Phosphorylation of the Inositol 1,4,5-Trisphosphate Receptor

CYCLIC GMP-DEPENDENT PROTEIN KINASE MEDIATES cAMP AND cGMP DEPENDENT PHOSPHORYLATION IN THE INTACT RAT AORTA*

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The effects of cyclic GMP (cGMP) and activation of cGMP-dependent protein kinase (PKG) on the phosphorylation of the inositol 1,4,5-trisphosphate (IP₃) receptor were examined in intact rat aorta using the technique of back phosphorylation. Aorta treated with the nitric oxide donors, S-nitroso-N-acetylpenicillamine and sodium nitroprusside, or the selective PKG activator, 8-(4-para-chlorophenylthio)-cGMP (8-CPT-cGMP), demonstrated increased IP₃ receptor phosphorylation in situ, which was both time- and concentration-dependent with a stoichiometry of 0.5 mol of phosphate/mol of receptor above control. Treatment of aorta with the adenylyl cyclase activator, forskolin, also demonstrated increased phosphorylation of the IP₃ receptor on the PKG site, although the selective cAMP-dependent protein kinase activator, 8-(4-para-chlorophenylthio)-cAMP (8-CPT-cAMP), did not increase the phosphorylation of the IP₃ receptor. Moreover, the PKG selective inhibitor, KT 5823, inhibited both sodium nitroprusside and forskolin-induced IP₃ receptor phosphorylation more potently than the selective cAMP-dependent protein kinase inhibitor, KT 5720, suggesting that PKG mediates the increase in IP₃ receptor phosphorylation by both cyclic nucleotides in intact aorta. These results provide further support for the notion that PKG is activated by cyclic nucleotides and that PKG performs a critical role in cyclic nucleotide-dependent relaxation of blood vessels.

Nitrovasodilators, nitric oxide (NO),¹ and natriuretic peptides relax vascular smooth muscle through the generation of cGMP (for reviews, see Refs. 1 and 2). The mechanisms by which cGMP causes vascular smooth muscle relaxation are not well understood. Studies have shown that the effects of cGMP are likely mediated through the activation of the cGMP-dependent protein kinase (PKG), and that activation of this kinase leads to the reduction of cytoplasmic Ca²⁺ ([Ca²⁺]ᵢ) in vascular smooth muscle cells (for review, see Ref. 3). Reduction of [Ca²⁺]ᵢ by cGMP has been attributed to several mechanisms including activation of Ca²⁺-ATPases to increase Ca²⁺ uptake or extrusion from the cytoplasm, inhibition of IP₃ formation by inhibition of phospholipase C activation, inhibition of G protein coupling to phospholipase C, inhibition of Ca²⁺ release by the sarcoplasmic reticulum (SR), and activation of Ca²⁺-activated K⁺ channels (for review, see Ref. 3, and references cited). All of these mechanisms appear to contribute to the reduction of [Ca²⁺]ᵢ in different smooth muscle tissues.

Several proteins have been reported to be phosphorylated in response to PKG activation either in vitro or in the intact cell which may contribute to the reduction of [Ca²⁺]ᵢ. Although this list is far from complete, several potentially important substrates include the vasodilator-activated phosphoprotein (4, 5), Go6 (6), the α-subunit of the Ca²⁺-activated K⁺ channel (7), phosphomodulin (3), and the type I inositol 1,4,5-trisphosphate receptor (8, 9). Studies using confocal laser scanning microscopy to determine the cellular distribution of PKG suggest that the enzyme is found in the SR (10) where both phosphomodulin and the IP₃ receptor are localized.

The role of phosphorylation of the IP₃ receptor by cGMP has been studied with respect to the inhibition of agonist-evoked Ca²⁺ release from the SR (8, 9, 11). Several forms of the IP₃ receptor have been identified (12), but the protein isolated and characterized from smooth muscle type I (13) is structurally and functionally similar to the protein isolated from the brain (14). The IP₃ receptor is known to be phosphorylated by several kinases in vitro including cAMP-dependent protein kinase (15), protein kinase C, and Ca²⁺-calmodulin-dependent protein kinase II (16), and by tyrosine kinases during T cell activation (17). The role of phosphorylation of the type I IP₃ receptor is not well understood. It is possible that phosphorylation may regulate Ca²⁺ gating or other regulatory features of the protein.

