Selective phosphorylation of the IP$_3$R-I in vivo by cGMP-dependent protein kinase in smooth muscle

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Submitted 18 September 2002; accepted in final form 5 November 2002

Murthy, Karnam S., and Huiping Zhou. Selective phosphorylation of the IP$_3$R-I in vivo by cGMP-dependent protein kinase in smooth muscle. *Am J Physiol Gastrointest Liver Physiol* 284: G221–G230, 2003; 10.1152/ajpgi.00401.2002.—This study examined the expression of inositol 1,4,5-trisphosphate (IP$_3$) receptor (IP$_3$R) types and PKG isoforms in isolated gastric smooth muscle cells and determined the ability of PKG and PKA to phosphorylate IP$_3$Rs and inhibit IP$_3$-dependent Ca$^{2+}$ release, which mediates the initial phase of agonist-induced contraction. PKG-Ia and PKG-Iß were expressed in gastric smooth muscle cells, together with IP$_3$-R-associated cG-kinase substrate, a protein that couples PKG-Iß to IP$_3$R-I. IP$_3$R-I and IP$_3$R-III were also expressed, but only IP$_3$R-I was phosphorylated by PKA and PKG in vitro and exclusively by PKG in vivo. Sequential phosphorylation by PKA and by PKG-Ia in vitro showed that PKA phosphorylated the same site as PKG (presumably S$^2765$) and an additional PKG-specific site (S$^569$). In intact muscle cells, agents that activated PKG or both PKG and PKA induced IP$_3$R-I phosphorylation that was reversed by the PKG inhibitor (8R,9S,11S)-(−)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,1H,2,7b,11a-trizadizo-benzo9-(a,g)cycloocta(c,d,e)-trinden-1-one. Agents that activated PKG induced IP$_3$R-I phosphorylation in permeabilized but not intact muscle cells, implying that PKA does not gain access to IP$_3$R-I in intact muscle cells. The pattern of IP$_3$R-I phosphorylation in vivo and in vitro was more consistent with phosphorylation by PKG-Ia. Phosphorylation of IP$_3$R-I in microsomes by PKG, PKA, or a combination of PKG and PKA inhibited IP$_3$-induced Ca$^{2+}$ release to the same extent, implying that inhibition was mediated by phosphorylation of the PKG-specific site. We conclude that IP$_3$R-I is selectively phosphorylated by PKG-I in intact smooth muscle resulting in inhibition of IP$_3$-dependent Ca$^{2+}$ release.

A close parallelism exists between inhibition of agonist-induced inositol 1,4,5-trisphosphate (IP$_3$)-dependent Ca$^{2+}$ release by cAMP- and cGMP-dependent protein kinases (PKA and PKG) and inhibition of muscle contraction (i.e., relaxation). PKA and/or PKG can regulate Ca$^{2+}$ mobilization by acting on various molecular targets, including IP$_3$ generation (19, 33, 34) and sarcoplasmic IP$_3$ receptors (IP$_3$R)/Ca$^{2+}$ channels (9, 10), which determine Ca$^{2+}$ release, plasmalemmal and sarcoplasmic Ca$^{2+}$/ATPase pumps, which determine Ca$^{2+}$ uptake into intracellular stores or efflux from the cell (2, 14), and plasmalemmal Ca$^{2+}$ and K$^+$ channels, which regulate membrane polarity and Ca$^{2+}$ influx via voltage-dependent Ca$^{2+}$ channels (25). Activation of PKG or PKA inhibits agonist-induced Ca$^{2+}$ release in intact visceral smooth muscle cells (17, 19) and IP$_3$-induced Ca$^{2+}$ release in permeabilized visceral smooth muscle cells (17). Inhibition of agonist-induced Ca$^{2+}$ release could result from inhibition of IP$_3$ formation, whereas inhibition of IP$_3$-induced Ca$^{2+}$ release in permeabilized muscle probably reflects phosphorylation of the IP$_3$R by either kinase. Studies in vascular smooth muscle suggest that both kinases are capable of phosphorylating IP$_3$R in vitro, whereas only PKG phosphorylates IP$_3$R in vivo (9, 10). Permeabilization may allow access of PKA to the IP$_3$R that is normally denied to this kinase in intact smooth muscle cells.

