cAMP inhibits IP$_3$-dependent Ca$^{2+}$ release by preferential activation of cGMP-primed PKG

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Murthy, Karnam S. cAMP inhibits IP$_3$-dependent Ca$^{2+}$ release by preferential activation of cGMP-primed PKG. Am J Physiol Gastrointest Liver Physiol 281: G1238–G1245, 2001.—The singular effects and interplay of cAMP- and cGMP-dependent protein kinase (PKA and PKG) on Ca$^{2+}$ mobilization were examined in dispersed smooth muscle cells. In permeabilized muscle cells, exogenous cAMP and cGMP inhibited inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca$^{2+}$ release and muscle contraction via PKA and PKG, respectively. A combination of cAMP and cGMP caused synergistic inhibition that was exclusively mediated by PKG and attenuated by PKA. In intact muscle cells, low concentrations (10 nM) of isoproterenol and sodium nitroprusside (SNP) inhibited agonist-induced, IP$_3$-dependent Ca$^{2+}$ release and muscle contraction via PKA and PKG, respectively. A combination of isoproterenol and SNP increased PKA and PKG activities: the increase in PKA activity reflected inhibition of phosphodiesterase 3 activity by cGMP, whereas the increase in PKG activity reflected activation of cGMP-primed PKG by cAMP. Inhibition of Ca$^{2+}$ release and muscle contraction by the combination of isoproterenol and SNP was preferentially mediated by PKG. In light of studies showing that PKG phosphorylates the IP$_3$ receptor in intact and permeabilized muscle cells, whereas PKA phosphorylates the receptor in permeabilized cells only, the results imply that inhibition of IP$_3$-induced Ca$^{2+}$ release is mediated exclusively by PKG. The effect of PKA on agonist-induced Ca$^{2+}$ release probably reflects inhibition of IP$_3$ formation.

smooth muscle; relaxation; protein kinase; 1,4,5-trisphosphate

THE MAIN RELAXANT NEUROPEPTIDES in the gut, vasoactive intestinal peptide (VIP) and pituitary adenyl cyclase-activating peptide (PACAP), stimulate cAMP and cGMP in smooth muscle cells and activate both cAMP- and cGMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) (23). The neuropeptides interact with VIP-PACAP (VPAC)$_2$ receptors to activate adenyl cyclase and with the natriuretic peptide clearance receptors (NPR-C) to activate sequentially nitric oxide synthase and soluble guanylyl cyclase (28). The resultant generation of cAMP and cGMP leads to a complex interplay of cyclases and cyclic nucleotide-dependent kinases and phosphodiesterases (PDE3, PDE4, and PDE5) that alter the levels of cAMP and cGMP and their relative affinities for PKA and PKG (7, 8). cAMP is formed in much greater abundance than cGMP (~10-fold) and can cross-activate PKG at high concentrations (7, 13, 14, 19, 22, 23). In the presence of cGMP, the affinity of cAMP for PKG and its ability to cross-activate this kinase are greatly enhanced, endowing PKG with the major role in cellular responses (2, 7, 11, 17).

PKG can act on various targets to regulate Ca$^{2+}$ mobilization and induce relaxation in vascular or visceral smooth muscle (1, 7, 20). Evidence exists that PKG I inhibits the formation of the Ca$^{2+}$-mobilizing messenger inositol 1,4,5-trisphosphate (IP$_3$) by phosphorylating various isoforms of phospholipase C (PLC)-β (44); 2 inhibits IP$_3$-dependent Ca$^{2+}$ release by phosphorylating the IP$_3$ receptor/Ca$^{2+}$ channel (15, 16); 3 stimulates plasmalemmal and sarcoplastic Ca$^{2+}$-ATPase pumps, thereby increasing Ca$^{2+}$ uptake into the stores and stimulating Ca$^{2+}$ efflux from the cells (3); and 4 inhibits Ca$^{2+}$ channel activity and stimulates K$^+$ channel activity, causing membrane hyperpolarization and a reduction of Ca$^{2+}$ influx into the cell via voltage-dependent Ca$^{2+}$ channels (32, 39).