Regardless of the role of phosphorylation of the IP₃ receptor, few studies have demonstrated phosphorylation of this protein in the intact cell in response to second messenger-activated pathways.

In this study we show that the phosphorylation of the IP₃ receptor occurs in the intact rat aorta in response to elevation of either cAMP or cGMP using the technique of back phosphorylation to quantitate the stoichiometry of phosphorylation. Furthermore, these results demonstrate that PKG catalyzes the phosphorylation of the IP₃ receptor in response to both cGMP and cAMP elevation.

EXPERIMENTAL PROCEDURES

Materials—Sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), 8-para-chlorophenylthio cyclic AMP (8-CPT-cAMP), and protease inhibitors were obtained from Sigma. Forskolin, KT 5823, and KT 5720 were obtained from Calbiochem-Behring Corp. 8-para-Chlorophenylthio cyclic GMP (8-CPT-cGMP) was purchased from Calbiochem-Behring Corp. Calbicol A was purchased from LC Laboratories. Protein

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Phosphorylation of IP₃ Receptor by PKG

A-agarose was from Life Technologies, Inc. (Grand Island, NY). [γ-³²P]ATP was purchased from DuPont NEN. The IP₃ receptor antibody was a gift from Dr. Alan Saltzman (Rhone Poulenc Rorer).

Preparation and Incubation of Rat Aorta—Male Sprague-Dawley rats (250–280 g) were sacrificed by CO₂ asphyxiation, and the aorta were rapidly excised anteriorly below the aortic arch and posteriorly above the bifurcation into the external iliac. Loose fat and connective tissue were carefully stripped, the aorta opened, and the endothelium removed by gentle scraping. The aortic strips were submerged in 5 ml of Krebs-Ringer bicarbonate (KRB) buffer containing 118 mM NaCl, 4.7 mM KC1, 1.1 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 1.5 mM CaCl₂, and 5 mM glucose aerated with 95% O₂, 5% CO₂ at 37°C and shaken in a water bath to ensure proper diffusion. The strips were equilibrated for 60 min and incubated with different agents for varying lengths of time. The strips were rapidly removed from the medium, blotted once, and frozen in liquid N₂ and stored at −80°C for later use. Immunoprecipitation and Western Blot Analysis of IP₃ Receptor from Rat Aortic Microsomes—The pulverized aortae were homogenized with 1 ml of 50 mM Tris-Cl, pH 7.7, 0.3 M sucrose, 1 mM EDTA, 100 mM sodium fluoride, 1 mM sodium orthovanadate, 50 mM tetrasodium pyrophosphate, 1 mM phenylmethylsulfonfyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin A, 10 mM calcium A (Buffer A) for 1 h at 4°C. The homogenate was centrifuged at 10,000 × g for 5 min. The supernatant was centrifuged at 100,000 × g for 1 h. The microsomal pellet was stored at −80°C until used. The microsomal pellet was solubilized with 0.5 ml of Buffer B (Buffer A) for 1 h on ice. The supernatant was replaced with 1% Triton X-100 on ice using a glass Teflon homogenizer and incubated for 1 h on ice. The samples were centrifuged 14,000 × g for 15 min. The supernatants were removed and proteins were estimated.