Two PKG isoforms, PKG-Ia and PKG-Iß, have been implicated in IP$_3$R-I phosphorylation. Although both isoforms are frequently colocalized, PKG-Ia appears to be more widely distributed in human, bovine, rabbit, and murine tissues (5, 7, 11, 29, 35). PKG-Ia is 10-fold more sensitive to activation by cGMP and is more susceptible to cross-activation by cAMP (12, 29). Both kinases recognize in vivo substrates by interaction with their distinctive NH$_2$-terminal leucine-zipper amino acid sequences (1, 32). A 125-kDa protein, IP$_3$-R-associated cG-kinase substrate (IRAG), has been recently identified that binds to both the IP$_3$R and the distinctive NH$_2$-terminal sequence of PKG-Iß (1, 28). In cells expressing IP$_3$R-I, IRAG, and PKG-Iß, all three proteins are immunoprecipitated by antibodies to each. Reconstitution studies in COS-7 cells suggest that phosphorylation of IRAG at Ser$^{690}$ is a prerequisite for IP$_3$R phosphorylation and inhibition of IP$_3$-dependent Ca$^{2+}$ release by PKG-Iß (28). However, recent studies in native mouse aortic smooth muscle cells that normally express IRAG and both PKG-I isoforms have raised doubts regarding the functional role of PKG-Iß (3). Transfection of PKG-Ia into smooth muscle cells derived from PKG-Ia- and -ß-deficient mice restored the ability of PKG activators [nitric oxide

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(NO) donors and 8-bromo-cGMP to inhibit agonist-induced \( \text{Ca}^{2+} \) transients, whereas transfection of PKG-I\( \beta \) had no effect (3).  

In the present study, we have examined the expression of IP3R types and PKG isoforms in freshly dispersed and cultured gastric smooth muscle cells and determined the ability of PKG and PKA to phosphorylate IP3Rs in vivo and inhibit IP3-dependant \( \text{Ca}^{2+} \) release from isolated sarcoplasmic microsomes. The results indicate that PKG and PKA induce in vitro phosphorylation of IP3R-I expressed in smooth muscle cells. IP3R-I phosphorylation in vivo is mediated exclusively by PKG and is partly responsible for inhibition of IP3-dependent \( \text{Ca}^{2+} \) release.

**MATERIALS AND METHODS**

Preparation of dispersed gastric smooth muscle cells. Dispersed gastric smooth muscle cells were prepared by sequential enzymatic digestion, filtration, and centrifugation as described previously (16, 17, 19, 20). The partly digested strips were washed twice 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-\( \mu \)l Nitex (Tetko, Briarcliff Manor, NY) and centrifuged twice at 350 \( \times \) g for 10 min. For permeabilization, dispersed smooth muscle cells were treated for 5 min with saponin (35 \( \mu \)g/ml) and resuspended in low-\( \text{Ca}^{2+} \) (100 nM) medium as previously described (16, 17). In some experiments, the cells were placed in culture in Dulbecco’s Modified Eagle’s medium containing 10% fetal bovine serum until they attained confluence.

RT-PCR analysis of IP3R types, PKG isoforms, and IRAG. Specific primers were designed for IP3R-I, -II, and -III based on homologous sequences in human, bovine, and mouse cDNAs. The sequences in human, bovine, and mouse cDNAs, for PKG-I and PKG-\( \alpha \) based on the sequence of rabbit PKG-I\( \alpha \) cDNA, and for PKG-I\( \beta \) based on homologous sequences in human, bovine, and mouse cDNAs. The sequences of the primers are listed in Table 1.

