Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle

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The regulation of cGMP-specific phosphodiesterase (PDE) 5 and soluble guanylate cyclase (GC) by cGMP- and cAMP-dependent protein kinases (PKG and PKA respectively) was examined in gastric smooth muscle. The NO donor, sodium nitroprusside (SNP), stimulated PDE5 phosphorylation and activity, which was blocked by the selective PKG inhibitor, KT5823, resulting in an elevation of cGMP levels. Activation of PKA either directly by Sp-5,6-dichloro-1- β -D-ribofuranosyl benzimidazole 3',5'-cyclic monophosphothioate, or via isoproterenol- and forskolin-dependent increase in cAMP, also caused an increase in PDE5 phosphorylation and activity, but only in the presence of cGMP; consistent with the dependence of PDE5 phosphorylation and activity on cGMP binding to allosteric sites in the regulatory domain of PDE5. The selective PKA inhibitors, myristoylated

protein kinase inhibitor and H-89, blocked the increase in PDE5 phosphorylation and activity induced by PKA. SNP also stimulated soluble GC phosphorylation and activity. KT5823 abolished phosphorylation and augmented soluble GC activity, implying feedback inhibition of soluble GC by PKG-dependent phosphorylation. Phosphorylation by PKG was direct and could be induced *in vitro*. Activation of PKA had no effect on soluble GC. Thus cGMP levels are regulated by PKG- and PKA-dependent activation of PDE5 and PKG-specific inhibition of soluble GC.

Key words: cyclic nucleotides, protein kinase A, protein kinase G, soluble guanylate cyclase.

INTRODUCTION

The cyclic nucleotides cGMP and cAMP play important roles in mediating relaxation of vascular and visceral smooth-muscle cells [1–3]. In the gut, the main relaxant neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase (AC)-activating peptide (PACAP), are co-localized with NO synthase (NOS) in a subset of myenteric neurons that project mainly to the circular muscle layer [4]. Relaxation is mediated by diffusion of NO from nerve terminals and by NO generation within smooth-muscle cells via the action of VIP and PACAP [5,6]. VIP and PACAP initiate dual signalling cascades by interacting with cognate VPAC₂ receptors coupled via G_s to AC, and with the single-transmembrane natriuretic peptide clearance receptor, NPR-C, coupled via G_{i1} and G_{i2} to the activation of endothelial NOS [7]. Activation of AC and NO-dependent soluble guanylate cyclase (GC) during nerve-induced relaxation leads to concurrent generation of cAMP and cGMP. The levels of cAMP and cGMP are regulated by the activities of specific phosphodiesterases (PDEs) [8–11]. The activities of PDEs and cyclases are highly regulated [9,12,13].

PDEs are a large group of structurally related enzymes that catalyse the hydrolysis of 3',5'-cyclic nucleotides to the corresponding inactive nucleoside 5'-monophosphate by cleaving the phosphodiester bond between the phosphorus and oxygen atoms at the 3'-position. Eleven families of PDEs have been identified with each family being the product of a separate gene and usually comprises several isoforms. PDEs differ in their primary structure, tissue distribution, affinities for cyclic nucleo-

tides and sensitivity to Ca²⁺ and various inhibitors [8,9,11,14]. PDE4, PDE7 and PDE8 are highly specific for cAMP, whereas PDE5, PDE6 and PDE9 are highly specific for cGMP; other PDEs (PDE1, PDE2, PDE3, PDE10 and PDE11) exhibit dual specificity [8,9,11,14]. The activity of PDEs is regulated by substrate availability, upon the increase in the levels of one or both cyclic nucleotides, various intracellular signals (e.g. Ca²⁺ and calmodulin) and feedback phosphorylation via cAMP- and cGMP-dependent protein kinases (PKA and PKG respectively) [15–20].

PDE5 is the predominant cGMP-specific PDE expressed in skeletal, cardiac and smooth muscle [8,21]. PDE5 contains two allosteric cGMP-specific binding sites in its regulatory N-terminal domain, in addition to specific cGMP-binding sites in its C-terminal domain [22–24]. Binding of cGMP to the allosteric sites is essential for regulatory phosphorylation of PDE5 by PKG or PKA [20,25,26]. The three cloned isoforms of PDE5 (PDE5A1–3) have similar catalytic activity but variable tissue distribution [27]. PDE5A1 and PDE5A2 are co-expressed in canine atrial, ventricular and vascular smooth muscle [28]. PDE5A1 is also expressed in canine pyloric and colonic smooth muscle [28]. The fact that PDE5 expression parallels that of PKG suggests that PDE5 is widely distributed in smooth muscle of the gut [8,21].

The levels of cGMP in smooth muscle of the gut reflect continued synthesis by soluble GC and breakdown by PDE, presumably PDE5. Soluble GC is a haem-containing, heterodimeric protein consisting of α - and β -subunits; each subunit has an N-terminal regulatory domain and a C-terminal catalytic

Abbreviations used: PDE, phosphodiesterase; GC, guanylate cyclase; AC, adenylate cyclase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; SNP, sodium nitroprusside; PKI, protein kinase inhibitor; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; PKC, protein kinase C; VIP, vasoactive intestinal peptide; PACAP, pituitary AC-activating peptide; IBMX, isobutylmethylxanthine; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; cBIMPS, Sp-5,6-dichloro-1- β -D-ribofuranosyl benzimidazole 3',5'-cyclic monophosphothioate; NOS, NO synthase; ECL[®] (Amersham Pharmacia Biotech), enhanced chemiluminescence.

