Role of pp60c-src and p44/42 MAPK in ANG II-induced contraction of rat tonic gastrointestinal smooth muscles

RAJINDER N. PURI, YA-PING FAN, AND SATISH RATTAN
Department of Medicine, Division of Gastroenterology and Hepatology, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received 18 January 2002; accepted in final form 25 March 2002

Puri, Rajinder N., Ya-Ping Fan, and Satish Rattan. Role of pp60c-src and p44/42 MAPK in ANG II-induced contraction of rat tonic gastrointestinal smooth muscles. Am J Physiol Gastrointest Liver Physiol 283: G390–G399, 2002; 10.1152/ajpgi.00025.2002.—We examined the role of mitogen-activated protein kinase (p44/42 MAPK) in ANG II-induced contraction of lower esophageal sphincter (LES) and internal anal sphincter (IAS) smooth muscles. Studies were performed in the isolated smooth muscles and cells (SMC). ANG II-induced changes in the levels of phosphorylation of different signal transduction and effector proteins were determined before and after selective inhibitors. ANG II-induced contraction of the rat LES and IAS SMC was inhibited by genistein, PD-98059 [a specific inhibitor of MAPK kinases (MEK 1/2)], herbimycin A (a pp60c-src inhibitor), and antibodies to pp60c-src and p120 ras GTPase-activating protein (p120 rasGAP). ANG II-induced contraction of the tonic smooth muscles was accompanied by an increase in tyrosine phosphorylation of p120 rasGAP. These were attenuated by genistein but not by PD-98059. ANG II-induced increase in phosphorylations of p44/42 MAPKs and caldesmon was attenuated by both genistein and PD-98059. We conclude that pp60c-src and p44/42 MAPKs play an important role in ANG II-induced contraction of LES and IAS smooth muscles.

Caldesmon is a physiological substrate of MAPKs and inhibits the ATPase activity of actomyosin complex in smooth muscles (31). Phosphorylated caldesmon positively regulates smooth muscle contraction by relieving the inhibition of actomyosin ATPase activity (18, 31) and, consequently, promoting smooth muscle contraction.

The purpose of this investigation is to identify critical events in the signal transduction mechanisms of ANG II-induced contraction of LES and IAS smooth muscles...
muscles. Results of the present investigation show that the activation of pp60src and p44/42 MAPKs play an important role in ANG II-induced contraction of the tonic smooth muscles of the LES and IAS.

MATERIALS AND METHODS

Preparation of smooth muscle strips. Smooth muscle strips were prepared as described previously (17). Adult Sprague Dawley rats (of either sex, −250 g) were anesthetized with pentobarbital sodium (50 mg/kg ip). Laparotomy was performed, and the LES and IAS smooth muscle tissues were excised and transferred immediately to a beaker containing oxygenated (95% O2, 5% CO2) Krebs solution (in mM): 118.07 NaCl, 4.69 KCl, 2.52 CaCl₂, 1.16 MgSO₄, 1.01 NaH₂PO₄, 25 NaHCO₃, and 11.10 glucose. The smooth muscle tissues were carefully freed of all extraneous structures, opened, and pinned flat with the mucosal side up on a dissecting tray containing oxygenated Krebs solution. The mucosal and submucosal layers along with the serosal connective tissue and visible blood vessels were removed by sharp dissection. The smooth muscle strips (1 × 10 mm) were secured at both ends with silk sutures and transferred to 2-ml muscle baths containing oxygenated Krebs solution at 37°C. One end of each smooth muscle strip was secured at the bottom of the muscle bath, and the other end was attached to a force transducer (model FT03; Grass Instruments, Quincy, MA) connected to a PowerLab recorder (CB Sciences, Milford, MA). Only those smooth muscle strips that developed a spontaneous steady tone and relaxed in response to electrical field stimulation were used for these experiments.

For some studies, the smooth muscle tissues were treated with 1 × 10⁻⁷ M ANG II (maximal effective concentration) alone or after different inhibitors and frozen by clamping at the point of maximal effect of the agonist. For controls, tissues were not treated with any agent. These tissues were subjected to protein extraction for the Western blot studies as outlined below.