RNA (5 \( \mu \)g) prepared from cultured rabbit gastric smooth muscle cells was reversibly transcribed and amplified by PCR under standard conditions (2 mM MgCl2, 200 \( \mu \)M dTTP, and 2.5 units of Taq polymerase) in a final volume of 50 \( \mu \)l containing 100 ng of each primer. The PCR products were separated by electrophoresis in 1.2% agarose gel in the presence of ethidium bromide, visualized by ultraviolet fluorescence, and recorded by a Chemilager 4400 Fluorescence system. PCR products were purified by gel extraction kit and sequenced.

**Western blot analysis of IP3R.** The expression of IP3Rs was determined by Western blot as described previously (18) using homogenates prepared from freshly dispersed gastric smooth muscle cells. Proteins were resolved by 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated for 12 h at 4°C with specific antibodies for IP3R-I, -II, or -III and then for 1 h with secondary antibody. The bands were identified by enhanced chemiluminescence.

In vivo phosphorylation and immunoprecipitation of IP3R-I. Phosphorylation of IP3R-I was determined from the amount of \( ^{32} \text{P} \) incorporated after immunoprecipitation with specific IP3R-I antibody as previously described for other proteins (18). Ten milliliters of smooth muscle cell suspension (4 \( \times \) 10^6 cells/ml) were incubated with \( ^{32} \text{P} \)orthophosphate for 4 h at 31°C. Samples (1 ml) were reincubated with PKA or PKG activators for 1 min in the presence or absence of the specific PKG or PKA inhibitors. The reaction was terminated with an equal volume of lysis buffer. The cell lysates were separated from the insoluble material by centrifugation at 13,000 \( g \) for 15 min at 4°C, precleared with 40 \( \mu \)l of protein A-sepharose, and incubated overnight with IP3R-I antibody at a final concentration of 5 \( \mu \)g/ml. Protein A/G-sepharose was then added for 1 h, and the mixture was centrifuged for 5 min. The immunoprecipitates were washed four times with the lysis buffer and reuspended in Laemmli buffer and boiled for 15 min. The proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. \( ^{32} \text{P} \)IP3R-I was visualized by autoradiography, and the amount of radioactivity in the bands was counted. Immunoblot analysis was performed on the membranes after autoradiography to determine comigration of IP3R-I with the corresponding radioactive bands.

**In vitro phosphorylation of IP3R-I.** IP3R-I immunoprecipitates were washed five times with lysis buffer and phosphorylated in the presence of exogenous PKG-I\( \alpha \) holenzyme (0.5 \( \mu \)M) or the catalytic subunit of PKA (0.5 \( \mu \)M). Phosphorylation with the catalytic subunit of PKA was performed in a medium containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM EDTA, 10 \( \mu \)M cAMP, 6 mM \( \text{Ca}^{2+} \), 12 mM \( \beta \)-glycerophosphate, and 20 \( \mu \)M sodium vanadate. Phosphorylation with the purified holenzyme of PKG-I\( \alpha \) was performed in the presence of PKI\( 14 \rightarrow 22 \) amide and 10 \( \mu \)M cGMP instead of cAMP. Phosphorylation was initiated by the

### Table 1. Sequence of PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP3R-I</td>
<td>Forward: 5’ TCTGACCTTCTCCGATTAACAAGTCC 3’</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ GGACATGCGGGCAAAAGGCTCTTCCGCG 3’</td>
<td></td>
</tr>
<tr>
<td>IP3R-II</td>
<td>Forward: 5’ GACCCCAACAGATAACACG 3’</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ CATGCGGTTGTTTGATGC 3’</td>
<td></td>
</tr>
<tr>
<td>IP3R-III</td>
<td>Forward: 5’ TGAACCTGGGCAGCCAGCC 3’</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ GTCTCCAAAGGTCTGTGAGC 3’</td>
<td></td>
</tr>
<tr>
<td>PKG-I( \alpha )</td>
<td>Forward: 5’ ATGGATCGACCTGGAGGAAGAC 3’</td>
<td>474</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ GTGCCACCTGGAGCTCTC 3’</td>
<td></td>
</tr>
<tr>
<td>PKG-I( \beta )</td>
<td>Forward: 5’ TGACCTGGCTCTCTACCCC 3’</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ TGACCTGGCTCTCTACCCC 3’</td>
<td></td>
</tr>
<tr>
<td>IRAG</td>
<td>Forward: 5’ AGCAGGGCTTCTCAAAATCGC 3’</td>
<td>524</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ GTGATGATGCTGGAGGAAGAC 3’</td>
<td>378</td>
</tr>
</tbody>
</table>