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Figure 1 Expression of PDE5 and soluble GC in freshly dispersed gastric and intestinal smooth-muscle cells

Lysates were prepared separately from smooth-muscle cells isolated from the circular muscle layer of the stomach (Gastric) and the circular (C) and longitudinal (L) muscle layers of the small intestine of rabbit. The proteins were resolved by SDS/PAGE and transferred on to nitrocellulose membranes. Recombinant PDE5 was used as a standard. The proteins were detected with polyclonal anti-PDE5 or anti-[soluble GC] antibodies respectively and the bands revealed by ECL[®].

domain [12,13,29]. The haem moiety acts as an intracellular receptor for NO and determines the ability of NO to activate the enzyme and generate cGMP [30].

The regulation of PDE5 and soluble GC activities has not been characterized in gastrointestinal smooth muscle. In the present study, the mechanisms by which PKG and PKA regulate PDE5 and/or soluble GC activity in gastric smooth muscle were examined. The results indicate that PKG-dependent phosphorylation enhances PDE5 activity and inhibits soluble GC activity, thereby attenuating the levels of cGMP. Concurrent activation of PKA also attenuates cGMP levels by enhancing PDE5 activity.

EXPERIMENTAL

Materials

[α -³²P]GTP, [³²P]P_i, [γ -³²P]ATP, and ¹²⁵I-labelled cGMP were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Collagenase and soybean trypsin inhibitor were purchased from Worthington Biochemical (Freehold, NJ, U.S.A.). Western blotting, chromatography materials and protein assay kit were from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The catalytic subunit of PKA, PKG, protein phosphatases 1 and 2A, recombinant bovine PDE5 and the polyclonal anti-PDE5 antibody were purchased from Calbiochem (La Jolla, CA, U.S.A.). The polyclonal anti-[soluble GC] antibody was from Alexis Corporation (San Diego, CA, U.S.A.). *Crotalus atrox* snake venom, cGMP and all other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of dispersed gastric smooth-muscle cells

Dispersed gastric smooth-muscle cells were prepared by sequential enzymic digestion, filtration and centrifugation as described previously [5,31,32]. Briefly, strips of circular muscle were dissected and incubated at 31 °C for 30 min in Hepes medium [25 mM Hepes, 120 mM NaCl, 4 mM KCl, 2.6 mM KH₂PO₄, 0.6 mM MgCl₂, 14 mM glucose and 2.1 % (v/v) Eagle's essential amino acid mixture (pH 7.4)] containing type II collagenase (0.1 %, w/v) and soybean trypsin inhibitor (0.1 %, w/v). 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 a 500 μ m Nitex mesh (Tetko Inc., Briarcliff Manor, NY, U.S.A.) and centrifuged twice at 350 g for 10 min at 25 °C.

Assay for PDE5 activity

PDE5 activity was measured in immunoprecipitates of PDE5 by the method of Wyatt et al. [33]. PDE5 was immunoprecipitated from lysates of dispersed gastric smooth-muscle cells (3×10^6 cells/ml) using an anti-PDE5 antibody, and the immunoprecipitates were washed in a solution of 50 mM Tris/HCl (pH 7.5), 200 mM NaCl and 5 mM EDTA. The immunoprecipitates were then incubated for 15 min at 30 °C in a reaction mixture containing 100 mM Mes (pH 7.5), 10 mM EDTA, 0.1 M magnesium acetate, 0.9 mg/ml BSA, 20 μ M cGMP and [³H]cGMP. The samples were boiled for 3 min, chilled for 3 min and then incubated at 30 °C for 10 min in 20 mM Tris/HCl (pH 7.5) containing 10 μ l of *C. atrox* snake venom (10 μ g/ μ l). The samples were added to DEAE-Sephacel A-25 columns and the radioactivity in the effluent was counted. The results were expressed as c.p.m./mg of protein.

In experiments using phosphatases 1 and 2A, the immunoprecipitates were washed with a solution of 50 mM Tris/HCl (pH 7.5), 0.5 mM EDTA, 5 mM 2-mercaptoethanol and 0.1 % (v/v) Triton X-100 and incubated for 20 min at 30 °C with the purified protein phosphatase 1 (0.3 μ g) and 2A (0.3 μ g) in the presence or absence of okadaic acid (10 μ M) and calyculin A (10 μ M). The phosphatases were then removed by further washes with 50 mM Tris/HCl (pH 7.5) and PDE5 phosphorylation and activity were measured.

Assay for soluble GC activity

Soluble GC activity was measured using [α -³²P]GTP as the substrate. Gastric muscle cells were treated with 1 μ M sodium nitroprusside (SNP) in the presence of isobutylmethylxanthine (IBMX; 1 mM) and zaprinast (10 μ M). Crude homogenates of muscle cells were incubated in a solution of 50 mM Tris/HCl (pH 7.4), 2 mM cGMP, 0.1 mM GTP, 1 mM IBMX, 5 mM MgCl₂, 100 mM NaCl, 5 mM creatine phosphate, 50 units/ml creatine kinase and 0.5 mM [α -³²P]GTP (approx. 0.2 μ Ci) for 15 min at 37 °C. The reaction was terminated by the addition of 2 % (v/v) SDS/45 mM GTP/1.5 mM cGMP. [α -³²P]GMP was separated from [α -³²P]GTP by sequential chromatography on Dowex AG50W-4X and alumina columns. The results were expressed as pmol cGMP/mg of protein.

