4) or NO (19 ± 1 µm; P < 0.001, n = 4) was not affected by treatment of the cells with L-NNA (100 µM), whereas the contraction induced by VIP (21 ± 1 µm; P < 0.001, n = 4) was partly inhibited (10 ± 2 µm) (Fig. 2); the residual contraction was attributed to stimulation of Ca$^{2+}$ influx by VIP. Previous studies (25) had shown that L-NNA had no effect on Ca$^{2+}$ release induced by NO or 8-BrcGMP.

As expected from measurements of 24Ca$^{2+}$ release, both 8-BrcGMP and NO increased [Ca$^{2+}$i], measured in fura 2-loaded cells (8-BrcGMP to 140 ± 55 nM, P < 0.05, and NO to 89 ± 6 nM, P < 0.01, respectively, above a basal level of 56 ± 2 nM Ca$^{2+}$); the increase in [Ca$^{2+}$i] was observed only after treatment of the cells with PKA and PKG inhibitors (Fig. 3). VIP increased [Ca$^{2+}$i] (160 ± 20 nM above basal level; P < 0.01, n = 4) in the absence of protein kinase inhibitors; the increase was caused by Ca$^{2+}$ influx and was abolished by nifedipine (11 ± 6 nM; not significant). The increase in VIP-induced [Ca$^{2+}$i] was significantly aug-
mented (P < 0.02) after treatment of the cells with PKA and PKG inhibitors (314 ± 50 nM; Fig. 3).

After treatment with thapsigargin to deplete the Ca$^{2+}$ stores (30-min incubation with 2 µM thapsigargin in zero Ca$^{2+}$ plus EGTA followed by resuspension of the cells in control 2 mM Ca$^{2+}$ medium), there was no increase in [Ca$^{2+}$i], with 8-BrcGMP and NO in the presence of PKA and PKG inhibitors, whereas the increase in [Ca$^{2+}$i] induced by VIP reverted to control levels reflecting Ca$^{2+}$ influx (123 ± 16 nM above basal levels; Fig. 3).

The pattern of muscle cell contraction paralleled the changes in [Ca$^{2+}$i]. In the presence of PKA and PKG inhibitors, all three agents induced muscle cell contraction (Fig. 3). The contraction induced by 8-BrcGMP (18 ± 2 µm decrease in cell length; P < 0.01, n = 4) and NO (19 ± 1 µm; P < 0.001, n = 4) was abolished after treatment of the cells with thapsigargin, whereas the contraction induced by VIP (21 ± 1 µm; P < 0.001, n =

pmol/10$^6$ cells), whereas the increase in cGMP induced by VIP (0.51 ± 0.01 pmol/10$^6$ cells; P < 0.001, n = 4) was abolished by L-NNA (0.03 ± 0.04 pmol/10$^6$ cells).

Fig. 1. Stimulation of Ca$^{2+}$ release by 8-bromo-cGMP (8-BrcGMP) in dispersed gastric smooth muscle cells. Muscle cells were incubated in a medium containing 45Ca$^{2+}$ (10 µCi/ml) and antimycin (10 µM), and Ca$^{2+}$ uptake into nonmitochondrial Ca$^{2+}$ stores was measured at intervals for 60 min when a steady state was attained. 8-BrcGMP was then added, and net Ca$^{2+}$ efflux was measured after 30 s in presence or absence of cAMP-dependent protein kinase (PKA) inhibitor, H-89 (1 µM), and cGMP-dependent protein kinase (PKG) inhibitor, KT-5823 (1 µM). Net Ca$^{2+}$ efflux, reflecting Ca$^{2+}$ release from nonmitochondrial stores, was expressed as percent decrease in steady-state 45Ca$^{2+}$ cell content. Steady-state 45Ca$^{2+}$ cell content in these experiments was 2.29 ± 0.28 nmol/10$^6$ cells. Values are means ± SE of 4 experiments.