Some but not all of these properties are shared by PKA. Thus PKA inhibits agonist-stimulated IP$_3$ formation mediated by PLC-β but does not inhibit voltage-dependent Ca$^{2+}$ channel activity or stimulate Ca$^{2+}$ uptake into the stores (5, 21, 26, 27). PKA inhibits agonist-stimulated Ca$^{2+}$ release in intact smooth muscle cells, but whether this effect represents inhibition of IP$_3$ formation only or also inhibition of IP$_3$-dependent Ca$^{2+}$ release is uncertain. PKA, like PKG, can inhibit IP$_3$-dependent Ca$^{2+}$ release in permeabilized smooth muscle cells, but its effect in intact smooth muscle cells has not been determined. Our recent studies (25) indicate that PKG can phosphorylate the IP$_3$ receptor in both intact and permeabilized smooth muscle cells, whereas PKA phosphorylates the IP$_3$ receptor in permeabilized smooth muscle cells only. Thus inhibition of agonist-stimulated Ca$^{2+}$ release in intact smooth muscle cells by PKA could reflect inhibition of IP$_3$ formation (and/or stimulation of IP$_3$ inactivation) rather than inhibition of IP$_3$-dependent Ca$^{2+}$ release (26).
PKA and PKG can also induce relaxation by acting on targets downstream of Ca\(^{2+}\) mobilization. Both protein kinases can decrease myosin light chain (MLC) phosphorylation during the initial phase of contraction by inhibiting Ca\(^{2+}\)/calmodulin-dependent activation of MLC kinase and/or by activating MLC phosphatase via telokin (18, 22, 35, 37). During the sustained (tonic) phase of contraction, which is Ca\(^{2+}\)-independent, both kinases decrease MLC phosphorylation by inhibiting the activity of the monomeric G protein RhoA (30, 33).

In the present study, we sought to examine the singular effects and the interplay of PKA and PKG on IP\(_3\)-dependent Ca\(^{2+}\) release and smooth muscle contraction. The effects of exogenous cAMP and cGMP, alone and in combination, on Ca\(^{2+}\) release and smooth muscle contraction induced by exogenous IP\(_3\) were determined in permeabilized, dispersed smooth muscle cells. In parallel experiments, the effects of isoproterenol and sodium nitroprusside (SNP), alone and in combination, on agonist (cholecystokinin octapeptide; CCK-8)-stimulated, IP\(_3\)-dependent Ca\(^{2+}\) release and muscle contraction were determined in intact smooth muscle cells. When used alone at low concentrations (10 nM), isoproterenol selectively stimulates cAMP and activates PKA, whereas SNP selectively stimulates cGMP and activates PKG (23). The results show that when used separately, isoproterenol and exogenous cAMP inhibited Ca\(^{2+}\) release and muscle contraction (i.e., induced relaxation) via PKA, whereas SNP and exogenous cGMP elicited the same effects via PKG. When isoproterenol and SNP were used in combination, both PKA and PKG activities increased but inhibition of Ca\(^{2+}\) release and muscle contraction was preferentially mediated by PKG. When exogenous cAMP and cGMP were used in combination, inhibition of Ca\(^{2+}\) release and muscle contraction was preferentially mediated by PKG and attenuated by PKA.

**MATERIALS AND METHODS**

**Preparation of dispersed muscle cells.** Muscle cells were dispersed from the circular muscle layer of the rabbit stomach as described previously (23, 28). Briefly, muscle strips were incubated for 30 min at 31°C in a HEPES medium containing 0.1% collagenase (type II) and 0.1% soybean trypsin inhibitor. The medium consisted of (in mM) 115 NaCl, 5.8 KCl, 2.1 KH\(_2\)PO\(_4\), 2 CaCl\(_2\), 0.6 MgCl\(_2\), and 25 glucose as well as 2.1% essential amino acid mixture (pH 7.4). The partly digested tissues were washed with 50 ml of enzyme-free medium, and the muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through 500-μm Nitex and centrifuged to eliminate broken cells and organelles.