IP3R, inositol 1,4,5-trisphosphate receptor; IRAG, IP3R-associated cGK kinase substrate.
addition of [γ-32P]ATP (12 Ci/mmol) and terminated after 30 min by the addition of 40 mM EDTA and an equal volume of Laemmli buffer. The proteins were resolved, and the immunoblots were analyzed as described above for in vivo phosphorylation.

In experiments involving sequential phosphorylation by PKA and PKG, IP3R-I was initially phosphorylated in the presence of one kinase and nonradioactive ATP for 30 min, after which the immunoprecipitates were washed with Tris buffer saline containing Tween 20. A second incubation was then initiated by addition of the other kinase in the presence of [γ-32P]ATP, and the mixture was incubated for 30 min. The samples were analyzed for IP3R-I phosphorylation as described above.

Back phosphorylation of IP3R. A back-phosphorylation approach was also used to determine in vivo phosphorylation of IP3R-I (10). IP3R-I immunoprecipitates derived from control smooth muscle cells and cells treated with activators of PKA or PKG were phosphorylated in vitro using [γ-32P]ATP in the presence of exogenous PKG-I holoenzyme as described in In vitro phosphorylation of IP3R. The amount of 32P incorporated into the immunoprecipitated IP3R-I derived from control muscle cells was taken as 100%, and the amount of 32P incorporated into the immunoprecipitated IP3R-I derived from cells treated with PKA or PKG activators was calculated as a percentage of the control value. The decrease in 32P incorporation after treatment with PKA and PKG activators reflected endogenous phosphorylation by PKA or PKG.

Ca2+ release from smooth muscle microsomes. The effect of IP3R-I phosphorylation on IP3-induced Ca2+ release was measured in microsomes prepared from freshly dispersed smooth muscle cells and preloaded with 45Ca2+ as described previously (16). One-milliliter samples (0.5 mg) of the microsomal suspension were incubated for 60 min at 31°C with 45Ca2+ (10 μCi/ml), ATP (1 μM), and ATP regenerating system when a steady state of Ca2+ uptake was reached (16). After the addition of IP3, 100-μl samples were removed at 15 s and added to 25 μl of quench medium consisting 0.633 M formalin and 50 mM EDTA (pH 7.0). The samples were centrifuged at 13,000 g for 5 min, and the pellets were washed twice with the same medium and extracted with 50 μl of trichloroacetic acid for measurement of 45Ca2+.

Materials. [γ-32P]ATP and [32P]orthophosphate were obtained from Amersham Pharmacia Biotech (Piscataway, NJ); collagenase and soybean trypsin inhibitor were from Worthington Biochemical (Freehold, NJ); 8-4-(chlorophenylthio) guanosine 3’,5’-cyclic monophosphate (8-pCPT-cGMP) and 5,6-dichloro-1-β-d-ribofuranosyl benzimidazole 3’,5’-cyclic monophosphothioate, Sp-isomer (cBIMPS) were from Alexis Biochemicals (San Diego, CA); IP3 was from Calbiochem (San Diego, CA); Western blotting and chromatography material and protein assay kit were from Bio-Rad Laboratories (Hercules, CA); antibody to IP3R types I, II, and III were from Santa Cruz (Santa Cruz, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

Expression of IP3R types, PKG isoforms, and IRAG in gastric smooth muscle. PKG-Iα and PKG-Iβ were detected by RT-PCR in primary cultures of gastric smooth muscle cells and after first passage using specific primers based on conserved sequences in rabbit for PKG-Iα and in human, bovine, and mouse for PKG-Iβ (Fig. 1).