Isolation of SMC from rat LES and IAS. SMC from the tonic smooth muscle tissues were isolated essentially by the method described previously (6). Rat LES and IAS smooth muscle strips were cut into small pieces (1 mm cubic pieces) and incubated at 37°C for two successive 1-h periods in oxygenated Krebs solution containing collagenase (0.01% for LES and 0.013% for IAS) and soybean trypsin inhibitor (0.01%). After each incubation, the mixture was filtered through a 500-μm Nitex mesh. Tissue trapped on the mesh was rinsed with 25 ml (5 × 5 ml) collagenase-free Krebs solution. Tissue was finally incubated in collagenase-free Krebs solution at 37°C, and dispersion of the cells (0–1 h) was monitored periodically by examining a 10-μl aliquot of the mixture under a microscope. SMC were harvested by filtration through Nitex mesh. Filtrate containing the cells was centrifuged at 350 × g for 10 min at room temperature. Cells in the pellet were resuspended in Krebs solution at a cell density of 3 × 10⁶ cells/ml.

Permeabilization of LES and IAS SMC. Permeabilization of LES and IAS SMC was accomplished by the method previously used in our laboratory (17). SMC from rat LES and IAS were permeabilized by incubating them in cytosolic solution (in mM): 20 NaCl, 100 KCl, 5 MgSO₄, 0.96 Na₂HPO₄, 25 NaHCO₃, 1 EGTA, 0.48 CaCl₂, and 1% BSA with saponin (75 μg/ml) for 3 min at room temperature. Cell suspension was centrifuged at 350 × g for 10 min. The pellet was suspended in a cytosolic solution supplemented with antimycin A (10 μM), ATP (1.5 mM), phosphocreatine (5 mM), and creatine phosphokinase (10 U/ml) and centrifuged at 350 g for 10 min. Cells were washed twice with the modified cytosolic solution to remove saponin and resuspended in the fresh, modified cytosolic solution.

Measurement of cell length by scanning micrometry. Aliquots (30 μl) of SMC from the rat tissues were incubated with various agonists in the absence or presence of specific inhibitors. Incubations were terminated by the addition of 1% acrolein. The mean length of 30 cells chosen at random in each set was determined by micrometry using phase contrast microscopy. Images were stored digitally, and the cell lengths were measured by the Image-Pro Plus version 4.0 program (Media Cybernetics, Silver Spring, MD). Digital data were transferred directly to the Microsoft Excel computer program. Data are presented as percentage shortening of the SMCs after different treatments in each set as means ± SE compared with that of controls (untreated cells).

Gel electrophoresis and Western blot analysis. Protein extracts from rat LES and IAS smooth muscles were prepared by cutting the tissues into 1–2 mm cubic pieces and by incubating them in a lysis buffer (1% SDS, 1 mM ortophenylendiamine, and 10 mM Tris, pH 7.4) at 90°C for 3 min. Incubation mixtures were homogenized, followed by centrifugation at 16,000 g for 15 min at 4°C. Protein in the supernatants was estimated by Lowry’s method. Solutions of the protein extracts were prepared by mixing them with an equal volume of 2× sample buffer (125 mM Tris, pH 6.8, 10% glycerol, 2% β-mercaptoethanol, and 0.006% bromophenol blue). These samples then were heated in a boiling water bath for 3 min. Protein samples (40 μg protein/20 μl) were subjected to SDS-PAGE by the Laemmli method (28). Unless otherwise noted, a discontinuous gel system utilizing 4% stacking gel, pH 6.8, and 10% running gel, pH 8.8 (or as stated), was used in all experiments involving gel electrophoresis.

Proteins in the gels were electroblotted onto a nitrocellulose membrane (NCM) at 100 V for 1 h at 4°C. NCMs were then transferred into a blocking buffer containing Tris-buffered saline (TBS) (20 mM Tris and 137 mM NaCl, pH 7.4). NCMs were then rinsed with a washing buffer containing TBS and 0.1% Tween 20 (TBS-Tween), treated with a primary antibody for 1 h with gentle agitation, and washed three times with TBS-Tween. NCMs were subsequently treated with an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h with gentle agitation and washed three times with TBS-Tween. All primary and secondary antibodies were used at dilutions described in the respective figure legends. Membranes were blotted semidry by placing them between two filter papers and were then developed with the enhanced chemiluminescense Western blotting reagents according to the instructions provided by the supplier (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized by exposing the membranes to X-ray films, which were then scanned (model SNAPSHOT 310; Agfa, Ridgefield Park, NJ). Protein bands were quantitated by densitometric analysis by using the Image-Pro Plus version 4.0 program.

Data analyses. Data were calculated as means ± SE by using the Sigma Plot computer program for personal computers. Differences between groups were examined by Student’s t-test (P value) with P < 0.05 considered to be statistically significant.