Phosphorylation of PDE5 and soluble GC

Phosphorylation of PDE5 or soluble GC was determined from the amount of ³²P incorporated into the enzyme after immunoprecipitation with anti-PDE5 or anti-[soluble GC] antibodies respectively. A suspension (10 ml) of dispersed smooth-muscle cells (4×10^6 cells/ml) were prelabelled with 0.5 mCi/ml of [³²P]P_i for 3 h. Samples (1 ml) were incubated with 1 nM SNP for 1 min in the presence or absence of the specific PKG inhibitor, KT5823, or the specific PKA inhibitors, myristoylated protein kinase inhibitor (PKI) or H-89. For soluble GC phosphorylation, samples were incubated with SNP in the presence of IBMX (1 mM) and zaprinast (10 μ M). The reaction was terminated with an equal volume of lysis buffer [1 % (v/v) Triton X-100, 0.5 % (w/v) SDS, 0.75 % (w/v) deoxycholate, 10 mM EDTA, 1 mM PMSF, 10 μ g/ml leupeptin, 100 μ g/ml aprotinin, 10 mM sodium pyrophosphate, 50 mM NaF and 0.2 mM sodium vana-

Figure 2 Concentration-dependent phosphorylation of PDE5 induced by SNP in smooth muscle

Gastric smooth-muscle cells were incubated with various concentrations of SNP (1 pM–1 μ M) for 1 min. Cells were lysed and immunoprecipitated with an anti-PDE5 antibody. (A) Phosphorylation of PDE5 was identified in immunoprecipitates by autoradiography from cells labelled with 32 P as described in the Experimental section. The level of basal (b) phosphorylation is indicated. (B) The membrane from (A) was immunoblotted with the anti-PDE5 antibody to determine the co-migration of the radioactive bands with PDE5. (C) The radioactivity in the bands from (A) were measured and the basal level values (425 ± 65 c.p.m./mg of protein) were subtracted. Results are expressed as c.p.m./mg of protein and are means $\pm$ S.E.M. of four experiments.

date, final concentrations], and placed on ice for 30 min. The cell lysates were separated from the insoluble material by centrifugation at 13000 g for 15 min at 4 $^{\circ}$ C, precleared with 40 μ l of Protein A–Sepharose and incubated with anti-PDE5 or anti-[soluble GC] antibodies for 2 h at 4 $^{\circ}$ C. Protein A–Sepharose (40 μ l) was added to the lysates and incubated for another 1 h. The immunoprecipitates were washed five times with 1 ml of wash buffer [10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.5 % (v/v) Triton X-100], extracted with sample buffer [62.5 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 25 % (v/v) glycerol, 0.01 % Bromophenol Blue] and boiled for 15 min. The samples were then resolved by SDS/PAGE using 10 % (w/v) polyacrylamide gels. After transfer of the proteins on to PVDF membranes, 32 P-labelled PDE5 or soluble GC were visualized by autoradiography and the amount of radioactivity in the bands was counted. In some experiments, immunoblot analysis was performed on the membranes after autoradiography to determine co-migration of the immunoblot bands for PDE5 and soluble GC with the corresponding radioactive bands.

A back phosphorylation approach was used to determine *in situ* phosphorylation of soluble GC. Gastric muscle cells were treated with SNP (1 μ M) or isoproterenol (100 μ M) in the presence of 1 mM IBMX and 10 μ M zaprinast for 60 s. Immuno-

precipitates of soluble GC were washed with a solution of 50 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$ and 0.1 % (v/v) Triton X-100 and then incubated with either purified type I PKG (PKG-I) (0.5 μ g) and 1 μ M cGMP or with the catalytic subunit of PKA (0.5 μ g), and 100 μ M [γ - 32 P] ATP (5 μ Ci/sample) for 10 min at 30 $^{\circ}$ C. The samples were treated with sample buffer, boiled for 15 min and resolved by SDS/PAGE using a 10 % (w/v) polyacrylamide gel. After transfer of the proteins on to PVDF membranes, 32 P-labelled soluble GC was visualized by autoradiography and the amount of radioactivity in the bands was counted. The decrease in 32 P incorporation in the soluble GC band in samples treated with 1 μ M SNP or 100 μ M isoproterenol were compared with control phosphorylation.

Western blot for PDE5 and soluble GC

The expression of PDE5 and soluble GC was determined by Western blot as described previously [32]. Homogenates prepared from dispersed gastric muscle cells were solubilized on ice for 1 h in a solution of 20 mM Tris/HCl (pH 8.0), 1 mM dithiothreitol, 100 mM NaCl, 0.5 % (w/v) SDS, 0.75 % (w/v) deoxycholate, 1 mM PMSF, 10 μ g/ml leupeptin and 100 μ g/ml aprotinin. The suspension was centrifuged at 13000 g for 5 min at 4 $^{\circ}$ C. Proteins

in the supernatant were resolved by SDS/PAGE (10% gel) and electrophoretically transferred on to nitrocellulose membranes. The membranes were incubated for 12 h at 4 °C with anti-PDE5 (1:1500) or anti-[soluble GC] (1:1000) antibodies and then for 1 h with a horseradish-peroxidase-conjugated secondary antibody. The bands were identified by enhanced chemiluminescence (ECL[®]; Amersham Pharmacia Biotech).

RIA for cGMP

cGMP production was measured by RIA as described previously [3,7,31]. Briefly, muscle cells (3×10^6 cells) were treated with SNP (0.1–100 nM) for 1 min and the reaction terminated with 10% trichloroacetic acid. The samples were centrifuged and the supernatants extracted three times with water-saturated diethyl ether. The resulting aqueous phase was lyophilized and reconstituted in 500 μ l of 50 mM sodium acetate (pH 6.2). The samples were acetylated with triethylamine/acetic anhydride (2:1, v/v) for 30 min and cGMP was measured in duplicate using 100 μ l aliquots. The results were expressed as pmol/mg of protein.