Two major steps of Ca$^{2+}$ release, influx, and contraction were described in legend to Fig. 1. 8-BrcGMP (10 µM), NO (1 µM), or VIP (1 µM) was then added, and net Ca$^{2+}$ efflux was measured after 30 s in presence or absence of PKA and PKG inhibitors (1 µM each) and in presence of NO synthase (NOS) inhibitor, N$^G$-nitro-L-arginine (L-NNA; 100 µM). Cells were treated for 10 min with inhibitors before addition of 8-BrcGMP, NO, or VIP. Net Ca$^{2+}$ efflux (A) was expressed as percent decrease in steady-state 45Ca$^{2+}$ cell content (2.08 ± 0.23 nmol/10$^6$ cells in these experiments). Contraction (B) was measured by scanning micrometry and expressed in cell length from control (control cell length: 97 ± 3 µm). Values are means ± SE of 3–4 experiments. **P < 0.01, inhibition of VIP-induced Ca$^{2+}$ release and contraction by L-NNA.

Fig. 2. Ca$^{2+}$ release and contraction induced by 8-BrcGMP, nitric oxide (NO), and vasoactive intestinal peptide (VIP) in dispersed smooth muscle cells. Muscle cells were loaded with 45Ca$^{2+}$ as described in legend to Fig. 1. 8-BrcGMP (10 µM), NO (1 µM), or VIP (1 µM) was then added, and net Ca$^{2+}$ efflux was measured after 30 s in presence or absence of PKA and PKG inhibitors (1 µM each) and in presence of NO synthase (NOS) inhibitor, N$^G$-nitro-L-arginine (L-NNA; 100 µM). Cells were treated for 10 min with inhibitors before addition of 8-BrcGMP, NO, or VIP. Net Ca$^{2+}$ efflux (A) was expressed as percent decrease in steady-state 45Ca$^{2+}$ cell content (2.08 ± 0.23 nmol/10$^6$ cells in these experiments). Contraction (B) was measured by scanning micrometry and expressed in cell length from control (control cell length: 97 ± 3 µm). Values are means ± SE of 3–4 experiments. **P < 0.01, inhibition of VIP-induced Ca$^{2+}$ release and contraction by L-NNA.
induced increase in NOS activity was not altered by treatment with protein kinase inhibitors (728 ± 116 cpm/10^6 cells).

Treatment of the cells with L-NNA (100 µM) abolished NOS activity induced by all three agents, whereas treatment with thapsigargin abolished only the NOS activity induced by NO and 8-BrcGMP (Fig. 4). The results implied that activation of NOS by 8-BrcGMP and NO was mediated by cGMP-dependent Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores. The resultant increase in [Ca^{2+}]i activated a constitutive NOS in smooth muscle (26).

Identity of cGMP-sensitive Ca^{2+} stores. In permeabilized smooth muscle cells treated with PKA and PKG inhibitors, Ca^{2+} release induced by 10 µM cGMP (26 ± 2% decrease in ^{45}Ca^{2+} steady-state cell content; P < 0.001, n = 4) or 1 µM NO (25 ± 2%; P < 0.001, n = 4) was not affected by 10 or 100 µg/ml heparin (range: 24 ± 2 to 27 ± 4%) or by 10 and 100 µM ruthenium red (range: 22 ± 2 to 26 ± 2%). The corresponding contractions induced by cGMP (22 ± 5 µm decrease in cell length; P < 0.02, n = 4) and NO (21 ± 4 µm; P < 0.01, n = 4) were also not affected by heparin (range: 21 ± 3 to 24 ± 4 µm) or ruthenium red (20 ± 4 to 24 ± 3 µm). In contrast, Ca^{2+} release (32 ± 3% decrease in ^{45}Ca^{2+} cell content; P < 0.01, n = 4) and contraction (28 ± 5 µm decrease in cell length; P < 0.01, n = 4) induced by 1 µM IP_3 were abolished by heparin (10 µg/ml). The lack of effect of ruthenium red or heparin on cGMP-stimulated Ca^{2+} release suggested that cGMP did not activate IP_3 receptor/Ca^{2+} channels or ryanodine receptor/Ca^{2+} channels and may have acted on distinct, nonmitochondrial Ca^{2+} stores.