Dispersed muscle cells were permeabilized by incubation for 10 min with 35 μg/ml of saponin in a medium containing (in mM) 20 NaCl, 100 KCl, 5 MgSO\(_4\), 1 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 0.34 CaCl\(_2\), and 1 EGTA with 1% bovine serum albumin (23, 27). The cells were centrifuged at 350 g for 5 min, washed free of saponin, and resuspended in the same medium with 1.5 mM ATP and ATP-regenerating system (5 mM creatine phosphate and 10 U/ml creatine phosphokinase).

**cAMP- and cGMP-dependent protein kinase assay.** PKA and PKG activities were measured by the method of Jiang et al. (13) as described previously (23). PKA activity was measured in a volume of 60 μl containing 50 mM Tris, 10 mM MgCl\(_2\), 100 μM [γ-\(^{32}\)P]ATP, 50 μM keptomide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), and 0.25 mg/ml of bovine serum albumin. The assay was done in the presence or absence of 10 μM cAMP and was initiated by the addition of 20 μl of cell supernatants to the reaction mixture. The results were corrected for cAMP-independent kinase activity by separate measurements in samples containing 2 μM PKA inhibitor protein kinase inhibitor(6–22) amide [PKI(6–22) amide]. Protein kinase activity was calculated as picomoles of phosphate transferred into keptomide per minute per microgram of protein and expressed as the ratio of activity in the presence or absence of 10 μM cAMP (−cAMP/+cAMP). A similar procedure was followed for the assay of PKG activity, and the synthetic heptapeptide histone H2B [Arg-Lys-Arg-Ser-Arg-Ala-Glu (RKRRAAE)] was used as phosphate-accepting substrate. The assay was performed in the presence of PKI(6–22) amide and in the presence or absence of 10 μM cGMP. Enzyme activity was calculated as picomoles per milligram of protein and expressed as the ratio of activity in the presence or absence of 10 μM cGMP (−cGMP/+cGMP).

**Radioimmunoassay for cAMP.** cAMP levels were measured by radioimmunoassay as described previously (23, 26, 28). Dispersed muscle cells (3 × 10\(^6\) cells) were stimulated for 1 min with 10 nM isoproterenol alone or in combination with trequinsin or SNP, and the reaction was terminated with 10% trichloroacetic acid. The samples were centrifuged, and the supernatant was extracted with diethyl ether and then lyophilized. For assay, lyophilized samples were acetylated with triethylamine-acetic anhydride (2:1) for 30 min. cAMP was measured in duplicate using 100-μl aliquots, and the results were expressed as picomoles per milligram of protein.

**Measurement of Ca\(^{2+}\) release in dispersed muscle cells.** Ca\(^{2+}\) release was measured in intact and permeabilized muscle cells by an adaptation of the method of Poggioli and Putney (31) as described previously (23). The cells were incubated with \(^{45}\)Ca\(^{2+}\) (10 μCi/ml), and Ca\(^{2+}\) uptake was measured at intervals for 90 min when a steady state was attained. After 90 min, IP\(_3\) was added and the reaction was terminated after 15 s. Cyclic nucleotides or relaxant agents were added 60 s before IP\(_3\). The decrease in \(^{45}\)Ca\(^{2+}\) content, representing net Ca\(^{2+}\) efflux, was expressed as nanomoles per 10\(^6\) cells.