IRAG was also detected by RT-PCR in primary cultures of rabbit smooth muscle cells and after first passage using specific primers based on conserved sequences in human and bovine, including a primer based on the sequence that specifically binds PKG-Iβ (Fig. 1). The partial amino acid sequence was 91–92% similar to human and bovine IRAG.

Both IP3R-I and -III but not IP3R-II were detected by RT-PCR in primary cultures of gastric smooth muscle cells and after first passage using primers based on conserved sequences in human, bovine, and rat (Fig. 2). Western blot analysis of lysates derived from freshly dispersed smooth muscle cells confirmed expression of IP3R-I and -III but not -II (Fig. 2). The partial amino acid sequence of IP3R-I was 92–94%, and that of IP3R-III was 90–94%, similar to those of human and rat receptors.

In vitro phosphorylation of IP3R-I by PKA and PKG. Exogenous PKG-Iα holoenzyme and the catalytic subunit of PKA phosphorylated IP3R-I in immunoprecipi...
states obtained from lysates of dispersed smooth muscle cells (Fig. 3). Although the extent of IP$_3$R-I phosphorylation was greater with PKA than with PKG, phosphorylation by both kinases in combination was not greater than that by PKA alone (Fig. 3). The relationship between phosphorylation by PKA and PKG was further examined by the sequential addition of the two kinases. Incubation with nonradioactive ATP in the presence of PKA for 30 min prevented further phosphorylation of IP$_3$R-I by PKG. In contrast, incubation with nonradioactive ATP in the presence of PKG for 30 min did not prevent additional phosphorylation of the receptor by PKA (Fig. 4). The extent of additional phosphorylation by PKA was similar to the difference between phosphorylation by PKA and PKG added separately (cf. Figs. 3 and 4). The pattern implied that PKA phosphorylated the same residue(s) as PKG as well as additional PKA-specific residue(s).

In vitro phosphorylation of IP$_3$R-I by PKG. Several agents that activate PKA, PKG, or both kinases were used alone and in conjunction with selective inhibitors of PKA (myristoylated PKI$_{14 - 22}$ amide) or PKG ([8R,9S,11a]-(-)-9-methoxy-carbamyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,1H,-2,7b,11a-triazidobenzo[9a,g]cycloocta[cd,e]-trinden-1-one (KT5823)) to determine the ability of PKA and PKG to phosphorylate IP$_3$R-I in vivo. Direct measurement of PKA and PKG activities in these muscle cells have shown that, at concentrations of 1 μM and less, myristoylated PKI selectively inhibits PKA activity, whereas KT5823 selectively inhibits PKG activity (15, 17, 18). The kinase activators included selective activators of PKG [8-pCPT-cGMP and sodium nitroprusside (SNP)], selective activators of PKA (cBIMPS and a low concentration of isoproterenol, <1 μM), and activators of both PKA and PKG [vasoactive intestinal peptide (VIP) and high concentrations of forskolin or isoproterenol, >1 μM] (8, 15, 17). As shown previously, VIP interacts with distinct receptors to stimulate cAMP and cGMP and activate both kinases (17, 20); high concentrations of forskolin and isoproterenol generate high levels of cAMP that activate PKA and cross-activate PKG (8, 17).

Two sets of parallel studies were performed: in one set, the cells were first labeled with $^32$P and then treated with various agents before immunoprecipitation.
PKA and PKG, increased IP₃-R-I phosphorylation by 304 ± 25% above the basal level; the increase was abolished by KT5823 but was not affected by myristoylated PKI (Fig. 9A). With each of these agents (cBIMPS, forskolin, isoproterenol, and VIP) identical results were obtained using the technique of back phosphorylation (Figs. 7B, 8B, and 9B). These results indicated that, in vivo, only PKG is capable of phosphorylating IP₃-R-I, raising the possibility that PKA, which is capable of phosphorylating the receptor in vitro, may not gain access to the receptor in vivo. This notion was
examined in permeabilized smooth muscle cells. In these cells, selective activation of PKA by cBIMPS or a low concentration of isoproterenol (1 mM) increased phosphorylation of IP3-R-I by 300 ± 23 and 345 ± 39% above the basal level (basal: 612 ± 86 cpm); phosphorylation by both agents was selectively inhibited by myristoylated PKI but was not affected by KT5823 (Fig. 10).