These notions were corroborated in studies using permeabilized muscle cells treated sequentially with cGMP and IP_3. After loading with ^{45}Ca^{2+} for 60 min, the

4) was partly inhibited; the residual contraction (11 ± 2 µm) reflected the effect of Ca^{2+} influx (Fig. 3). Effect of 8-BrcGMP, NO, and VIP on NOS activity. The changes in NOS activity were reflected by corresponding changes in NOS activity of dispersed smooth muscle cells. In the absence of PKA and PKG inhibitors, neither NO (1 µM) nor 8-BrcGMP (10 µM) stimulated NOS activity, whereas VIP (1 µM) caused a significant increase in NOS activity (716 ± 114 cpm/10^6 cells above basal level of 1,010 ± 133 cpm; P < 0.01, n = 4), similar to that previously reported for VIP and shown to be dependent on VIP-stimulated Ca^{2+} influx (Fig. 4) (29). In the presence of PKA and PKG inhibitors, both NO and 8-BrcGMP caused significant increases in NOS activity (520 ± 49 and 297 ± 43 cpm/10^6 cells above basal level, respectively; P < 0.01, n = 4); the VIP-induced increase in NOS activity was not altered by
cells were treated with protein kinase inhibitors and thapsigargin. Addition of a maximally effective concentration of cGMP (10 µM) caused a prompt release of Ca²⁺ (26 ± 3% decrease in ⁴⁵Ca²⁺ cell content within 30 s; P < 0.01; n = 4); subsequent addition of IP₃ in the continued presence of cGMP caused a further release of Ca²⁺ (32 ± 2% decrease in ⁴⁵Ca²⁺ cell content within 15 s; P < 0.01, n = 4); the combined effect of cGMP and IP₃ caused a 58 ± 3% decrease in ⁴⁵Ca²⁺-steady-state cell content (Fig. 5). Similar additive effects were obtained when IP₃ was added first, followed by cGMP, and were evident when a longer interval (10 min) separated the addition of the two agents (Fig. 5). Addition of ionomycin (10 µM) caused further Ca²⁺ release, virtually depleting the Ca²⁺ stores (87 ± 4%). Addition of GTP (10 µM) to permeabilized gastric

smooth muscle cells did not elicit significant Ca²⁺ release (2.5% decrease in ⁴⁵Ca²⁺ cell content).

DISCUSSION

Three agents (8-BrcGMP, NO, and VIP) that normally cause relaxation of smooth muscle could be converted to contractile agents in the presence of PKA and PKG inhibitors. The agents, which have in common the ability to increase intracellular levels of cGMP, stimulated Ca²⁺ release from IP₃-insensitive sarcoplasmic Ca²⁺ stores, leading to an increase in [Ca²⁺i] and smooth muscle cell contraction.

The Ca²⁺ release induced by these agents was clearly attributable to cGMP. In the presence of protein kinase inhibitors, exogenous 8-BrcGMP stimulated Ca²⁺ release in a concentration-dependent fashion with an EC₅₀ of 0.4 ± 0.1 µM and a threshold concentration of 10 nM. The resultant increase in [Ca²⁺i] induced by 8-BrcGMP (or by NO that stimulated endogenous cGMP) was abolished after depletion of the Ca²⁺ stores with thapsigargin. The increase in [Ca²⁺i] induced by VIP reflected VIP-stimulated Ca²⁺ influx (29) as well as cGMP-dependent Ca²⁺ release: only the latter component was eliminated after treatment of the muscle cells with thapsigargin; the residual increase in [Ca²⁺i], reflecting Ca²⁺ influx was not affected by protein kinase inhibitors but was abolished by the dihydropyridine Ca²⁺ channel blocker, nifedipine (25, 29). The possibility that VIP-induced Ca²⁺ influx could have led to Ca²⁺-induced Ca²⁺ release was ruled out by the fact that L-NNA, which suppresses VIP-stimulated NOS activity and cGMP formation but has no effect on VIP-stimulated Ca²⁺ influx, suppressed VIP-induced Ca²⁺ release (25, 29). It is worth noting that the increase in [Ca²⁺i] induced by 8-BrcGMP and NO in the presence of protein kinase inhibitors led to activation of the constitutive smooth muscle NOS; the resultant NO formation was abolished by the NOS inhibitor, L-NNA.

