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Tyrosine phosphorylation in contraction of opossum esophageal longitudinal muscle in response to SNP

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Hirano, Ikuo, Rahul Kakkar, Joy K. Saha, Pawel T. Szymanski, and Raj K. Goyal. Tyrosine phosphorylation in contraction of opossum esophageal longitudinal muscle in response to SNP. *Am. J. Physiol.* 273 (*Gastrointest. Liver Physiol.* 36): G247–G252, 1997.—Sodium nitroprusside (SNP) has been shown to elicit a guanosine 3′,5′-cyclic monophosphate (cGMP)-mediated, indomethacin-sensitive contraction of the opossum esophageal longitudinal muscle. We examined the role of tyrosine phosphorylation in the signal transduction pathway of contractions induced by SNP and cGMP in longitudinal muscle strips in vitro. Force of isometric contractions was expressed as the percentage of responses to KCl (73 mM). SNP (100 μM)-induced contractions were 75 ± 5% before and 3 ± 2% after 50 μM genistein ($P < 0.005$) and 86 ± 16% before and 0 ± 0% after 50 μM tyrphostin B46. Contractions in response to 8-bromo-cGMP (8-BrcGMP; 1 mM) were 74 ± 15% before and 3 ± 2% after genistein ($P < 0.01$) and 63 ± 15% before and 18 ± 4% after tyrphostin B46 ($P < 0.05$). In contrast, KCl-induced contractions were 82 ± 8% and 96 ± 9% of the control value after genistein and tyrphostin B46 treatments, respectively ($P > 0.05$ for both). Carbachol contractions were partially suppressed by genistein (106 ± 8% vs. 79 ± 8%; $P < 0.05$) but unaffected by tyrphostin B46 (114 ± 10% vs. 107 ± 12%; $P > 0.05$). Western blot analysis revealed a 116-kDa phosphotyrosine protein in the control muscle strips. The level of this protein was increased to 206 ± 15% of control after SNP treatment. Both genistein and tyrphostin B46 blocked this increase. These studies show that contractions of the esophageal longitudinal muscle induced by SNP and cGMP utilize a signal transduction pathway different from that used by the depolarizing agent KCl and the muscarinic agonist carbachol. Contractions induced by SNP and cGMP involve tyrosine phosphorylation of a protein, possibly identified as a 116-kDa protein, as a key step in the signaling pathway.

tyrosine kinase; nitric oxide; cyclooxygenase; guanylate cyclase; genistein; tyrphostin

THE NITRIC OXIDE DONOR sodium nitroprusside (SNP) generally causes relaxation of smooth muscle via guanosine 3′,5′-cyclic monophosphate (cGMP) accumulation (13). In esophageal and other gastrointestinal longitudinal muscles, however, SNP produces a transient relaxation that is followed by a prolonged contraction (17). In some tissues, SNP-elicited contractions are cholinergically mediated (11). The SNP-induced contraction in the esophageal longitudinal muscle is mediated via cGMP as evidenced by the observation that the excitatory action of SNP is suppressed by a guanylate-cyclase inhibitor, LY-83583, and is mimicked by a cell permeable analog of cGMP, 8-bromo-cGMP (8-BrcGMP). Contractions elicited by SNP and 8-BrcGMP are suppressed by indomethacin, suggesting that excitatory eicosanoids are involved in their contractile actions (17). Excitatory eicosanoids have also been shown to mediate contractions in certain smooth muscles due to agonists such as epidermal growth factor (EGF) and angiotensin II (10). Moreover, the contractions elicited by these agonists have also been shown to be antagonized by genistein (2) and tyrphostin B46 (14), suggesting that the contractile actions of EGF and angiotensin II involve tyrosine phosphorylation of a signaling protein (7, 10). Recent investigations have speculated that this signaling protein may be a 116-kDa protein, Ras-GTPase-activating protein (Ras-GAP) (7).