Inhibition of IP3-induced Ca2+ release by IP3-R-I. The effect of IP3-R-I phosphorylation on IP3-induced Ca2+ release was determined in microsomes derived from freshly dispersed gastric smooth muscle cells. After the microsomes were loaded with 45Ca2+, they were treated with PKG-1α holoenzyme (0.5 μM) or the catalytic subunit of PKA (0.5 μM) for 15 min. Treatment with either kinase induced IP3-R-I phosphorylation. The addition of IP3 for 15 s elicited rapid concentration-dependent Ca2+ release (EC50 = 1 nM IP3) that was inhibited to the same extent by PKA or PKG (EC50 = 1 μM IP3 with either PKG or PKA), suggesting that phosphorylation of a common site by either kinase was responsible for inhibition of Ca2+ release (Fig. 11). An increase in phosphorylation induced by a higher concentration of the catalytic subunit of PKA (1 μM) or a combination of PKA and PKG-Iα did not elicit greater inhibition of Ca2+ release (50% inhibition of release by 1 μM IP3), implying that the site phosphorylated by PKG-Iα mediated inhibition of IP3-R-I activity. Treatment of the microsomes for 15 min with a selective antibody directed against the COOH terminal of IP3-R-I but not antibodies directed against the COOH terminal of IP3R-II or IP3R-III strongly inhibited IP3-induced Ca2+ release (Fig. 12) (23).

DISCUSSION

IP3Rs belong to a family of tetrameric Ca2+ channels that open on binding of IP3. Three receptor types have been identified (IP3-R-I, -II, -III) that exhibit similar structures consisting of an endoplasmic membrane-spanning Ca2+ channel domain flanked by a large cytosolic IP3-binding domain and a smaller cytosolic COOH-terminal domain (25, 26, 36). A regulatory domain, NH2 terminal to the Ca2+ channel, contains putative consensus sequences for phosphorylation by PKG (S1755) and PKA (S1755 and S1589) (4, 6, 9, 31, 37, 38, 40). These sequences (GRRS1755L and ARRS1589V) appear to be restricted to IP3-R-I, which is most abundant and has the widest central and peripheral distri-

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**Fig. 6.** Concentration-dependent phosphorylation of IP3-R-I by SNP. Phosphorylation of IP3-R-I was determined by the technique of back phosphorylation as described in MATERIALS AND METHODS and the legend to Fig. 5. As shown in the radioactive bands, maximal IP3-R-I back phosphorylation was observed in the basal state (B). Increasing in vivo phosphorylation with SNP concentration was reflected by a decrease of radioactivity in the bands. Immunoblots of the IP3-R-I bands are shown for loading control. Values are means ± SE of 5 experiments.