RESULTS

Expression of PDE5 and soluble GC in gastric smooth muscle

Western-blot analysis using specific anti-PDE5 and anti-[soluble GC] antibodies confirmed the presence of PDE5 and soluble GC proteins in extracts of dispersed smooth-muscle cells derived from the circular muscle layer of the stomach and from the longitudinal and circular muscle layers of the intestine (Figure 1). Previous studies [5] had shown that after dual centrifugation, the suspensions containing these cells are devoid of neural elements, as indicated by the absence of [³H]saxitoxin binding.

Figure 3 Inhibition of SNP-stimulated PDE5 activity by KT5823

Dispersed gastric smooth-muscle cells were treated with SNP (0.1 nM–0.1 μ M) for 1 min. PDE5 was immunoprecipitated from lysates of muscle cells and PDE5 activity was measured as described in the Experimental section using [³H]cGMP as the substrate. In some experiments, muscle cells were pretreated for 10 min with the PKG inhibitor, KT5823 (0.1 and 1.0 μ M), and PDE5 activity was measured in response to SNP. Basal level values (1854 ± 145 c.p.m./mg of protein) were subtracted and the results expressed as c.p.m./mg of protein. Values are means $\pm$ S.E.M. of four experiments.

Figure 4 PKG-dependent phosphorylation and activation of PDE5

Gastric muscle cells were incubated with SNP (1 nM) in the presence or absence of selective inhibitors of PKG [KT-5823 (KT), 1 μ M], PKA (myristoylated PKI and H-89, 1 μ M each), PKC [bisindolylmaleimide (Bis), 1 μ M], MEK [PD98059 (PD), 10 μ M] or p38 MAPK [SB203580 (SB), 1 μ M]. Cells were lysed and PDE5 was immunoprecipitated using an anti-PDE5 antibody. (A) Phosphorylation of PDE5 was identified in immunoprecipitates by autoradiography from cells labelled with ³²P as described in the Experimental section. (B) Western-blot analysis of PDE5 immunoprecipitates using the polyclonal anti-PDE5 antibody. (C) PDE5 activity in the immunoprecipitates was measured using [³H]cGMP as the substrate as described in the Experimental section. Results are expressed as c.p.m./mg of protein and are means $\pm$ S.E.M. of three to six experiments.

Phosphorylation-dependent activation of PDE5 by PKG

It has been shown previously [31] that SNP stimulates cGMP formation and activates PKG in a concentration-dependent fashion in dispersed gastric smooth-muscle cells. In the present study, it was shown that SNP stimulated PDE5 phosphorylation and PDE5 activity (Figures 2 and 3). Stimulation of PDE5 phosphorylation and activity was concentration-dependent with an EC₅₀ of approx. 1 nM (Figures 2 and 3). The selective PKG inhibitor, KT5823, inhibited PDE5 activity in a concentration-dependent fashion (Figure 3). KT5823 (1 μ M) inhibited PDE5 phosphorylation by $87 \pm 4\%$ ($P < 0.001$). The selective PKA inhibitors, H-89 (1 μ M) and myristoylated PKI (1 μ M), the selective protein kinase C (PKC) inhibitor, bisindolylmaleimide (1 μ M), the selective mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK) inhibitor, PD98059 (10 μ M), and the p38 MAPK inhibitor, SB203580 (1 μ M), had no effect (Figure 4). In previous studies [31,32], we have shown that, at concentrations of 0.1–1 μ M, KT5823 selectively inhibited PKG activity, whereas H-89 and PKI selectively inhibited PKA. A PDE5 inhibitor, zaprinast (10 μ M), abolished PDE5 activity (results not shown).

Dependence of PDE5 phosphorylation and activation on cGMP

A non-hydrolysable membrane-permeable analogue of cGMP, 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP), which select-

Figure 5 cGMP-dependent phosphorylation and activation of PDE5 by PKG

Gastric muscle cells were incubated with a selective activator of PKG, 8-pCPT-cGMP (pCPT; 10 μ M) in the presence or absence of SNP (1 nM) and a selective PKG inhibitor, KT5823 (KT; 1 μ M). Cells were lysed and PDE5 was immunoprecipitated using an anti-PDE5 antibody. In some experiments, immunoprecipitates were incubated with phosphatases 1 and 2A (Pase) in the presence or absence of okadaic acid (OA; 10 μ M) and calyculin A (CaA; 10 μ M). (A) Phosphorylation of PDE5 was identified in immunoprecipitates by autoradiography from cells labelled with 32 P as described in the Experimental section. (B) Western-blot analysis of PDE5 immunoprecipitates using the anti-PDE5 antibody. (C) PDE5 activity in the immunoprecipitates was measured using [3 H]cGMP as the substrate as described in the Experimental section. Results are expressed as c.p.m./mg of protein and are means $\pm$ S.E.M. of five experiments. ** P < 0.01 compared with SNP alone; # P < 0.01 compared with SNP in the presence of 8-pCPT-cGMP.

ively activates PKG, did not activate PDE5 when present alone, but significantly augmented SNP-stimulated PDE5 activity and phosphorylation by $61 \pm 3\%$ (P < 0.01) and $52 \pm 4\%$ (P < 0.01) respectively (Figure 5), implying that binding of cGMP to PDE5 was required for phosphorylation and activation of PDE5 by PKG. Stimulation of PDE5 and activity by 8-pCPT-cGMP in the presence of SNP was abolished by KT5823 (1 μ M). Addition of purified phosphatase 1 and 2A to PDE5 immunoprecipitates inhibited PDE5 activity and phosphorylation by $74 \pm 6\%$ (P < 0.01) and $61 \pm 4\%$ (P < 0.01) respectively, and the inhibition was completely reversed by a combination of okadaic acid (10 μ M) and calyculin A (10 μ M) (Figure 5).