We performed studies in the opossum esophageal longitudinal muscle to determine whether a tyrosine kinase pathway is also involved in the contractile actions of SNP and cGMP. Our findings suggest that the contractile actions of SNP may involve tyrosine phosphorylation of a 116-kDa protein in the signal transduction pathway. This pathway is distinct from the signaling cascades involved in contractions in response to the depolarizing agent KCl and the muscarinic agonist carbachol.

METHODS

Tissue preparation. Experiments were performed in opossums (*Didelphis virginiana*). The animals were anesthetized by intra-abdominal injection of pentobarbital sodium (40 mg/kg). The abdomen was opened by midline incision. The esophagus was removed, placed on a petri dish, and bathed in 37°C Krebs solution that was gassed with 95% O_2-5% CO_2. The esophagus was then cleared of the attached tissues, opened longitudinally, and pinned to a Sylgard (Dow Corning)-based petri dish. The mucosa was removed by sharp dissection.

Isometric force recordings. Longitudinally oriented unstretched muscle strips ~1.5 mm in width and 3–4 mm in length were prepared for the study. One end of each strip was tied to a stainless steel tissue holder, and the other was connected to an isometric force displacement transducer (Gould-Statham UC2 and Grass FT O3) by a silk thread. The transducers were mounted on two-dimensional manipulators. Muscle strips were suspended in 2-ml double-jacketed organ baths (Radnoti Glass) containing Krebs solution gassed

with 95% O_2-5% CO_2 mixture through a porous sintered disk at the bottom of the baths. Prewarmed water (37°C) was circulated through the outer jacket of the tissue bath via a constant temperature circulator (Haake FE2). The temperature of the Krebs solution in the organ bath was maintained within ±0.5°C. The Krebs solution contained (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot H_2O$, 0.6 $MgSO_4 \cdot 7H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 25 $NaHCO_3$, and 11.1 glucose.

Muscle contractions were recorded on a rectilinear polygraph (Beckman R-711) after amplification through a low noise bioamplifier (Beckman 9853H) or a digital chart recorder (AD Instruments MacLab 8e and an Apple Power Macintosh running AD Instruments Chart v3.4.2 digital acquisition software) after amplification through a transducer amplifier (AD Instruments ETH-400). Longitudinal muscle strips were equilibrated at an applied tension of ~1.5–2 g for 1 h. This amount of tension stretched the tissues to ~150% of the original length and placed them near the length at which longitudinal muscle strips were found to develop maximal active tension (17). The Krebs solution was changed every 15 min. The force generated by the excitatory agents was expressed as a percentage of the maximal contractions in response to 73 mM KCl.

Western blots. The muscle strips used for Western blot assays were prepared and treated in an identical manner to the contraction studies. The strips were removed from the organ baths immediately before reaching maximal contraction in response to the various agonists and then clamped with liquid nitrogen-cooled forceps. The strips were immediately transferred into Eppendorf tubes immersed in liquid nitrogen and then stored at −80°C. The muscle strips were later ground in a liquid nitrogen-cooled mortar and pestle and homogenized, using a Pyrex 7727 glass homogenizer in buffer solution consisting of 0.5 M NaCl, 40 mM tris(hydroxymethyl)-aminomethane (Tris)·HCl, pH 7.2, 2% sodium dodecyl sulfate (SDS), 5 mM EDTA, 10 mM NaF, 2 mM NaN_3, and 2 mM phenylmethylsulfonyl fluoride (PMSF). Samples were heated at 100°C for 5 min and centrifuged at 10,000 *g* for 5 min. Supernatants were collected and stored in a −80°C freezer.