The supernatants were precleared by mixing with 30 μl of Protein A-agarose prebound to 5 μg of rabbit IgG for 1 h at 4°C. The samples were pulse centrifuged and 2 μl of IP₃ receptor antibody (antiserum raised in rabbit to the peptide TFFREADDHDHYSQG which corresponds to amino acid residues 1916-1929 from the type I IP₃ receptor) were added to each supernatant and rotated for 4 h at 4°C. After 4 h, 30 μl of prewashed Protein A-agarose were added to each sample and rotated overnight at 4°C. The samples were pulse centrifuged and the supernatants were discarded. The pellets were washed three times with Buffer B and twice with 40 mM Tris-Cl, pH 7.4, 0.1% Triton X-100. The immunoprecipitated IP₃ receptor was separated on SDS-PAGE, transferred to nitrocellulose membrane, and identified by Western blot analysis using IP₃ receptor specific antibodies and Enhanced Chemiluminescence detection system (Amersham Life Sciences).

Phosphorylation of the Immunoprecipitated IP₃ Receptor—For the back phosphorylation experiments, the immunoprecipitated samples were phosphorylated using PKG by adding 30 μl of phosphorylation buffer containing 40 mM Tris-Cl, pH 7.4, 0.1% Triton X-100, 10 mM HEPES-Cl, 50 μM PKG (40 nM final concentration), 0.5 mM cGMP, 1 μM caliculin A (Buffer A) for 1 h at 4°C. The immunoprecipitated IP₃ receptor was separated on SDS-PAGE, transferred to nitrocellulose membrane, and identified by Western blot analysis using IP₃ receptor specific antibodies and Enhanced Chemiluminescence detection system (Amersham Life Sciences).

Cyclic GMP and AMP were determined by radiomunoassay (20) and protein was determined by Bradford assay (21) using bovine serum albumin as a standard. Bovine lung PKG was purified to apparent homogeneity by affinity chromatography on 8-hexylamine CAMP-agarose as described (22).

RESULTS

Immunoprecipitation of the IP₃ Receptor from Intact Rat Aorta—In order to study the phosphorylation of the type I IP₃ receptor by PKG in the intact smooth muscle cell, we utilized the technique of back phosphorylation originally described by Forn and Greengard (19). Because both the site and stoichiometry of PKG-dependent IP₃ receptor phosphorylation is known, it was possible to quantitate the extent of phosphorylation by PKG in the intact cell. To do this, it was necessary to isolate the IP₃ receptor from intact aortic tissue in order to both visualize and quantitate phosphate incorporation. Hence, the IP₃ receptor from rat aorta treated with different agents was immunoprecipitated using antisera against an N-terminal peptide from the type I IP₃ receptor, back phosphorylated in vitro using PKG and [γ-³²P]ATP, and resolved by SDS-PAGE. The data shown in Fig. 1 demonstrate that the IP₃ receptor was immunoprecipitated by the antibody and not by the preimmune serum. The band above 199 kDa was identified as the IP₃ receptor by Western blot analysis using IP₃ receptor specific antibodies. The phosphorylation of the IP₃ receptor in response to cGMP Elevating Agents—Treatment of intact rat aorta with NO donor drugs which activate soluble guanylate cyclase resulted in the stoichiometric phosphorylation of the IP₃ receptor in the intact tissue. As shown in Fig. 2, SNP (1 μM) produced a time-dependent increase in the phosphorylation of the IP₃ receptor to a stoichiometry of approximately 0.5 mol of phosphate/mol of receptor above baseline. The endogenous phosphorylation was maximal at 2 min. SNP increased the levels of cGMP from 0.35 ± 0.14 to 5.32 ± 1.34 pmol/mg of protein, while the cAMP level was unchanged (1.93 ± 0.92 to 2.33 ± 0.82 pmol/mg of protein). Phosphorylation of IP₃ receptor was also increased in a concentration-dependent manner when rat aortas were treated with a different NO donor and cGMP elevating agent, SNAP. As shown in Fig. 3, the half-maximally effective concentration of SNAP for stimulating IP₃ receptor phosphorylation was approximately 0.25 μM. This is a concentration of SNAP similar to that which many laboratories have demonstrated...
produces half-maximal relaxation of contracted rat aortic strips. These results demonstrate that elevation of cGMP levels using concentrations of NO-donor drugs that produce vascular relaxation produce stoichiometric endogenous phosphorylation of the type I IP₃ receptor in intact rat aorta.