Chemicals and drugs. ANG II (human) and herbimycin A (Hb A) were obtained from Calbiochem (San Diego, CA). Tyrosine kinase inhibitor genistein and the MAPK kinase (MEK) inhibitor PD-98059 were obtained from Research Biochemicals International (Natick, MA) and Biomol (Plymouth, PA).
Anti-p60c-src, HR3 were obtained from Transduction Labs (Lexington, KY). Polyclonal antibodies, and anti-mouse antibody conjugated with HRP were obtained from Transduction Labs (Lexington, KY). Anti-pp60c-src polyclonal antibody was obtained from Chemicon International (Temecula, CA). Anti-phosphoserine, anticaldesmon monoclonal antibodies, and anti-mouse antibody-HRP were obtained from Sigma. Anti-phospho-p44/42 MAPK monoclonal antibody was purchased from New England Biolabs (Boston, MA). Anti-rabbit antibody-HRP, enhanced chemiluminescence Western blotting reagents kit, and X-ray hyperfilm were purchased from Amersham Pharmacia Bio-tech.

RESULTS

Effects of tyrosine kinase inhibitor genistein and MAPK kinase inhibitor PD-98059 on ANG II-induced contraction of LES and IAS SMC. ANG II-induced contractions of the SMC were comparable with that of bethanechol chloride, a muscarinic receptor agonist. ANG II-induced contraction of SMC from LES and IAS was antagonized by preincubation of the SMC with genistein (2) and PD-98059 (12) (\( P < 0.05; n = 4 \) animals; Fig. 1). In control experiments, the basal lengths of the LES and IAS SMC were 54.6 ± 3.4 and 39.8 ± 2.5 μm, respectively. Genistein and PD-98059 by themselves had no significant effect on the basal lengths of the SMC. In the LES and IAS SMC, ANG II caused a 15.0 ± 2.1 and 27.0 ± 2.7% shortening that was subsequently antagonized to 5.0 ± 1.0 and 13.1 ± 1.8%, respectively, by genistein. Tyrophostin, another tyrosine kinase inhibitor, had effects similar to those found with genistein (data not shown). Results suggest that ANG II-induced contraction of LES and IAS SMC involves a tyrosine kinase signal transduction pathway leading to the activation of p44/42 MAPKs.

Relative distribution of phospho-p44/42 MAPKs in the basal state and after ANG II treatment in LES and IAS smooth muscles: effect of genistein and PD-98059. We examined the effect of ANG II on the levels of phosphorylated (phospho)-p44/42 MAPKs before and after the specific inhibitors of tyrosine kinase and p44/42 MAPKs. Western blots show the presence of phospho-p44/42 MAPKs in the basal state and an increase in their levels by ANG II treatment (Fig. 2). ANG II caused a two- to threefold increase in the levels of phosphorylation of p44 MAPK in the LES and IAS, respectively. Corresponding increases in phospho-p43 MAPK levels were approximately five- and threefold, respectively. Data further showed that ANG II-induced increases in phospho-p44/42 MAPKs were significantly attenuated by genistein and PD-98059 (\( P < 0.05; n = 4 \) animals). Genistein or PD-98059 alone had no effect on the basal levels of phospho-p44/42 MAPKs in these tissues (data not shown).

Effect of Hb A on ANG II-induced contraction of SMC from LES and IAS. Hb A is an irreversible and specific inhibitor of the src family of tyrosine kinases (50) and by itself had no significant effect on the length of the SMC from LES and IAS. ANG II-induced contraction of SMC from LES and IAS is comparable with that of bethanechol chloride. These data show that contraction of the SMC is significantly attenuated by the inhibitors of tyrosine kinase (genistein) and p44/42 mitogen-activated protein kinases (MAPKs) (PD-98059) (\( P < 0.05; n = 4 \) animals).

Effect of pp60-src and p120 rasGAP antibodies on ANG II-induced contraction of SMC from LES and IAS. Antibodies to pp60-src and p120 rasGAP do not penetrate the plasma membrane readily and require permeabilization of the SMC. We first determined that the permeabilization procedure by itself had no signifi-
significant effect on the SMC contraction caused by the agonists bethanechol chloride and ANG II (not significant (NS); P > 0.05; n = 4 animals; Figs. 4, A and B). ANG II and bethanechol chloride caused 18 ± 1.0 and 25 ± 2.1% shortening of the LES SMC in nonpermeabilized SMC, respectively. Corresponding values for the IAS SMC were 15 ± 1.4, 19 ± 2, and 22 ± 2.2% shortening, respectively.