Protein content was determined by BCA protein assay according to the protocol of the manufacturer (Pierce, Rockford, IL), using bovine serum albumin (BSA) as a standard. The level of tyrosine phosphorylation was determined as follows: tissue material in the amount of 30 μg/lane was separated with the use of a 6–20% gradient SDS-polyacrylamide gel electrophoresis (12). The proteins were then electrophoretically transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). The membranes were incubated in Tris-buffered saline solution, pH 7.2, plus 0.5% Tween 20 and 2% of BSA for 60 min at room temperature, to block any nonspecific-binding sites. To identify phosphotyrosine residues, we then incubated the Immobilon-P membranes with a mouse monoclonal anti-phosphotyrosine antibody, PY-20 (ICN, Irvine, CA), in dilution of 1:2,500 for 1 h at room temperature. The membranes were washed three times in the buffer solution without BSA. Peroxidase-conjugated affinity pure goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) was diluted 1:75,000 and added to the membranes that were further incubated for 1 h at room temperature. Substrates were visualized using enhanced chemiluminescence Western blotting detection reagents, according to the manufacturer's instructions (Amersham, Arlington Heights, IL), and exposing the membranes to autoradiographic film (Kodak X-OMAT; XAR-5). The quantification of phosphotyrosine residues in proteins was performed by densitometry of autoradiographic film using a Sony XC-77 charge-coupled device video camera interfaced with a Macintosh computer. Gel images were analyzed with the program Image from the National Institutes of Health, Research Series Branch.

Chemicals and reagents. SNP, 8-BrcGMP, carbachol, and genistein were obtained from Sigma Chemical (St. Louis, MO). Tyrphostin B46 was from Calbiochem-Novabiochem (La Jolla, CA). Materials for electrophoresis were from Bio-Rad.

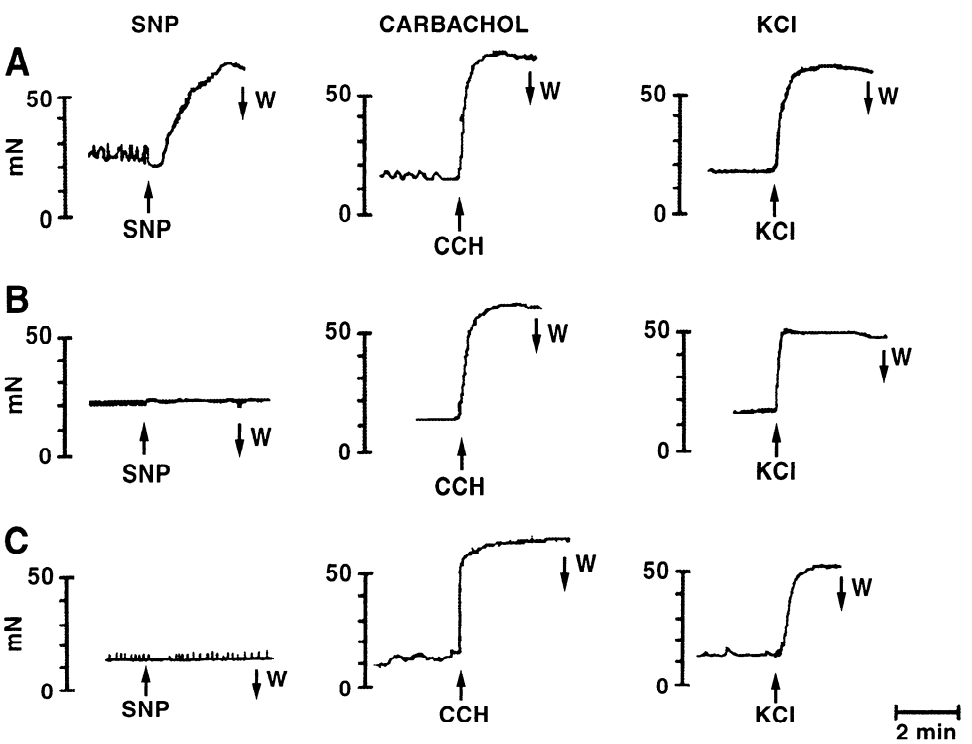

Fig. 1. Examples of effects of genistein (*B*) and tyrphostin B46 (*C*) on contractile response of opossum esophageal longitudinal smooth muscle to 100 μM sodium nitroprusside (SNP), 1 μM carbachol (CCH), and 73 mM KCl. Note that KCl and CCH cause strong contraction without preceding relaxation, whereas SNP causes relaxation followed by contraction. Genistein (50 μM) or tyrphostin B46 (50 μM) nearly abolished contraction elicited by SNP without similarly affecting contractions to KCl or CCH. *A*: control. W, wash.