Similarly, a non-hydrolysable membrane-permeable analogue of cAMP, Sp-5,6-dichloro-1- β -D-ribofuranosyl benzimidazole 3',5'-cyclic monophosphothioate (cBIMPS), which selectively activates PKA, did not activate PDE5 when present alone, but significantly augmented SNP-stimulated PDE5 activity and phosphorylation by $53 \pm 4\%$ (P < 0.001) and $45 \pm 5\%$ (P < 0.01) respectively, implying that PKA, like PKG, was capable of phosphorylating and activating PDE5, but only in the presence of cGMP (Figure 6). Myristoylated PKI and H-89 blocked only

Figure 6 cGMP-dependent phosphorylation and activation of PDE5 by PKA

Dispersed gastric smooth-muscle cells were incubated with cBIMPS (10 μ M), a selective activator of PKA, in the presence or absence of SNP (1 nM) and selective PKA (myristoylated PKI and H-89; 1 μ M each) and PKG (KT-5823; 1 μ M) inhibitors. Cells were lysed and PDE5 was immunoprecipitated using an anti-PDE5 antibody. (A) Phosphorylation of PDE5 was identified in immunoprecipitates by autoradiography from cells labelled with 32 P as described in the Experimental section. (B) Western-blot analysis of PDE5 immunoprecipitates using the anti-PDE5 antibody. (C) PDE5 activity in the immunoprecipitates was measured using [3 H]cGMP as the substrate as described in the Experimental section. Results are expressed as c.p.m./mg of protein and are means $\pm$ S.E.M. of five experiments. ** P < 0.01 compared with SNP alone; # P < 0.01, compared with SNP in the presence of cBIMPS.

the increase in PDE5 activity and phosphorylation over that induced by SNP alone, providing further evidence that PKA can phosphorylate and activate PDE5. A combination of PKI and KT5823 abolished PDE5 activity and phosphorylation induced by cBIMPS in the presence of SNP (Figure 6).

The ability of PKA to activate PDE5 in the presence of cGMP was corroborated in studies with isoproterenol (1 μ M). At this concentration, isoproterenol generates cAMP levels in vascular and visceral smooth muscle that selectively activate PKA without cross-activating PKG [1,3,31,34]. Isoproterenol increased PDE5 activity stimulated by various concentrations of SNP (0.1 nM–0.1 μ M); only the increase above that induced by SNP alone was abolished by myristoylated PKI (Figure 7).

A similar pattern was observed with forskolin (1 μ M), which increased PDE5 activity stimulated by various concentrations of SNP; the increase in PDE5 activity was abolished by myristoylated PKI (Figure 8). The increase in PDE5 activity induced by forskolin caused a decrease in SNP-stimulated cGMP levels; the decrease in cGMP levels was reversed by myristoylated PKI (Figure 8). Thus an increase in PDE5 activity, resulting from the activation of PKA by forskolin, occurred despite a decrease in the component of PDE5 activity resulting from the activation of PKG by reduced levels of cGMP.

Figure 7 Augmentation of SNP-stimulated PDE5 activity by isoproterenol

Dispersed gastric muscle cells were treated with SNP (0.1 nM–0.1 μ M) alone (Control) and in the presence of isoproterenol (ISOP; 1 μ M) or isoproterenol plus myristoylated PKI (ISOP + PKI; 1 μ M). Cells were lysed and PDE5 was immunoprecipitated using an anti-PDE5 antibody. PDE5 activity in the immunoprecipitates was measured using [3 H]cGMP as the substrate as described in the Experimental section. Isoproterenol augmented PDE5 activity induced by SNP, shifting the concentration-response curve to the left ($P < 0.01$ at all concentrations of SNP). Results are expressed, after subtraction of the basal level values (1757 ± 289 c.p.m./mg of protein), as c.p.m./mg of protein and are means $\pm$ S.E.M. of four experiments.

Attenuation of cGMP levels by PKG-dependent activation of PDE5

Inhibition of PDE5 activity by KT5823 significantly augmented SNP-stimulated cGMP levels by $88 \pm 13\%$ (basal cGMP, 0.25 ± 0.05 pmol/ 10^6 cells; 1 μ M SNP, 0.47 ± 0.06 pmol/ 10^6 cells; SNP plus 1 μ M KT5823, 0.68 ± 0.05 pmol/ 10^6 cells) (Figure 9). Similarly, inhibition of PDE5 activity by zaprinast (10 μ M) augmented SNP-stimulated cGMP levels by $118 \pm 15\%$ ($P < 0.001$) (Figure 9). The results imply that activation of PKG leads to activation of PDE5 and more extensive breakdown of cGMP. Thus the observed levels of cGMP reflect its sustained breakdown by activated PDE5. As noted above, the breakdown of cGMP increases in the presence of cAMP when PDE5 activity is further enhanced by PKA.