**Fig. 7.** PKG-dependent phosphorylation of IP3-R-I by forskolin. A: gastric smooth muscle cells were labeled with 32P and then treated with forskolin (10 μM) or the PKA activator 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole 3',5-cyclic monophosphothioate, Sp-iso-mer (cBIMPS; 10 μM) in the presence or absence of myristoylated PKI (1 μM) or KT5823 (1 μM). [32P]IP3-R-I was immunoprecipitated, and radioactivity in the bands was expressed as cpm. Immunoblots of the IP3-R-I bands are shown for loading control. B: back phosphorylation: the cells were first treated with forskolin or cBIMPS in the presence or absence of myristoylated PKI or KT5823. After immunoprecipitation, IP3-R-I was phosphorylated in vitro in the presence of PKG-Iα holoenzyme and [γ-32P]ATP. Control cells were treated in similar fashion without forskolin or cBIMPS. Radioactive bands are not shown for back phosphorylation. Values are means ± SE of 4 experiments each in A and B. No IP3-R-I phosphorylation was observed with cBIMPS. With both techniques, significant increase in phosphorylation (P < 0.01) by forskolin was selectively suppressed by KT5823.
Two experimental strategies that yielded similar results were used to characterize PKA- and PKG-specific phosphorylation. In one, PKG and PKA were selectively activated in the presence or absence of specific kinase inhibitors in cells prelabeled with $^{32}$P and IP$_3$R-I was immunoprecipitated to determine the extent and specificity of phosphorylation. In the other, unlabeled cells were treated in similar fashion and immunoprecipitated IP$_3$R-I was subsequently phosphorylated by PKG-I$_{o}$. PKG was selectively activated by the cGMP analog 8-pCPT-cGMP and SNP, whereas PKA was selectively activated by the cAMP analog cBIMPS and low concentrations of isoproterenol (1 $\mu$M). PKG and PKA were also activated concurrently by VIP, which interacts with VPAC2 receptors to stimulate cAMP and with the natriuretic peptide clearance receptors to stimulate sequentially NO and cGMP formation (20); and 2) high concentrations of forskolin (10 $\mu$M) or...
isoproterenol (100 μM), which stimulates cAMP formation in amounts that activate PKA and cross-activate PKG (8, 13, 17). These experiments showed clearly that activation of PKA alone by cBIMPS or 1 μM isoproterenol did not induce IP3R-I phosphorylation in vivo. When PKA was activated concurrently with PKG by VIP, forskolin, or 100 μM isoproterenol, IP3R-I phosphorylation was selectively inhibited by KT5823, implying that it was mediated by PKG. Previous studies in vascular smooth muscle also showed that IP3R-I phosphorylation induced by forskolin reflected cross-activation of PKG, because phosphorylation was strongly inhibited by KT5823 and was only weakly inhibited by KT5720, a preferential inhibitor of PKA (10). When used at low concentrations (1 μM and less), KT5823 and myristoylated PKI inhibit selectively PKG and PKA, respectively, as shown in previous studies by direct measurement of PKA and PKG activities in gastric smooth muscle cells (15, 17).

In permeabilized smooth muscle cells, unlike intact cells, PKA phosphorylated IP3-R-I, and the phosphorylation was reversed by selective PKA inhibitor (Fig. 10). As shown previously, IP3-induced Ca2+ release in permeabilized gastric smooth muscle cells was inhibited by PKG and PKA, and the inhibition was reversed by selective PKG and PKA inhibitors, respectively (17). In light of the present study, we propose that permeabilization enables PKA to gain access to and phosphorylate IP3-R-I and thus inhibit Ca2+ release.

Both isoforms of PKG-I (PKG-Iα and PKG-Iβ) were identified by RT-PCR in cultured gastric smooth muscle cells. Although both PKG-Iα and -Iβ are coexpressed, the former appears to be the predominant isoform in the peripheral tissues of most mammalian species, including human, bovine, rabbit, and mouse (5, 7, 11, 29, 35). As noted above, PKG-Iα is more
sensitive to activation by cGMP and is susceptible to cross-activation by cAMP (12, 29). The two isoforms recognize in vivo substrates by binding to distinct NH$_2$-terminal leucine-zipper amino acid sequences. PKG-I$\alpha$ binds the “regulatory myosin light chain phosphatase-targeting subunit” and skeletal muscle tropo- nin T via its NH$_2$-terminal sequence (32, 39), whereas PKG-I$\beta$ binds a recently identified substrate, IRAG, via its distinct NH$_2$-terminal sequence (1, 28). IRAG appears to couple PKG-I$\beta$ to IP$_3$-R-I; reconstitution studies in COS-7 cells suggest that phosphorylation of IRAG at Ser$^{696}$ is a prerequisite for IP$_3$-R-I phosphorylation and inhibition of IP$_3$-dependent Ca$^{2+}$ release by PKG-I$\beta$ (28). However, more recent studies in native mouse aortic smooth muscle cells that normally express both PKG-I$\alpha$ and -I$\beta$ have raised doubts regarding the functional role of PKG-I$\beta$ in inhibition of IP$_3$-dependent Ca$^{2+}$ release (3). Transfection of PKG-I$\alpha$ into smooth muscle cells derived from mice deficient in both PKG-I isoforms restored the ability of PKG activators (NO donors and 8-bromo-cGMP) to inhibit agonist-induced Ca$^{2+}$ transients, whereas transfection of PKG-I$\beta$ had no effect (3).