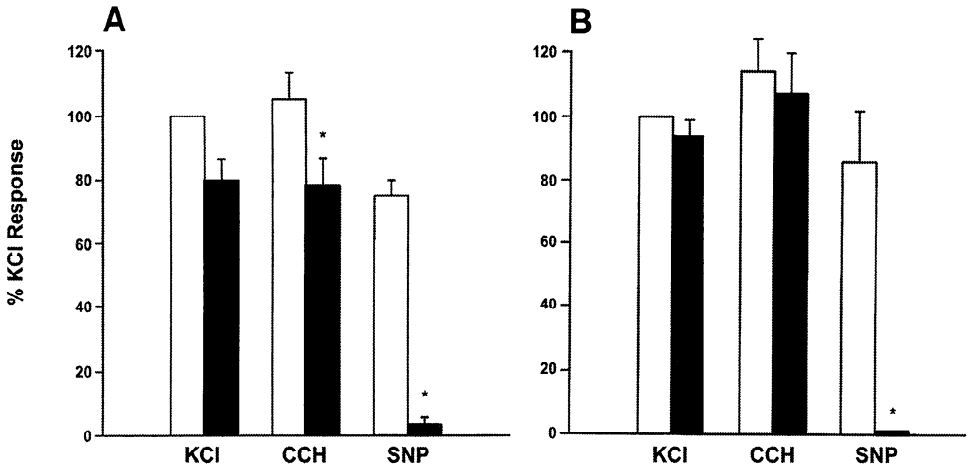

Fig. 2. Effects of genistein (*A*) and tyrphostin B46 (*B*) on contraction elicited by KCl, CCH, and SNP. Bars are means ± SE of contraction amplitudes expressed as % of 73 mM-KCl-elicited contractions for each strip. Note that 50 μM genistein or 50 μM tyrphostin B46 had no significant effect on KCl contractions. Genistein slightly suppressed CCH (1 μM)-elicited contraction, but tyrphostin B46 had no significant effect. In contrast, genistein and tyrphostin B46 nearly abolished SNP (100 μM)-elicited contractions. *A* and *B*: open bars, control; filled bars, treatment. *P < 0.005.

Statistics. Student's *t*-test was used for statistical analysis with $P < 0.05$ taken as an indication of a statistically significant difference.

RESULTS

Effects of KCl, carbachol, and SNP. Depolarizing concentrations of KCl (73 mM) caused maximal contraction of the esophageal longitudinal muscle that averaged 72 ± 5 mN (n = 35 strips from 19 animals). Carbachol and SNP produced concentration-dependent contraction. A cumulative concentration-response curve showed that threshold concentration for carbachol contraction was 10 nM with maximal effect at 1 μM. SNP produced maximal contraction at 100 μM. Contractions caused by these agonists were normalized to KCl contraction in each strip. Carbachol (1 μM) and SNP (100 μM) resulted in an average response of 109 ± 6% (n = 11 strips from 6 animals) and 82 ± 10% (n = 13 strips from 7 animals) of KCl contraction, respectively.