**Measurement of relaxation in dispersed muscle cells.** Relaxation was measured in intact muscle cells contracted with CCK-8 (1 nM) or in permeabilized muscle cells contracted with IP\(_3\) (1 μM) (23, 27). Intact cells were treated for 60 s with isoproterenol and/or SNP followed by CCK-8 for 30 s. Permeabilized muscle cells were treated for 60 s with cAMP and/or cGMP followed by addition of IP\(_3\) for 15 s. The reaction was terminated with 1% acrolein. The length of 50 cells treated with CCK-8 or IP\(_3\) was measured in sequential microscopic fields by scanning micrometry, and the contractile response was expressed as the decrease (in μm) in mean cell length from control. Relaxation was expressed as percent increase in the length of cells contracted with CCK-8 or IP\(_3\).

**Materials.** [γ-\(^{32}\)P]ATP was from NEN Life Science Products (Boston, MA); collagenase type II from Worthington (Freehold, NJ); IP\(_3\), trequinsin, and PKI(6–22) amide from Calbiochem (San Diego, CA); and cAMP, cGMP, and all other chemicals from Sigma (St. Louis, MO).
RESULTS

Synergistic inhibition of IP3-induced muscle contraction by combinations of cAMP and cGMP in permeabilized smooth muscle. Inhibition of IP3-induced contraction (i.e., relaxation) was measured in permeabilized smooth muscle cells in response to various concentrations of cAMP or cGMP, alone and in combination. Concentration responses for cAMP and cGMP alone and in various combinations are shown in Figs. 1 and 2. No relaxation was observed with 0.01 or 0.1 μM cAMP or cGMP alone. However, a combination of 0.1 μM cAMP with either 0.01 μM or 0.1 μM cGMP elicited significant responses of 10 ± 2% (P < 0.01) and 19 ± 3% (P < 0.01), respectively (Fig. 1). A similar synergistic effect was observed when 0.1 μM cGMP was combined with either 0.01 μM or 0.1 μM cAMP (8 ± 3% and 16 ± 2%, respectively; Fig. 2). Synergistic effects were also observed at higher concentrations: the response to a combination of 1 μM cGMP and 1 μM cAMP (39 ± 2%) or 10 μM cAMP and 10 μM cGMP (86 ± 4%) was significantly greater than the corresponding additive response (17 ± 4% and 49 ± 5%, respectively; Figs. 1 and 2). In permeabilized muscle cells exposed to steady-state levels of cyclic nucleotides in the medium, the synergistic effect could not be attributed to an increase in the levels of cAMP resulting from inhibition of cAMP-specific PDE3 by cGMP.

Selective inhibitors were used to determine whether synergistic relaxation reflected cross-activation of PKA or PKG. Relaxation induced by 1 μM cAMP alone was inhibited by PKI(6–22) amide, a selective inhibitor of PKA, but was not affected by KT5823, a selective inhibitor of PKG (Fig. 3). Conversely, relaxation induced by 1 μM cGMP was inhibited by KT5823 but not by PKI (Fig. 3). The synergistic response to a combination of 1 μM cAMP and 1 μM cGMP, however, was augmented by PKI(6–22) amide and abolished by KT5823. The results implied that in the presence of cGMP relaxant effects induced by cAMP were mediated by PKG, consistent with increase in the affinity of PKG for cAMP in the presence of cGMP (7, 11, 17). The increase in relaxation induced by inhibition of PKA suggested the existence of a competitive interaction between PKA and PKG on a common cellular target.
PKG when activated by endogenous cAMP and cGMP.

cells to determine the effects and interplay of PKA and

Ca\(^{2+}\) contraction by combinations of cAMP- and cGMP-

Ca\(^{2+}\) by a combination of 1 M cAMP and/or 1 mM cGMP in the presence or absence of the PKA inhibitor PKI(6–22) amide (1 mM) or the PKG inhibitor KT5823 (1 mM). Inhibition of Ca\(^{2+}\) release was expressed as a percentage of Ca\(^{2+}\) release induced by IP\(_3\) (0.72 \pm 0.04 nmol/10\(^6\) cells). Inhibition of Ca\(^{2+}\) release by a combination of cAMP and cGMP was significantly augmented by PKI(6–22) amide (Fig. 4). Inhibition of Ca\(^{2+}\) release induced by a combination of isoproterenol and SNP (59 \pm 6\%) was not affected by myristoylated PKI(14–22) amide (Fig. 5).