Phosphorylation of the IP₃ Receptor in Response to Forskolin—To study further the phosphorylation of the type I IP₃ receptor, the effects of activating the cAMP-dependent protein kinase signaling pathway was examined. As shown in Fig. 4, SNP and forskolin increased the endogenous phosphorylation of the IP₃ receptor. On the other hand, angiotensin II (0.1 μM), an activator of both Ca²⁺ and protein kinase C pathways in vascular smooth muscle, neither elevated cyclic nucleotides (2.97 and 0.43 pmol/mg of protein of cAMP and cGMP, respectively) nor increased endogenous phosphorylation of serine 1755 of the IP₃ receptor. Forskolin, however, increased cAMP levels (to 8.22 ± 2.23 pmol/mg of protein) but not cGMP levels (0.52 ± 0.12 pmol/mg of protein) and produced an increase in the phosphorylation of serine 1755 on the type I IP₃ receptor to 0.5 mol of phosphate/mol of receptor above control in a concentration dependent manner. On the other hand, 8-CPT-cGMP, a selective PKG activator, increased the endogenous phosphorylation of the IP₃ receptor to 0.5 mol of phosphate/mol of receptor above control in a concentration dependent manner. KT 5823 and KT 5720, have differing selectivities for inhibiting selective inhibitors of PKA and PKG. KT 5823 is selective for PKG (Kᵢ = 234 nM), whereas KT 5720 is selective for PKA (Kᵢ = 56 nM). As shown in Fig. 7, a 20-min preincubation of rat aortae with increasing concentrations of KT 5823 inhibited SNP-dependent phosphorylation of the IP₃ receptor with the half-maximally effective concentration of the inhibitor being approximately 350 nM. KT 5720 was significantly less potent with...
a half-maximally effective concentration of approximately 1 μM. Interestingly, KT 5823 more selectively inhibited forskolin-dependent phosphorylation of the IP$_3$ receptor than did KT 5720 with a half-maximally effective concentration of approximately 300 nM compared with approximately 1 μM KT 5720 (Fig. 7). Indeed, the effects of KT 5823 were similar for inhibiting both forskolin- and SNP-dependent phosphorylation of the IP$_3$ receptor, suggesting that PKG mediates the phosphorylation of this protein in response to elevations in either cAMP or cGMP.

**DISCUSSION**

The data reported in this article demonstrate that both cGMP and cAMP elevating agents increased in a stoichiometric fashion the endogenous phosphorylation of the type 1 IP$_3$ receptor in intact rat aorta. Using the purified protein, our laboratory has shown that PKG catalyzes the phosphorylation of the type 1 IP$_3$ receptor isolated from rat cerebellum on serine 1755. The sequence surrounding this site contains the canonical site for phosphorylation by both PKA and PKG (i.e. RRXS) and in addition contains an aromatic residue in the +4 position to the phosphorylatable serine. Colbran et al. (25) have reported that aromatic residues at this position enhance the selectivity for PKG-mediated phosphorylation compared with PKA-mediated phosphorylation. Nevertheless, studies on the phosphorylation of the purified cerebellum IP$_3$ receptor by Ferris et al. (15) indicate that PKA was capable of phosphorylating both serine 1755 and serine 1589, and we have confirmed this study in our own laboratory (8). Despite these studies, there is no information available on whether or not PKG or PKA catalyze the phosphorylation of the type 1 IP$_3$ receptor in the intact cell.

In order to begin to examine the role of PKG in the phosphorylation of this protein in vivo, we used the technique of back phosphorylation to investigate endogenous phosphorylation. This method is quite useful for determining the endogenous stoichiometry of phosphorylation if the site of phosphorylation for the kinase is known and the protein is readily isolated from tissues and cells. Such approaches have recently been used to study the phosphorylation of phospholamban in rat aorta (26), voltage-sensitive sodium channels in neurons (27), and L-type ($\alpha_1$) calcium channels in rat skeletal muscle cells (28). The results reported here demonstrate that NO-generating vasodilator drugs stimulate time- and concentration-dependent increases in the phosphorylation of the IP$_3$ receptor from rat aorta. Furthermore, the concentrations that stimulate IP$_3$ receptor phosphorylation are similar to those reported by several laboratories including our own that produce relaxation of vascular muscle strips. However, it is important to point out that it is unclear what the role of phosphorylation of this protein is in the relaxation process.