Influence of tyrosine kinase inhibitors. Genistein (50 μM) reversibly abolished all spontaneous tonic and phasic contractions. Because of the absence of spontaneous tone in the presence of genistein, the effect of tyrosine kinase inhibition on the SNP-induced transient relaxation could not be evaluated. Genistein (50 μM) also almost abolished the contractile effect of SNP (Fig. 1). The SNP-induced contraction was 61 ± 7 mN before and 2.6 ± 1.6 mN after genistein ($P < 0.05$, n = 5 strips from 3 animals). The SNP contractions normalized to KCl contraction were 75 ± 5% before and 3 ± 5% after genistein ($P < 0.005$, n = 5 strips from 3 animals) (Fig. 2). In contrast, 50 μM genistein did not significantly affect the contraction in response to KCl and slightly inhibited the response to carbachol (Fig. 1). The mean KCl response was 55 ± 7 mN before and 42 ± 3 mN (80 ± 7% of control) after genistein ($P > 0.05$, n = 5 strips from 3 animals). The mean carbachol contraction was 77 ± 14 mN before and 55 ± 7.4 mN after genistein ($P < 0.05$, n = 6 strips from 3 animals). The carbachol response normalized to KCl contraction was 106 ± 8% of KCl contraction before and 79 ± 8% after genistein ($P < 0.05$, n = 6 strips from 3 animals) (Fig. 2). This mild inhibitory effect could be due to the suppressant action of genistein on L-type calcium channels (9).

Tyrphostin B46 (50 μM), a tyrosine kinase inhibitor structurally unrelated to genistein, also reversibly abolished the spontaneous contractions, while minimally affecting the KCl-induced contraction that was 93 ± 6%

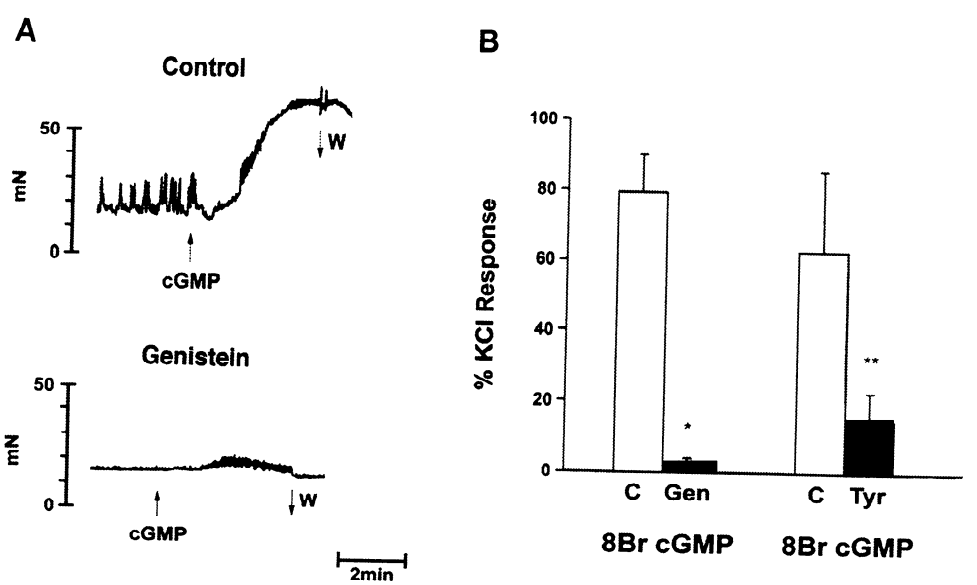

Fig. 3. Effect of 8-bromo-cGMP (8-BrcGMP) on longitudinal muscle and influence of tyrosine kinase inhibitors. *A*: example of biphasic action of 8-BrcGMP (cGMP; 1 mM). Contraction is almost abolished by 50 μM genistein. *B*: means ± SE of 8-BrcGMP-elicited contractions expressed as a % of 73 mM KCl response. Note that genistein (Gen) and tyrphostin B46 (Tyr) markedly suppressed contractions. C, control; W, wash. *$P < 0.01$; **$P < 0.05$.