Similar results were obtained for inhibition of CCK-stimulated, IP\(_3\)-dependent Ca\(^{2+}\) release. Ca\(^{2+}\) release measured during the first minute after stimulation with 1 nM CCK-8 was similar to that elicited by exogenous IP\(_3\) (0.68 \pm 0.05 nmol/10\(^6\) cells) (23, 27). Isoproterenol and SNP inhibited CCK-stimulated Ca\(^{2+}\) release by 15 \pm 3\% and 15 \pm 2\%, respectively (P < 0.01). Inhibition of Ca\(^{2+}\) release by isoproterenol was blocked by myristoylated PKI(14–22) amide but not by KT5823, whereas inhibition of Ca\(^{2+}\) release by SNP was blocked by KT5823 but not by myristoylated PKI(14–22) amide (Fig. 6).

Synergistic inhibition of IP\(_3\)-induced Ca\(^{2+}\) release by combinations of exogenous cAMP and cGMP in permeabilized smooth muscle. The synergistic effect of combinations of cAMP and cGMP on relaxation (i.e., inhibition of IP\(_3\)-induced contraction) was reflected in the inhibition of IP\(_3\)-induced Ca\(^{2+}\) release. Ca\(^{2+}\) release, measured as the decrease in steady-state 45Ca\(^{2+}\) cell content during the first minute of stimulation with IP\(_3\), was 0.72 \pm 0.04 nmol/10\(^6\) cells. cAMP (1 mM) and cGMP (1 mM) inhibited IP\(_3\)-induced Ca\(^{2+}\) release by 10 \pm 2\% and 13 \pm 2\%, respectively (P < 0.01). Inhibition of Ca\(^{2+}\) release by cAMP was blocked by PKI(6–22) amide but not by KT5823, whereas inhibition of Ca\(^{2+}\) release by cGMP was blocked by KT5823 but not by PKI(6–22) amide (Fig. 4). Inhibition of Ca\(^{2+}\) release by a combination of 1 mM cAMP and 1 mM cGMP (29 \pm 4\%) was significantly increased by PKI(6–22) amide (42 \pm 5\%, P < 0.05) but was abolished by KT5823 (Fig. 4). The results suggest that in permeabilized muscle cells, the IP\(_3\) receptor is a target for inhibitory phosphorylation by either PKG or PKA (15, 25). In the presence of cGMP, however, phosphorylation induced by cAMP or cGMP is mediated by PKG only. The increase in the inhibition of Ca\(^{2+}\) release induced by PKI(6–22) amide suggests that, when present with PKG, PKA attenuates the interaction of PKG with the IP\(_3\) receptor.

Inhibition of agonist-induced Ca\(^{2+}\) release and muscle contraction by combinations of cAMP- and cGMP-stimulating agents in intact smooth muscle. Experiments were done in intact, nonpermeabilized muscle cells to determine the effects and interplay of PKA and PKG when activated by endogenous cAMP and cGMP.

![Graph](image)

**Fig. 4.** Effects of selective PKA and PKG inhibitors on inhibition of IP\(_3\)-induced Ca\(^{2+}\) release by cAMP and/or cGMP in permeabilized muscle cells. Peak Ca\(^{2+}\) release was measured as described in MATERIALS AND METHODS. Inhibition of Ca\(^{2+}\) release measured during the first minute after stimulation with 1 nM CCK-8 was similar to that elicited by exogenous IP\(_3\) (0.68 \pm 0.05 nmol/10\(^6\) cells) (23, 27). Isoproterenol and SNP inhibited CCK-stimulated Ca\(^{2+}\) release by 15 \pm 3\% and 15 \pm 2\%, respectively (P < 0.01). Inhibition of Ca\(^{2+}\) release by isoproterenol was blocked by myristoylated PKI(14–22) amide but not by KT5823, whereas inhibition of Ca\(^{2+}\) release by SNP was blocked by KT5823 but not by myristoylated PKI(14–22) amide (Fig. 6).