Pretreatment of the permeabilized LES and IAS SMC with pp60c-src and p120 rasGAP antibodies caused significant antagonism of ANG II-induced contraction (P < 0.05; Fig. 4, C and D). Antibodies by themselves had no significant effect on the basal cell lengths of the SMC (NS; P > 0.05; Fig. 4, C and D). These data suggest the role of pp60c-src and p120 rasGAP in ANG II-induced contraction of the tonic smooth muscles of the LES and IAS.

Relative distribution of phospho-p120 rasGAP in the LES and IAS smooth muscles in the basal state and after ANG II. The presence of p120 rasGAP in the LES and IAS smooth muscles was determined first by Western blot studies with an appropriate antibody. Western blot studies utilizing anti-phosphotyrosine monoclonal antibody were carried out to determine the presence of tyrosine phospho-p120 rasGAP in rat LES and IAS, in the basal state, and after ANG II treatment. ANG II (1 × 10^-7 M) caused an increase in phospho-p120 rasGAP in both the LES and IAS tissues (Fig. 5). PD-98059, a specific inhibitor of p44/42 MAPKs, had no significant effect, but the tyrosine kinase inhibitor genistein caused an attenuation of ANG II-induced increase in the levels of p120 rasGAP phosphorylation in these smooth muscles.

Effect of genistein and PD-98059 on ANG II-induced phosphorylation of caldesmon in LES and IAS smooth muscles. Caldesmon, an actin-binding protein, negatively regulates actomyosin ATPase activity and smooth muscle contraction. Caldesmon phosphorylation by p44/42 MAPKs at serine residues relieves this inhibition. Western blots using an appropriate anti-
phosphoserine antibody showed the presence of both phosphorylated 87- and 150-kDa caldesmons in the LES and IAS smooth muscles in the basal state and their significant increase after ANG II (P < 0.05; Fig. 6). PD-98059 and genistein caused significant attenuation in ANG II-mediated increase in the levels of phosphorylated 87- and 150-kDa caldesmons (P < 0.05; Fig. 6). Data suggest the role of tyrosine phosphorylation signaling pathway in activating p44/42 MAPKs, which led to increased caldesmon phosphorylation. This may be partly responsible for the smooth muscle contraction after ANG II treatment.

DISCUSSION

These studies show that a part of ANG II-induced contraction of the SMC from the tonic smooth muscles of the LES and IAS is dependent on pp60^src and p120 ras GAP antibodies on ANG II-induced contraction of SMC from LES and IAS. Data show that permeabilization of SMC from LES (A) and IAS (B) has no significant effect on their ability to contract in response to different agonists [not significant (NS); P > 0.05; n = 4 animals]. Effect of pp60^src and p120 ras GAP antibodies on ANG II-induced contraction of SMC from LES and IAS are shown in C and D, respectively. Permeabilized SMC from LES and IAS were incubated with antibodies to pp60^src and p120 ras GAP for 1 h at room temperature followed by ANG II treatment for 5 min. Note that pp60^src and p120 ras GAP antibodies cause significant attenuation of LES and IAS SMC contraction by ANG II (°P < 0.05; n = 4). These antibodies alone caused no significant effect on the basal SMC lengths (data not shown).

ANG II-induced activation of pp60^src represents an important early upstream signaling event in the contraction of LES and IAS smooth muscles. This is evident from the findings that genistein, a tyrosine kinase inhibitor, and Hb A, an irreversible inhibitor of pp60^src causes attenuation of ANG II-induced contraction of LES and IAS SMC. In addition, pp60^src antibody causes significant attenuation of ANG II-induced contraction of the LES and IAS SMC. In addition, these treatments block intermediary events before smooth muscle contraction, such as ras GAP phosphorylation (monitored by p120^ras GAP phosphorylation at tyrosine residues), p44/42 MAPK activation (monitored by p44/42 MAPK phosphorylation), and phosphorylation of caldesmon at serine residues. Earlier studies from our laboratory show that ANG II-induced contraction of the sphincteric smooth muscle is mediated primarily via the activation of AT1 receptor, because it was antagonized by AT1 antagonist losartan (16). Our data are consistent with those in different smooth muscle systems, which suggest that the activation of the AT1 receptors by ANG II followed by activation of pp60^src represents a critical event in
the ras-related signal transduction pathway (14, 25, 26, 33, 43, 45). Involvement of p44/42 MAPKs in ANG II-induced contraction of the SMC was identified recently with the use of knockout mice and retrovirally transduced VSMC (known to express pp60c-src) (24). We speculate that stimulation of pp60c-src tyrosine kinase may set off activation of the ras-raf-MEK-MAPK signaling cascade responsible for a part of ANG II-induced contraction of rat LES and IAS smooth muscles. Activation of pp60c-src may play an important role in signal transduction mechanisms associated with Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent contraction of smooth muscles. In the Ca\(^{2+}\)-dependent pathway, G protein-coupled receptor (GPCR)-mediated activation of G\(_{\alpha}\) leads to an increase in intracellular Ca\(^{2+}\) concentration, which activates nonreceptor tyrosine kinases such as PYK2, a Ca\(^{2+}\)-sensitive proline-rich tyrosine kinase (42). PYK2 can associate with pp60c-src through src homology domain 3. Activation of PYK2 by ANG II in rat VSMC has been shown to promote increased PYK2-pp60c-src complex formation (42). This process combined with a series of additional steps leads to the activation of p44/42 MAPKs. In a Ca\(^{2+}\)-independent pathway, GPCR may couple to pp60c-src through \(\beta\)-arrestin 1, an adapter protein (35). This signaling process.