Of particular interest in this study is the fact that elevations of cAMP using forskolin produced phosphorylation of the same residue as elevations in cGMP. However, treatment of rat aortas with 8-CPT-cGMP, but not 8-CPT-cAMP, resulted in the phosphorylation of the IP$_3$ receptor. Because these cyclic nucleotide analogs are relatively selective for activating their respective kinases, these results do not support a role for PKA.

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**Fig. 5.** Phosphorylation of the IP$_3$ receptor in intact rat aorta in response to cGMP and cAMP analogs. Rat aortae were incubated with varying concentrations of 8-CPT-cGMP and 8-CPT-cAMP for 30 min and the phosphorylation of the IP$_3$ receptor was analyzed as described in the legend to Fig. 2. Results are plotted as the mean ± S.E. of four determinations.

**Fig. 6.** Time course of phosphorylation of the IP$_3$ receptor in intact rat aorta treated with cGMP and cAMP analogs. Rat aortae were incubated with 50 μM 8-CPT-cGMP (●) or 50 μM 8-CPT-cAMP (●) for different time intervals and the phosphorylation of the IP$_3$ receptor was analyzed as described in the legend to Fig. 2. Data are plotted as the mean ± S.E. of four determinations.

**Fig. 7.** Inhibition of endogenous phosphorylation of the IP$_3$ receptor by selective kinase inhibitors. Rat aortae were treated with varying concentrations of the selective inhibitor for PKG, KT 5823 for 20 min, or varying concentrations of the selective inhibitor for PKA, KT 5720 for 20 min, followed by 1 μM SNP for 2 min or 1 μM forskolin for 5 min. The IP$_3$ receptor was immunoprecipitated, phosphorylated, and analyzed as described under "Experimental Procedures." The percent inhibition of endogenous phosphorylation stimulated by SNP or forskolin with the respective inhibitors was plotted as the mean ± S.E. of four determinations for the SNP experiment and two determinations for forskolin experiment.
in catalyzing the phosphorylation of serine 1755 on the protein. Additional evidence which indicates that PKG, but not PKA, mediates the phosphorylation of serine 1755 on the IP₃ receptor was obtained using the KT compounds. Pretreatment of aorta with KT 5823, a selective inhibitor of PKG, inhibited IP₃ receptor phosphorylation to either SNP or forskolin more potently than did KT 5720, a selective inhibitor of PKA. Inhibition of IP₃ receptor phosphorylation by KT 5823 is well within the range of potency of this compound for inhibiting PKG activity. However, KT 5720 at higher concentrations also inhibits SNP- and forskolin-stimulated IP₃ receptor phosphorylation thereby demonstrating that this compound is capable of inhibiting PKG in the intact cell. These results highlight one important characteristic of protein kinase inhibitors, namely that none of the currently available inhibitors are completely specific for a particular protein kinase. It is therefore important to perform proper dose-response curves when using these compounds to facilitate the interpretation of the data in these types of experiments. Taken together, the data using cyclic nucleotide analogs and protein kinase inhibitors suggest that PKG, but not PKA, mediates the phosphorylation of serine 1755 of the type I IP₃ receptor in intact rat aorta.