Ca²⁺ release and muscle cell contraction induced by cGMP and NO in permeabilized muscle cells were not affected by heparin, a blocker of IP₃ receptors/Ca²⁺ channels (18, 24), or ruthenium red, a blocker of ryano(lipopeptide receptors/Ca²⁺ channels (18, 19), whereas Ca²⁺ release induced by IP₃ under similar conditions was abolished by heparin. The lack of effect of heparin or ruthenium red implied that cGMP did not cause Ca²⁺ release by activating IP₃ receptors/Ca²⁺ channels or ryano(lipopeptide receptors/Ca²⁺ channels. The latter are confined to smooth muscle cells isolated from the longitudinal layer, which, unlike smooth muscle cells from the circular layer, possess high-affinity binding sites for ryano(lipopeptide and cyclic ADP-ribose but not for IP₃ (18, 19).

Furthermore, the effects of maximally effective concentrations of cGMP (10 µM) and IP₃ (1 µM; 25) on Ca²⁺ release were approximately equal and additive, independently of which agent was applied first or of the interval separating application (2–10 min; Fig. 5). Together, IP₃ and cGMP released ~60% of the Ca²⁺ stores. Addition of 10 µM ionomycin released a further 30% for a total of ~90% of Ca²⁺ store content. The pattern suggests that thapsigargin-sensitive Ca²⁺ stores discharged by IP₃

---

Fig. 5. Effect of sequential addition of cGMP and inositol 1,4,5-trisphosphate (IP₃) on Ca²⁺ release from permeabilized smooth muscle cells. Ca²⁺ release in muscle cells loaded with ⁴⁵Ca²⁺ was measured as described in METHODS. After steady-state Ca²⁺ uptake was attained, either cGMP (10 µM) or IP₃ (1µM) was added in presence of thapsigargin to prevent Ca²⁺ reuptake and in presence of PKA and PKG inhibitors (1 µM each). After a 2-min interval (A) or 10-min interval (B), the other agent was added. Ionomycin (IM; 10 µM) was added at end of experimental period. Net Ca²⁺ efflux was expressed as percent decrease in steady-state ⁴⁵Ca²⁺ cell content (2.48 ± 0.25 nmd/10⁶ cells). Values are means ± SE of 4 experiments.
and cGMP may be distinct, although possibly confluent, but it is not consistent with the notion that cGMP acted to enhance the activity of IP<sub>3</sub>-sensitive Ca<sup>2+</sup> channels, since the ability of IP<sub>3</sub> to induce Ca<sup>2+</sup> release was not affected by the presence of cGMP (Fig. 5).

The mechanism mediating Ca<sup>2+</sup> release by cGMP could be a cGMP-activated Ca<sup>2+</sup> channel or a cGMP-inhibited sarcoplasmic Ca<sup>2+</sup> pump. cGMP-gated cationic channels have been described in several locations (in retinal rods (6, 16) and olfactory neurons (30) and in pancreatic acinar cells (1, 31, 43)). The retinal cGMP-gated cationic channel contains a cGMP-binding domain that possesses substantial homology to the cGMP-binding domain of PKG (16). The structure of the cGMP-gated Ca<sup>2+</sup> channel in pancreatic acinar cells, thought to mediate sustained Ca<sup>2+</sup> influx following agonist stimulation, has not been determined.