The muscle cells were treated with isoproterenol (10 nM) or SNP (10 nM), alone or in combination. As shown previously, at these concentrations, isoproterenol selectively stimulates cAMP formation and activates PKA, whereas SNP selectively stimulates cGMP formation and activates PKG (23). Isoproterenol (10 nM) and SNP (10 nM) inhibited CCK-stimulated contraction (i.e., induced relaxation) in nonpermeabilized muscle cells by 22 \pm 2\% and 26 \pm 4\% (P < 0.01), respectively. Relaxation induced by isoproterenol was abolished by myristoylated PKI(14–22) amide but was not affected by KT5823, whereas relaxation induced by SNP was abolished by KT5823 but was not affected by myristoylated PKI(14–22) amide (Fig. 5). Relaxation induced by a combination of isoproterenol and SNP (59 \pm 6\%) was not affected by myristoylated PKI(14–22) amide (59 \pm 4\%) but was abolished by KT5823 (Fig. 5).

![Graph](image)

**Fig. 5.** Effects of selective PKA and PKG inhibitors on relaxation induced by isoproterenol and/or sodium nitroprusside (SNP) in nonpermeabilized muscle cells. Relaxation of dispersed muscle cells in response to 10 nM isoproterenol (Isop) and/or 10 nM SNP in the presence or absence of the PKA inhibitor myristoylated PKI(14–22) amide (1 mM) or the PKG inhibitor KT5823 (1 mM) was measured as described in MATERIALS AND METHODS. Relaxation was expressed as % inhibition of CCK-induced contraction (34.7 \pm 3.8 \mu m decrease from control cell length of 112.8 \pm 5.2 \mu m). Values are means \pm SE of 3–4 experiments.
cAMP-specific PDE3 by SNP-stimulated cGMP (29). As shown in Fig. 7, isoproterenol-stimulated cAMP levels were augmented in the presence of SNP to the same extent as in the presence of the selective PDE3 inhibitor trequinsin. Nevertheless, the effects of endogenous cAMP or cGMP when stimulated concurrently were mediated exclusively by PKG.

**Preferential activation of PKG by cAMP in presence of cGMP.** Conclusions based on the use of selective PKA and PKG inhibitors were corroborated by direct measurements of PKA and PKG. Isoproterenol (10 nM) increased PKA activity by 38 ± 3% above basal level ($P < 0.01$); the increase was selectively inhibited by myristoylated PKI(14–22) amide (Fig. 8). A smaller increase (19 ± 4%) in basal PKA activity elicited by SNP was also selectively inhibited by myristoylated PKI(14–22) amide (Fig. 8). A combination of 10 nM isoproterenol with 10 nM SNP increased PKA activity by 95 ± 4%, which is more than the double the increase in PKA activity elicited by isoproterenol alone; the PKA activity was selectively inhibited by myristoylated PKI(14–22) amide. The large increase in isoproterenol-stimulated PKA activity was attributed to increase in cAMP levels resulting from inhibition of PDE3 activity by SNP-stimulated cGMP (Fig. 7; Refs. 4, 29).

Isoproterenol had no effect on basal PKG activity, whereas SNP increased PKG activity by 101 ± 5% above basal level ($P < 0.01$; Fig. 9). In the presence of isoproterenol, SNP increased PKG activity by 190 ± 12%, which is more than the increase elicited by SNP.
alone ($P < 0.01$ for difference between response to SNP alone vs. SNP + isoproterenol; Fig. 9). PKG activity stimulated by SNP alone or in the presence of isoproterenol was selectively inhibited by KT5823 (Fig. 9).