The data described here also lend support to the concept that cyclic nucleotide-dependent protein kinases are not necessarily specifically activated by their respective nucleotides. The concept of “cross-activation” of PKG by cAMP was originally put forth by Francis et al. (29) and Lincoln et al. (30) for relaxation of vascular smooth muscle. Because the affinity of PKG for cAMP is relatively high (Kₐ = 2 μM) and the levels of cAMP usually exceed cGMP in tissues, it is likely based on theoretical considerations alone that cAMP activates PKG. Both Francis et al. (29) and our laboratory have confirmed these concepts experimentally in intact smooth muscle tissue and cells using different approaches. Furthermore, cross-activation of PKG by cAMP has been reported by Jiang et al. (31) in swine coronary arteries treated with forskolin. More recently, Cornwall et al. (32) have shown that the growth inhibitory actions of NO in adult rat aortic smooth muscle cells is due in part at least to the activation of PKA by cGMP. These findings clearly indicate that each cyclic nucleotide is capable of activating both cyclic nucleotide-dependent protein kinases in the intact cell.

Because PKA is expressed in rat aortic tissue, and PKA is capable of catalyzing the phosphorylation of the IP₃ receptor on serine 1755 in vitro at least, a question arises as to why PKA does not catalyze phosphorylation of the IP₃ receptor in the intact rat aorta. One possible explanation was provided by Cornwall et al. (10) who demonstrated that PKG, but not PKA, was found to be associated with the SR in rat aortic smooth muscle cells. The localization of PKG with substrate proteins such as the IP₃ receptor in the rat aortic smooth muscle cell SR could facilitate phosphorylation of these proteins. It would appear based on the above discussion that studies using cyclic nucleotide analogs in intact tissues should be interpreted cautiously since their effectiveness depends on several factors such as permeability, rate of hydrolysis, bio-accumulation, and interaction with several receptor proteins.

The role of phosphorylation of the IP₃ receptor is still not understood. Supattapone et al. (33) first demonstrated that PKA-mediated phosphorylation of the IP₃ receptor resulted in diminished potency of IP₃ in releasing Ca²⁺ from brain membrane fractions. Quinton and Dean (34) reported that PKA-dependent phosphorylation of platelet membranes substantially reduced the potency of IP₃ in releasing Ca²⁺ from this preparation. More recently, Cavallini et al. (35) demonstrated that prostacyclin and nitroprusside inhibited IP₃-evoked Ca²⁺ release in intact platelets. These authors suggested that cAMP and cGMP mediate a similar type of IP₃ receptor desensitization, perhaps as a result of PKG-mediated phosphorylation. There are also reports, however, which demonstrate that PKA-mediated phosphorylation of the IP₃ receptor increases the potency of IP₃ in releasing Ca²⁺ in platelets (36) and hepatocytes (37–40). This variation of results may be due to the capacity of PKA to catalyze the phosphorylation of additional sites on the type IP₃ receptor protein (i.e. serine 1589), or the tissue specific expression of different IP₃ receptor proteins.

In a recent report by Pfeifer et al. (6), the role of phosphorylation of the IP₃ receptor by PKG was questioned. In this study, the authors used Chinese hamster ovary cells transfected with cDNAs encoding PKG and concluded that PKG did not catalyze the phosphorylation of this protein. Because the type I IP₃ receptor is not uniformly expressed in cultured cells (41), and overexpressed PKG may not always be strategically localized with substrates, it is sometimes difficult to relate the findings from artificial heterologous cell systems with the physiological situation. It is also naïve to assume that PKG acts to lower [Ca²⁺]₀ by a singular mechanism in cells. At present, it is too early to speculate on the role of PKG-dependent phosphorylation of the IP₃ receptor protein. Studies using the purified receptor in reconstituted systems as well as vascular smooth muscle microsomes may help to elucidate the function of PKG-mediated phosphorylation of the receptor. Since the IP₃ receptor is phosphorylated by several other kinases and because of its complex nature, the regulation by phosphorylation in the intact cells may be different from the regulation of purified receptor in reconstituted systems.

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Phosphorylation of the Inositol 1,4,5-Trisphosphate Receptor: CYCLIC GMP-DEPENDENT PROTEIN KINASE MEDIATES cAMP AND cGMP DEPENDENT PHOSPHORYLATION IN THE INTACT RAT AORTA
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