Feedback inhibition of soluble GC activity by PKG

Our recent studies [35] have shown that PKA, but not PKG, can phosphorylate, and thus attenuate, the activity of Type V/VI AC expressed in gastric smooth muscle, and block further formation of cAMP. In the present study, the ability of PKG and PKA to phosphorylate soluble GC and attenuate its activity was examined. SNP acting as a NO donor stimulated soluble GC activity by $183 \pm 24\%$ above basal level within 60 s (Figure 10). Inhibition of PKG activity by KT5823 increased significantly ($P < 0.01$) the SNP-stimulated soluble GC activity to $364 \pm 32\%$ above basal level (Figure 10).

SNP stimulated soluble GC phosphorylation by 4-fold within 60 s (basal, 559 ± 83 c.p.m.; SNP, 2642 ± 318 c.p.m.) (Figure 11). Soluble GC phosphorylation was abolished by KT5823 (648 ± 76 ; not significantly different from basal), but was not

Figure 8 Augmentation of SNP-stimulated PDE5 activity and inhibition of cGMP formation by forskolin

Dispersed gastric muscle cells were treated with SNP (0.1 nM–0.1 μ M) alone (Control) and in the presence of forskolin (1 μ M) or forskolin plus PKI (1 μ M). Cells were lysed and PDE5 was immunoprecipitated using a polyclonal anti-PDE5 antibody. PDE5 activity (upper panel) was measured in PDE5 immunoprecipitates using [3 H]cGMP as described in the Experimental section, and cGMP levels (lower panel) were measured by RIA. Basal level values (1698 ± 214 c.p.m./mg of protein) were subtracted and PDE5 activity was expressed as c.p.m./mg of protein. cGMP levels were expressed as the percentage increase above basal level (0.32 ± 0.04 pmol/mg of protein). Forskolin augmented PDE5 activity and inhibited cGMP formation induced by SNP ($P < 0.05$ to $P < 0.01$ at various concentrations of SNP). Values are means $\pm$ S.E.M. of four experiments.

affected by myristoylated PKI, H-89, bisindolylmaleimide, PD98059 or SB203580 (Figure 11).

In contrast, neither cBIMPS (10 μ M) nor isoproterenol (1 μ M) alone induced soluble GC phosphorylation ($5 \pm 6\%$ above basal; not significant). A higher concentration of isoproterenol (100 μ M), capable of generating cAMP levels that cross-activate PKG [12,13,31], stimulated soluble GC phosphorylation which was selectively inhibited by KT5823, but was not affected by myristoylated PKI, H-89 or PD98059 (Figure 12).

Inhibition of phosphatase 1 and 2A activity by calyculin A (0.1–10 μ M) increased soluble GC phosphorylation in a concentration-dependent fashion (Figure 13). The increase in phosphorylation induced by 10 μ M calyculin A inhibited SNP-stimulated soluble GC activity by $29 \pm 5\%$ ($P < 0.02$) (SNP, 5.1 ± 0.2 pmol/mg of protein above basal levels; SNP plus calyculin A, 3.6 ± 0.3 pmol/mg of protein above basal levels). The ability of PKG to stimulate soluble GC phosphorylation directly was examined *in vitro* by a back-phosphorylation tech-

Figure 9 Augmentation of SNP-induced cGMP levels by selective inhibition of PKG or PDE5

Gastric muscle cells were treated with SNP ($1 \mu\text{M}$) for 1 min in the presence or absence of a selective PKG inhibitor, KT5823 ($1 \mu\text{M}$), or the PDE5 inhibitor, zaprinast ($10 \mu\text{M}$). cGMP levels were measured by RIA and the results expressed as pmol/mg of protein. Values are means $\pm$ S.E.M. of four to five experiments. ** $P < 0.01$ compared with SNP alone.

Figure 10 Feedback inhibition of soluble GC activity by PKG-dependent phosphorylation

Gastric muscle cells were treated with SNP ($1 \mu\text{M}$) in the presence or absence of the PKG inhibitor, KT5823 (KT; $1 \mu\text{M}$). (A) Cells were lysed and soluble GC (sGC) was immunoprecipitated using an anti-[soluble GC] antibody and phosphorylation of soluble GC was identified in immunoprecipitates by autoradiography from cells labelled with ^{32}P as described in the Experimental section. (B) The membrane from (A) was immunoblotted with the anti-[soluble GC] antibody to determine the co-migration of the radioactive bands with soluble GC. (C) Soluble GC (sGC) activity was measured in crude homogenates by the conversion of [^{32}P]GTP to [^{32}P]GMP as described in the Experimental section. All studies were performed in the presence of 1 mM IBMX and $10 \mu\text{M}$ zaprinast. Results were expressed as pmol cGMP/mg of protein and are means $\pm$ S.E.M. of four experiments. ** $P < 0.01$, compared with SNP alone.