**DISCUSSION**

The present study focused on the singular effects and interplay of PKA and PKG on IP$_3$-dependent Ca$^{2+}$ mobilization because of its crucial importance in initiating contraction in smooth muscle. Direct measurements and selective inhibitors of PKA and PKG activities were used to evaluate the role of each kinase in inhibiting IP$_3$-dependent Ca$^{2+}$ release. Activation of PKA and/or PKG was elicited by exogenous cAMP and cGMP in permeabilized smooth muscle or by isoproterenol and SNP in intact smooth muscle. The PKA inhibitors PKI(6–22) amide and myristoylated PKI(14–22) amide and the PKG inhibitor KT5823 were used at concentrations at which they selectively inhibited PKA and PKG, respectively (23, 24).

In previous studies (23), we showed that low concentrations of SNP stimulated cGMP and activated PKG in gastric smooth muscle cells, whereas equally low concentrations of isoproterenol ($<1 \mu$M) stimulated cAMP and activated PKA without cross-activating PKG. The present study confirmed preferential activation of PKA and PKG by low concentrations (10 nM) of isoproterenol and SNP, respectively, but also showed that treatment of smooth muscle cells with a combination of SNP and isoproterenol increased both PKA and PKG activities. The increase in PKA activity probably reflected an increase in cAMP resulting from inhibition of cAMP-specific PDE3 by SNP-stimulated cGMP (Fig. 7; Refs. 4, 8, 29). The increase in PKG activity, however, reflected an increase in the affinity of cAMP for PKG, resulting in greater activation of this kinase (7, 11, 17). The two main isoforms of PKG I expressed in gastrointestinal smooth muscle (70% PKG I$_a$ and 30% PKG I$_b$) undergo autophosphorylation and preferentially bind cGMP; autophosphorylation and the binding of cGMP enhance the affinity of cAMP for PKG by up to 10-fold (2, 7, 12). cAMP, which is generated in ~10 times greater abundance than cGMP, becomes the major activator of PKG when the two cyclic nucleotides are present together (7, 11, 13, 17, 23).

Despite the increase in both PKA and PKG activities induced by a combination of isoproterenol and SNP, the inhibition of agonist-induced, IP$_3$-dependent Ca$^{2+}$ release and muscle cell contraction (i.e., relaxation) in intact muscle cells was exclusively mediated by PKG (abolished by KT5823). Similarly, inhibition of IP$_3$-induced Ca$^{2+}$ release and muscle contraction in permeabilized muscle cells by a combination of exogenous cAMP and cGMP was exclusively mediated by PKG. The inhibitory effects of combinations of cAMP and cGMP on Ca$^{2+}$ release and contraction were synergistic, eliciting responses that were more than additive. The synergism could not be attributed to increase in the levels of cAMP resulting from inhibition of cAMP-specific PDE3 by cGMP, because the permeabilized smooth muscle cells were exposed to steady-state levels of exogenous cyclic nucleotides.

In permeabilized muscle cells, PKA stimulated by exogenous cAMP alone, or PKG stimulated by exogenous cGMP alone, was capable of inhibiting Ca$^{2+}$ release induced by exogenous IP$_3$. Their singular effects were blocked by the corresponding selective kinase inhibitor, implying that each kinase was independently capable of phosphorylating and thus inhibiting IP$_3$ receptor activity. However, the inhibitory effects during concurrent activation of PKA and PKG by a combination of exogenous cAMP and cGMP were exclusively mediated by PKG (abolished by KT5823) and attenuated by PKA [augmented by PKI(6–22) amide]. The pattern suggested that PKG and PKA act competitively in phosphorylating the IP$_3$ receptor in permeabilized muscle cells.