Although IRAG was expressed in gastric smooth muscle cells, the evidence suggests that PKG-I$\alpha$ rather than PKG-I$\beta$ and IRAG was involved in IP$_3$-R-I phosphorylation and IP$_3$-dependent Ca$^{2+}$ release. The results obtained with forskolin and high concentrations of isoproterenol imply that PKG-I$\alpha$ was involved in IP$_3$-R-I phosphorylation and IP$_3$-dependent Ca$^{2+}$ release (17), because this isoform is preferentially cross-activated by cAMP. Furthermore, the results of back phosphorylation with PKG-I$\alpha$ holoenzyme were identical to those obtained by activating endogenous PKG-I in vivo. In permeabilized smooth muscle cells, IP$_3$-R-I could be specifically phosphorylated by PKA using a selective activator of PKA (cBIMPS) or a low concentration of isoproterenol: phosphorylation of IP$_3$-R-I by PKA did not require IRAG, which does not bind to PKA. Phosphorylation of IP$_3$-R-I in micromoles by PKG-I$\alpha$ holoenzyme or the catalytic subunit of PKA inhibited Ca$^{2+}$ release induced by the addition of various concentrations of IP$_3$, implying that phosphorylation of the receptor was the proximate cause of inhibition of Ca$^{2+}$ release.

Forskolin and isoproterenol have been the agents of choice in evaluating the role of cAMP in mediating smooth muscle relaxation. This and other studies (8, 17) clearly show that, in intact smooth muscle cells, this cyclic nucleotide acts, at least in part, by cross-activating PKG-I to induce IP$_3$-R-I phosphorylation and inhibition of IP$_3$-dependent Ca$^{2+}$ release. Lower levels of cAMP that do not cross-activate PKG probably inhibit agonist-induced Ca$^{2+}$ release in intact smooth muscle cells by inhibiting IP$_3$ formation. Our recent studies in gastric smooth muscle cells indicate that although PKA and PKG do not directly phosphorylate and inhibit PLC-1$\alpha$, they can reduce the ability of Gq to activate PLC-1$\beta$ (21). Both kinases phosphorylate RGS4, which further accelerates the hydrolysis of Gq$_{\alpha}$-bound GTP (21). Thus inhibition of agonist-induced Ca$^{2+}$ release is the net outcome of at least two processes: a proximal process involving inhibition of IP$_3$ formation that can be evoked by both PKG and PKA and a more distal process involving phosphorylation of IP$_3$-R-I and inhibition of IP$_3$-induced Ca$^{2+}$ release that is PKG-specific. The precise contribution of each process has not been determined.

It is worth emphasizing that inhibition of Ca$^{2+}$ mobilization is relevant only to the initial transient contraction mediated by Ca$^{2+}$/calmodulin-dependent activation of myosin light chain kinase and phosphorylation of MLC$_{20}$. Sustained MLC$_{20}$ phosphorylation and contraction are largely Ca$^{2+}$ independent and reflect inhibition of MLC phosphatase via at least two pathways that involve activation of RhoA and its associated kinase (22, 30). Relaxation of sustained contraction results from inhibition of RhoA activity by both PKA and PKG (22, 27).

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-28300.

REFERENCES