Figure 11 PKG-dependent phosphorylation of soluble GC

Gastric muscle cells were treated with SNP ($1 \mu\text{M}$) in the presence or absence of selective inhibitors of PKG (KT-5823; $1 \mu\text{M}$), PKA (myristoylated PKI and H-89; $1 \mu\text{M}$ each), PKC (bisindolylmaleimide; $1 \mu\text{M}$), MEK (PD98059; $10 \mu\text{M}$) or p38 MAPK (SB203580; $1 \mu\text{M}$). All studies were performed in the presence of 1 mM IBMX and $10 \mu\text{M}$ zaprinast. Cells were lysed and soluble GC was immunoprecipitated using an anti-[soluble GC] antibody. (A) Phosphorylation of soluble GC was identified in immunoprecipitates by autoradiography from cells labelled with ^{32}P as described in the Experimental section. (B) Western-blot analysis of soluble GC immunoprecipitates using the anti-[soluble GC] antibody. (C) The radioactivity in the bands from (A) were measured and soluble GC (sGC) phosphorylation expressed as c.p.m./mg of protein. Values are means $\pm$ S.E.M. of four experiments.

nique. Immunoprecipitates of soluble GC from resting muscle cells were treated with purified PKG-I and cGMP or with the catalytic subunit of PKA in the presence of [γ - ^{32}P]ATP. Immunoprecipitates of soluble GC derived from cells stimulated with SNP ($1 \mu\text{M}$) or isoproterenol ($100 \mu\text{M}$) were treated in a similar fashion and the extent of ^{32}P incorporation into soluble GC compared with that obtained in unstimulated cells. As shown in Figure 14, soluble GC was directly phosphorylated by SNP and isoproterenol-stimulated PKG. In contrast, PKA did not stimulate soluble GC phosphorylation *in vitro*.

DISCUSSION

The emergence of NO as the main mediator of vascular and visceral smooth muscle relaxation has focused attention on the role of cGMP and PKG in this process. The relaxant neuropeptides, VIP and PACAP, which are co-released with NO in smooth muscle of the gut, act predominantly by regenerating NO in target smooth-muscle cells [6,7]. Although the neuropeptides also stimulate cAMP formation, increasing evidence suggests that cAMP may additionally influence relaxation by cross-activating PKG [1–3,31,34].

The levels of cAMP and cGMP in gastrointestinal smooth muscle are determined by the synthetic activities of AC and soluble GC respectively, and the degradative activities of specific PDEs, mainly cAMP-preferring PDE3, cAMP-specific PDE4 and cGMP-specific PDE5 [8,10,11,35]. In the present study,

Figure 12 Lack of effect of PKA on soluble GC phosphorylation

Gastric muscle cells were treated with SNP (1 μ M) or isoproterenol (ISOP; 100 μ M) in the presence or absence of selective inhibitors of PKG (KT5823; 1 μ M), PKA (myristoylated PKI and H-89; 1 μ M each) or MEK (PD98059; 10 μ M). All studies were done in the presence of 1 mM IBMX and 10 μ M zaprinast. Cells were lysed and soluble GC was immunoprecipitated using an anti-[soluble GC] antibody. (A) Phosphorylation of soluble GC was identified in immunoprecipitates by radiography from cells labelled with 32 P as described in the Experimental section. (B) Western-blot analysis of soluble GC immunoprecipitates using the anti-[soluble GC] antibody. (C) The radioactivity in the bands from (A) were measured and soluble GC (sGC) phosphorylation expressed as c.p.m./mg of protein. Values are means $\pm$ S.E.M. of four experiments.

Western-blot analysis showed that PDE5, which is widely expressed in vascular smooth muscle of various species [21,28], is also expressed in gastric smooth-muscle cells. PDE5 was activated via phosphorylation by PKG and, in the presence of cGMP, also via phosphorylation by PKA resulting in the attenuation of cGMP levels. Furthermore, PKG, but not PKA, phosphorylated soluble GC and inhibited its activity. Thus two PKG-dependent mechanisms are involved in attenuating the levels of cGMP in gastric smooth-muscle cells: enhanced breakdown resulting from stimulation of PDE5 activity by PKG-dependent phosphorylation of PDE5, and decreased synthesis resulting from inhibition of soluble GC activity by PKG-dependent phosphorylation of soluble GC. In the presence of both cAMP and cGMP, PDE5, but not soluble GC, is also phosphorylated by PKA, contributing to the decline in cGMP levels. These interactive pathways are depicted in Scheme 1.

The evidence supporting the dual regulation of cGMP can be summarized as follows. (1) SNP stimulated PDE5 phosphorylation and PDE5 activity in a concentration-dependent fashion. The selective PKG inhibitor, KT5823, inhibited PDE5 phosphorylation and PDE5 activity, whereas selective PKA, PKC, MEK or p38 MAPK inhibitors had no effect on PDE5 phosphorylation or activity. (2) The membrane-permeable analogues, 8-pCPT-cGMP and cBIMPS, which act as selective activators of PKG and PKA respectively, did not increase basal PDE5 activity, but they significantly augmented SNP-stimulated PDE5 activity and PDE5 phosphorylation. This implies that phosphorylation and activation of PDE5 by either

Figure 13 Concentration-dependent phosphorylation of soluble GC by calyculin A

Gastric muscle cells were treated with calyculin A (0.1–10 μ M). Cells were lysed and soluble GC was immunoprecipitated using an anti-[soluble GC] antibody. (A) Phosphorylation of soluble GC was identified in immunoprecipitates by autoradiography from cells labelled with 32 P as described in the Experimental section. (B) Western-blot analysis of soluble GC immunoprecipitates using the anti-[soluble GC] antibody. (C) The radioactivity in the bands from (A) were measured and soluble GC (sGC) phosphorylation expressed as c.p.m./mg protein. Values are means $\pm$ S.E.M. of three experiments.