In different tissues (9, 15, 16), PKA and PKG phosphorylate serine 1756 and/or serine 1589 of the type I IP$_3$ receptor. Komalavilas and Lincoln (15, 16) showed that, in aortic smooth muscle, PKG preferentially phosphorylates serine 1756 in vitro and in vivo, whereas PKA phosphorylates both serine residues in vitro only. Selective activators of PKG but not PKA elicited IP$_3$ receptor phosphorylation. Phosphorylation of the IP$_3$ receptor by forskolin resulted from cross-activation of PKG by high concentrations of cAMP and was blocked by a PKG inhibitor.

Our recent studies (25) on gastric smooth muscle cells using selective activators and inhibitors of PKA and PKG confirmed that PKG phosphorylates the IP$_3$ receptor in both intact and permeabilized smooth muscle cells, whereas PKA phosphorylates the IP$_3$ receptor in permeabilized smooth muscle cells only. The results suggest that PKA can gain access to the IP$_3$ receptor in permeabilized but not intact smooth muscle cells. These results, together with the earlier results of Komalavilas and Lincoln (15, 16), have an important bearing on interpretation of the effects of relaxant neurotransmitters and other agents in intact, nonpermeabilized smooth muscle cells.

In light of these results, the inhibition of agonist-stimulated, IP$_3$-dependent Ca$^{2+}$ release in intact gast
cAMP inhibits agonist-stimulated IP$_3$ formation by PKA rather than inhibition of IP$_3$-dependent Ca$^{2+}$ release. PKA can be targeted to the plasma membrane by specific anchoring proteins that enable it to phosphorylate membrane-bounded PLC-β activity (6, 10) but may not be targeted to the sarcoplasmic membrane to phosphorylate the IP$_3$ receptor. The results suggest that permeabilization facilitates access of PKA to the IP$_3$ receptor spanning the sarcoplasmic membrane, thus enabling PKA to phosphorylate the IP$_3$ receptor in vitro. This would explain why inhibition of IP$_3$-induced Ca$^{2+}$ release and contraction by exogenous cAMP in permeabilized smooth muscle cells was selectively mediated by PKA. It would also explain the competitive interaction between PKG and PKA in permeabilized muscle cells, in which the same serine residue on the IP$_3$ receptor is phosphorylated by both kinases, albeit with greater affinity by PKG. The inhibition of agonist-stimulated, IP$_3$-dependent Ca$^{2+}$ release by SNP or by a combination of SNP and isoproterenol in intact muscle cells was mediated by PKG and could result from inhibition of both IP$_3$ formation and IP$_3$-dependent Ca$^{2+}$ release. A model summarizing the results of this study and illustrating the crucial role of PKG in the inhibition of IP$_3$-dependent Ca$^{2+}$ release is shown in Fig. 10.

It is worth noting that many earlier studies in various types of smooth muscle showed that an increase in cAMP inhibits agonist-stimulated IP$_3$ formation and increase in cytosolic Ca$^{2+}$ levels (5, 36). However, the high levels of cAMP induced by micromolar concentrations of isoproterenol or forskolin, the agents of choice in many studies, can activate PKA and cross-activate PKG, obscuring the ability of PKA to inhibit IP$_3$ formation (13, 14, 23). The inhibition of IP$_3$ formation and/or Ca$^{2+}$ mobilization by PKA was unmasked in other studies by selective PKA inhibitors (23, 27).

Although a close parallelism exists between inhibition of Ca$^{2+}$ release and inhibition of muscle contraction (i.e., relaxation), other downstream processes besides inhibition of Ca$^{2+}$ release could contribute to PKA- or PKG-mediated relaxation, including inhibition of MLC kinase and stimulation of MLC phosphatase activities (18, 22, 35, 37). Sustained (tonic) contraction, which follows the initial Ca$^{2+}$-dependent contractile transient, is Ca$^{2+}$ independent and mediated by RhoA-dependent inhibition of MLC phosphatase (34). Our recent studies (30) suggest that relaxation of sustained muscle contraction results from inhibition of RhoA activity by both PKA and PKG, leading eventually to increase of MLC phosphatase activity and inhibition of MLC phosphorylation (30).

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