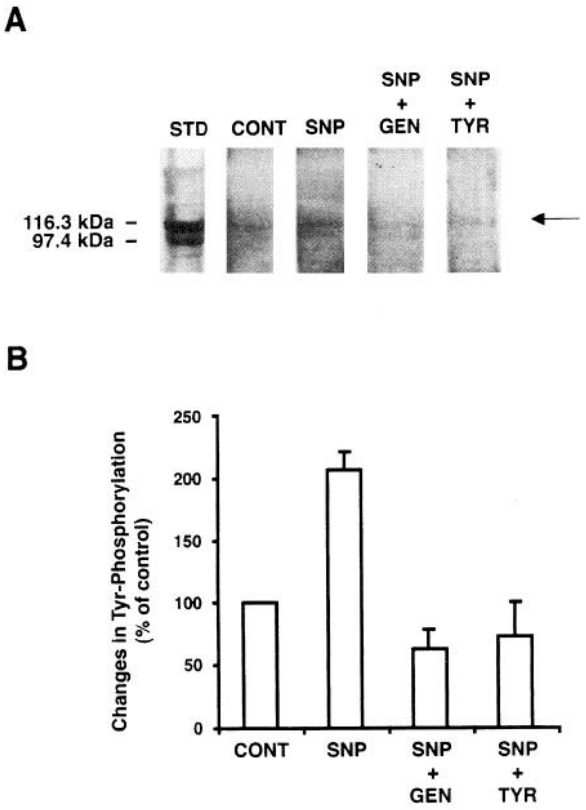

Fig. 4. Western blot analysis of tyrosine phosphorylation of 116-kDa protein in control and SNP-treated longitudinal muscle strips. *A*: position of 2 standard markers, β-galactosidase (116.3 kDa) and phosphorylase B (97.4 kDa), is shown in *lane 1* (STD). Control (CONT) strip is in *lane 2*, showing that a 116-kDa protein is present in untreated longitudinal muscle. *Lane 3* shows an increase in this band after treatment with SNP. *Lane 4* shows a faint band in a muscle strip that was treated with genistein (GEN) and SNP. Similarly, *lane 5* shows a diminished band at 116 kDa (indicated by arrow) after treatment with tyrphostin B46 (TYR) and SNP. *B*: summary of results of densitometric analysis of autoradiograms of Western blot. Control levels of phosphorylation (basal level) were taken to be 100% (*A*, *lane 2*). Level of phosphorylation in muscle strips after various treatments was expressed as %control. Note that SNP resulted in a more than twofold increase in 116-kDa phosphotyrosine to 206 ± 15% (triplicate determinations). Genistein (50 μM) reduced resting levels of phosphotyrosine to 62 ± 19% of control and abolished increase associated with SNP (206 ± 15% vs. 62 ± 16%, triplicate determinations). Tyrphostin B46 (50 μM) also blocked increase in 116-kDa phosphotyrosine elicited by SNP (206 ± 15% vs. 73 ± 28%, triplicate determinations).

of the control values ($P > 0.05$, $n = 6$ strips from 3 animals). Tyrphostin B46 (50 μM) insignificantly inhibited the contractile effect of carbachol. Carbachol-elicited contractions were 114 ± 10% and 107 ± 13% of the control KCl response before and after tyrphostin B46, respectively ($P > 0.05$, $n = 6$ strips from 3 animals). On the other hand, tyrphostin B46 (50 μM) completely abolished the response to SNP (100 μM) from an average contraction of 86 ± 16% of KCl contraction before to 0% after tyrphostin B46 ($n = 8$ strips from 4 animals) (Figs. 1 and 2).

Effect of 8-BrcGMP. 8-BrcGMP (1 mM) elicited a contraction of 44 ± 3 mN that was 70% of the KCl response ($n = 11$ strips from 6 animals). Genistein (50 μM) inhibited the 8-BrcGMP response from 79 ± 11% to 3 ± 1% of KCl-induced contraction ($P < 0.01$, $n = 6$ strips from 3 animals). Tyrphostin B46 (50 μM) inhibited contraction elicited by 8-BrcGMP from an average contraction of 63 ± 15% to a value of 18 ± 4% ($P < 0.05$, $n = 5$ strips from 3 animals) (Fig. 3).