Figure 14 Selective phosphorylation of soluble GC by PKG *in vitro*

Gastric muscle cells were treated with SNP (1 μ M) or isoproterenol (ISOP; 100 μ M) in the presence of IBMX (1 mM) and zaprinast (10 μ M) for 60 s. Soluble GC was immunoprecipitated and back phosphorylated with purified PKG-I together with 1 μ M cGMP or with the catalytic subunit of PKA as described in the Experimental section. (A) 32 P-labelled soluble GC was identified by autoradiography. (B) Western-blot analysis of soluble GC immunoprecipitates using the anti-[soluble GC] antibody. (C) The decrease in 32 P incorporation into the soluble GC band in samples treated with 1 μ M SNP or 100 μ M isoproterenol was compared with control phosphorylation and plotted as percentage increase in the endogenous phosphorylation of soluble GC. Values are means $\pm$ S.E.M. of three experiments.

Scheme 1 Regulation of cGMP levels by PKG- and PKA-dependent activation of PDE5 and PKG-specific inhibition of soluble GC

The increase in cGMP levels triggers its breakdown by PDE5. The activity of PDE5 is enhanced by PKG-dependent phosphorylation initiated by cGMP-specific binding to allosteric sites on PDE5. The binding of cGMP to these sites also enables PKA to phosphorylate PDE5 and enhance its activity leading to further breakdown of cGMP when both cAMP and cGMP are elevated. PKG-specific phosphorylation of soluble GC (sGC) attenuates the synthetic activity of this enzyme. Activation of PDE5 by PKG and PKA and inhibition of soluble GC by PKG conspire to maintain cGMP levels within the physiological range. AC, adenylyl cyclase.

protein kinase required the presence of cGMP. The increase in PDE5 activity and PDE5 phosphorylation induced by 8-pCPT-cGMP and cBIMPS was inhibited by KT5823 and myristoylated PKI respectively, providing further evidence that both PKG and PKA can phosphorylate and activate PDE5 in the presence of cGMP. (3) Isoproterenol and forskolin also augmented SNP-stimulated PDE5 activity; the increase in PDE5 activity above that elicited by SNP alone was inhibited by PKI. The increase in PDE5 activity induced by forskolin led to a decrease in SNP-stimulated cGMP levels. (4) SNP-induced soluble GC phosphorylation and stimulated soluble GC activity. KT5823 abolished phosphorylation and augmented soluble GC activity, implying that feedback inhibition of soluble GC activity by PKG-dependent phosphorylation contributed to a cessation of cGMP formation. Inhibition of soluble GC activity was direct and PKG-specific as shown *in vitro* by back-phosphorylation studies.

The regulatory domain in the N-terminal segment of PDE5 contains a phosphorylation site at Ser⁹² and two allosteric sites for binding of cGMP located in the N-terminal dimerization domain [20,25]. Phosphorylation of PDE5 by PKA, as shown by Burns et al. [36], and by PKG is substrate-dependent and requires the binding of cGMP to both allosteric sites [24–26]. A cGMP analogue, 8-pCPT-cGMP, that activates PKG, but does not bind to PDE5, did not activate PDE5. In the presence of cGMP, however, activators of PKG as well as activators of PKA (cBIMPS, isoproterenol and forskolin) stimulated PDE5 activity. Phosphorylation of other PDEs by PKA, however, does not require binding of cGMP to the regulatory domain. This applies to PDE1A2, cGMP-stimulated PDE2, cGMP-inhibited PDE3 and cAMP-specific PDE4, all of which are regulated by PKA-dependent phosphorylation in various tissues [9,10,16,17,19]. Our recent studies [35] in gastric smooth-muscle cells have shown that both PDE3 and PDE4 are activated by phosphorylation via PKA, but not PKG.

The activation of PDE5 by PKG results in more extensive breakdown of cGMP. The observed levels of cGMP after stimulation by SNP reflect the attenuation resulting from activation of PDE5; the initial levels could be restored upon inhibition of PKG by KT5823. Wyatt et al. [33] have shown that stimulation of membrane-bound GC in vascular smooth muscle

by atrial natriuretic peptide ('ANP') induced PKG-dependent phosphorylation and activation of PDE5, causing a decrease in cGMP levels.

The ability of PKA to phosphorylate and thus attenuate the activity of AC Type V/VI has been demonstrated in various tissues [37], including gastric smooth muscle where phosphorylation and inhibition of activity were shown to be PKA-specific [35]. In the present study, the first evidence that feedback phosphorylation of soluble GC and attenuation of its activity is PKG-specific is provided. The ability of PKG to phosphorylate soluble GC was confirmed *in vitro* by back-phosphorylation studies. Our results in smooth-muscle cells appear to be at variance with those of Ferrero et al. [38] in chromaffin cells. The experimental conditions, however, were different. In the present study the initial effects within 1 min of addition of SNP or analogues were examined, whereas in the studies of Ferrero et al. the measurements were made after incubations lasting up to 30 min, leaving open the possibility that additional time-dependent pathways may have been activated that led to stimulation of soluble GC activity.

It is worth noting that concurrent activation of AC and NO-dependent soluble GC leading to combined generation of cAMP and cGMP is the physiological norm in smooth muscle of the gut [3,6,31]. The resultant interplay of cyclic nucleotides, cyclic nucleotide-dependent protein kinases and PDEs, leads to a decrease in cGMP levels and an increase in cAMP levels (Scheme 1). When both cyclic nucleotides are present, cAMP enhances the breakdown of cGMP via PKA-dependent activation of PDE5, whereas cGMP decreases the breakdown of cAMP by inhibiting the activity of PDE3. The inhibitory feedback mechanisms targeting the synthetic enzymes are more specific, with PKG causing selective inhibition of soluble GC and PKA causing selective inhibition of AC. Furthermore, the autophosphorylation of activated PKG markedly increases its affinity for cAMP [39,40]. When present together, both cyclic nucleotides act preferentially by activating PKG.

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