Sarcoplasmic Ca<sup>2+</sup> channels and Ca<sup>2+</sup> pumps are known to be susceptible to regulatory phosphorylation by PKA and PKG, providing a basis for the requirement known to be susceptible to regulatory phosphorylation that PKG acts to inhibit IP<sub>3</sub>-mobilizing action of cGMP. Regulation of the IP<sub>3</sub> receptor type I, which is predominantly expressed in vascular smooth muscle and cerebellum, is mediated by PKG-dependent phosphorylation at serine-1755 (11, 17). Our previous studies on gastric smooth muscle cells have shown that both PKA and PKG regulate IP<sub>3</sub>-dependent Ca<sup>2+</sup> release and that PKG, in addition, stimulates Ca<sup>2+</sup> uptake by the sarcoplasmic Ca<sup>2+</sup> pump (27, 28). It seems possible, by analogy, that a cGMP-dependent Ca<sup>2+</sup> release mechanism is regulated by PKG- and PKA-dependent phosphorylation.

Unlike smooth muscle where PKG acts to inhibit IP<sub>3</sub>- and cGMP-dependent Ca<sup>2+</sup> release, Ca<sup>2+</sup> release in other cell types appears to be indirectly mediated by PKG. Phosphorylation of the IP<sub>3</sub> receptor in hepatocytes increases its sensitivity for Ca<sup>2+</sup> release and induces oscillatory Ca<sup>2+</sup> signals (36). Phosphorylation of ADP ribosyl cyclase by PKG results in synthesis of the Ca<sup>2+</sup>-mobilizing messenger, cyclic ADP-ribose; this mechanism is thought to underlie the ability of cGMP acting via PKG to initiate Ca<sup>2+</sup> mobilization at fertilization in the sea urchin egg (12, 13). A similar mechanism has been claimed for NO-induced Ca<sup>2+</sup> increase in interstitial cells of Cajal, but no direct evidence was provided for involvement of either cGMP or PKG (33).

The mechanism underlying the ability of cGMP to stimulate Ca<sup>2+</sup> release in gastric smooth muscle is different from the mechanism underlying the ability of GTP to stimulate Ca<sup>2+</sup> release in neuronal (NIE-115), smooth muscle (DDT1MF-2 and BC3H1), and fibroblast (WI-38) cell lines (5, 10, 14, 40). Ca<sup>2+</sup> release induced by GTP in these cells is additive to that of IP<sub>3</sub> and appears to be mediated by a product of GTP hydrolysis, since it could not be reproduced by nonhydrolyzable analogs of GTP (14). Ca<sup>2+</sup> release could not be elicited also by cGMP in permeabilized muscle cells or microsomes, making it unlikely that a Ca<sup>2+</sup>-mobilizing action of cGMP in these cell lines was masked by activation of PKG. In the present study on freshly dispersed gastric smooth muscle cells, however, Ca<sup>2+</sup> release was induced by cGMP in the absence of protein kinase activity but not by GTP.

The functional significance of a Ca<sup>2+</sup>-mobilizing action of cGMP may reside in greater Ca<sup>2+</sup> requirements during development. Lincoln and Cornell (21) have shown that repeated passage of vascular smooth muscle cells results in a substantial reduction in the expression of PKG. It is possible, although speculative, that PKG is either absent or minimally expressed during a stage in development when requirements for intracellular Ca<sup>2+</sup> are high and could thus be met by a Ca<sup>2+</sup>-mobilizing action of cGMP that is additive to that of the usual messenger, IP<sub>3</sub>.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-28300 and DK-15564. Address for reprint requests: G. M. Makhoul, PO Box 980711, Medical College of Virginia, Richmond, VA 23298-0711.

Received 18 November 1997; accepted in final form 13 January 1998.

REFERENCES


41. Wojcikiewicz, R. J. H. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. J. Biol. Chem. 270: 11678–11683, 1995.