Tyrosine phosphorylation of a 116-kDa protein. Unstimulated esophageal longitudinal muscle showed basal levels of several tyrosine phosphorylated proteins. SNP treatment was associated with increased tyrosine phosphorylation of a number of these proteins, including proteins with molecular masses of 14, 35, 86, 116, and 205 kDa. On the basis of recent data supporting the importance of a 116-kDa protein (7), we focused attention on this protein. SNP treatment showed levels of this 116-kDa phosphorylated protein that were 206 ± 15% of levels of the control strip ($P < 0.05$, triplicate determinations). Muscle strips treated with genistein alone, SNP and genistein, or SNP and tyrphostin B46 showed 116-kDa tyrosine phosphoprotein levels of 62 ± 19%, 62 ± 16%, and 73 ± 28% of the control values, respectively ($P < 0.05$ for SNP control vs. SNP plus tyrosine kinase inhibitors, triplicate determinations) (Fig. 4).

DISCUSSION

These studies confirm that SNP elicits a biphasic response consisting of transient relaxation followed by

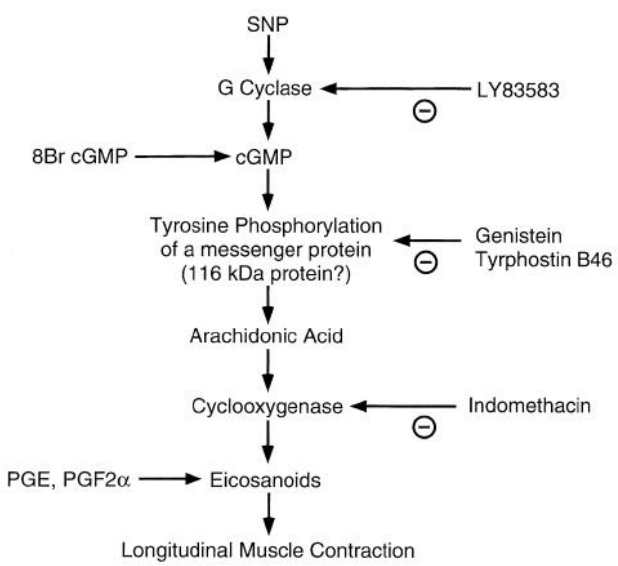

Fig. 5. Possible signaling pathway for SNP-induced contraction. Findings of this study and information available in literature are consistent with the following scheme: SNP stimulates guanylate cyclase (G cyclase) to cause cGMP accumulation, as evidenced by suppression of SNP contraction by guanylate cyclase inhibitor LY-83583 and mimicry of its effect by 8-BrcGMP (17). SNP or cGMP causes accumulation of 116-kDa phosphotyrosine (this study) by an unknown mechanism possibly due to inhibition of a tyrosine phosphatase (16). Accumulation of a tyrosine-phosphorylated 116-kDa protein stimulates production of eicosanoids of the cyclooxygenase pathway that may explain indomethacin sensitivity of SNP- or cGMP-elicited contractions (17). The mechanism by which phosphotyrosine may stimulate prostaglandin production is not known. In the gut, the major eicosanoids of the cyclooxygenase pathway are prostaglandins of E (PGE) and F ($PGF_{2\alpha}$) series (18), which both elicit contraction of esophageal longitudinal muscle (19).

a prominent contraction of the esophageal longitudinal muscle that is mediated by cGMP (17). The present studies further show that tyrosine kinase inhibitors abolish the longitudinal muscle contraction caused by SNP or 8-BrcGMP. The antagonistic effects of tyrosine kinase inhibitors indicate that tyrosine phosphorylation of a signaling protein is involved in SNP-elicited longitudinal muscle contraction. In contrast, the tyrosine kinase inhibitors do not similarly abolish contractions elicited by KCl or carbachol, suggesting that the action of SNP and cGMP involves a signal transduction pathway that is different from that utilized by KCl or carbachol. A possible signaling pathway for SNP-induced and cGMP-mediated contraction is shown in Fig. 5.

Phosphorylation of tyrosine residues in signaling proteins by tyrosine kinases is an important mechanism in the contraction of smooth muscles by many agonists that act on tyrosine kinase receptors, such as EGF and platelet-derived growth factor (3, 8, 10, 15, 20) as well as by certain G protein-coupled receptor agonists that may act to stimulate nonreceptor tyrosine kinases, such as angiotensin II, vasopressin, bombesin, and endothelins (10, 8). Recently, it has been shown that ethanol induces a rapid contractile response in gastric longitudinal muscle via a tyrosine kinase inhibitor-sensitive pathway (22).

The present study shows that SNP contraction is also accompanied by an increase in tyrosine phosphorylation of a 116-kDa protein that is abrogated by the tyrosine kinase inhibitors genistein and tyrphostin B46. These observations suggest that tyrosine phosphorylation of this 116-kDa protein may be involved in contraction of the longitudinal muscle induced by SNP and cGMP. The possible importance of a 116-kDa phosphotyrosine in the signaling cascade of certain contractile agonists was first proposed by Di Salvo and colleagues (7), who noted agonist-induced tyrosine phosphorylation of several substrates that included one with a molecular mass of 116 kDa. Di Salvo et al. (7) focused on the 116-kDa substrate due to the known involvement of a 116- to 120-kDa protein, Ras-GAP, in the signaling pathway that utilizes Ras, a G protein present in smooth muscle (1, 4). The identity of the 116-kDa protein isolated by Western blot electrophoresis was then confirmed to be Ras-GAP by reprobing the gels with antibodies specific for Ras-GAP (7). Measurements of intracellular calcium in isolated vascular smooth muscle cells demonstrated that tyrosine phosphorylation of Ras-GAP occurs before increases in calcium and declines before decreases in calcium, supporting a role for this signaling protein in contraction (7).

The mechanism by which SNP and cGMP increase tyrosine phosphorylation of the signaling protein is unclear. Tyrosine phosphorylation can be caused by stimulation of a tyrosine kinase or by suppression of the activity of tyrosine phosphatase. Studies using murine fibroblasts have demonstrated that both SNP and 8-BrcGMP stimulate tyrosine phosphorylation by inhibiting phosphotyrosine phosphatase activity (16). Studies are needed to define whether a similar mechanism for SNP is responsible for tyrosine phosphorylation in smooth muscle cells.

The contractile action of SNP and cGMP on the esophageal longitudinal muscle is suppressed by indomethacin, suggesting that an excitatory mediator is involved that is an eicosanoid of the cyclooxygenase pathway (17). Tyrosine kinase-dependent contractions in many other smooth muscle systems have been shown to be indomethacin sensitive (10, 21). It is unclear whether the tyrosine phosphorylation step occurs before or after the eicosanoid production. It is likely, however, that tyrosine phosphorylation precedes eicosanoid production. This is evidenced by studies suggesting that tyrosine phosphorylation stimulates phospholipase A_2 and diacylglycerol lipase, leading to enhanced arachidonic acid production in certain smooth muscles (5, 21). In the gut, the major eicosanoids of the cyclooxygenase pathway are prostaglandins E_2 and $F_{2\alpha}$ (18), which both contract the longitudinal muscle (19). The contractile effects of these excitatory prostaglandins are not significantly suppressed by tyrosine kinase inhibitors (20). Our preliminary unpublished data also show that the contractile effects of prostaglandin E_2 are not abolished by genistein. Excitatory eicosanoids elicit smooth muscle contraction through receptors that are coupled via a G protein to a phospholipase C/inositol trisphosphate/calcium/diacylglycerol pathway (6). Further studies are needed to substantiate the proposed signaling pathway of SNP-elicited longitudinal muscle contraction (Fig. 5).

In summary, this study shows that tyrosine phosphorylation of a 116-kDa protein may play a critical role in the signaling pathway of SNP-induced, cGMP-mediated and indomethacin-sensitive contraction of esophageal longitudinal muscle.

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