Fig. 5. Effect of PD-98059 and genistein on phospho-p120 rasGAP levels, in the LES and IAS smooth muscles, before and after ANG II. A: Western blots of these experiments. B: corresponding quantitative data by densitometric analyses. Western immunoblots of p120 rasGAP were prepared by using mouse monoclonal antibody to p120 rasGAP (primary antibody, 1:1,000) and anti-mouse IgG-HRP (secondary antibody, 1:1,000). Western immunoblots of phosphotyrosine p120 rasGAP were prepared by using mouse monoclonal anti-phosphotyrosine antibody (primary antibody, 1:1,000) and anti-mouse IgG-HRP (secondary antibody, 1:2,000). In these experiments SDS-PAGE was performed by using 7.5% running gels. ANG II causes a significant increase in the basal levels of phospho-p120 rasGAP (*\(P < 0.05; n = 4\) animals). Note a significant attenuation (\(\ast P < 0.05; n = 4\) animals) of ANG II-induced increase in p120 rasGAP phosphorylation (+\(P < 0.05; n = 4\) animals) by genistein, but not by PD-98059 [not significant (NS)].

Fig. 6. Effect of PD-98059 and genistein on phospho-caldesmon levels in the LES and IAS smooth muscles, before and after ANG II treatment. Western blots of such experiments with phosphorylated 87- and 150-kDa caldesmons are shown in A and C, respectively. Their corresponding densitometric analyses are given in B and D, respectively. Western immunoblots of caldesmon (both high and low caldesmons) were prepared by using mouse monoclonal antibody to caldesmon (primary antibody, 1:100) and anti-mouse IgG-HRP (secondary antibody, 1:2,000). ANG II causes a significant increase in the basal (control) levels of caldesmon phosphorylations (*\(P < 0.05; n = 4\) animals) that are significantly inhibited by PD-98059 and genistein (+\(P < 0.05; n = 4\) animals).
Fig. 7. Schematic diagram of signal transduction pathway involving pp60^src^ tyrosine (Tyr) kinase and p^{44/42} MAPK in ANG II-induced contraction of LES and IAS smooth muscles. Activation of AT_{1} receptor by ANG II causes an increase in intracellular Ca^{2+} concentration resulting in the activation of proline-rich tyrosine kinase-2 (PYK2) and pp60^src^. The latter causes increased tyrosine phosphorylation and complex formation with p^{44/42} rasGAP and p^{190} rhoGAP. Because present studies focused on a ras-related MAPK signal transduction pathway, only p^{120} rasGAP is followed here. The conversion of rasGDP to rasGTP is positively regulated by guanine exchange factors (GEFs). GAPs, on the other hand, downregulate ras. Tyrosine phosphorylation of p^{120} rasGAP by pp60^src^ may cause activation of ras by increased complex formation with other phosphoproteins such as p^{190} rhoGAP. This may downregulate rasGAP, turning off the negative regulation of ras. Activation of ras leads to activation of raf followed by the stimulation of MEK 1/2 and p^{44/42} MAPKs. This causes the phosphorylation of caldesmon, which is partly responsible for smooth muscle contraction. Pharmacological tools to block specific events are listed on the left side of the figure, and different parameters followed are represented on the right. ERK, extracellular signal-regulated kinase; MEK, MAPKK, MAPK kinase.

**Table:**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Intracellular Event</th>
<th>Functional Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Losartan</td>
<td>AT_{1} Receptor</td>
<td>SM and SMC contraction</td>
</tr>
<tr>
<td>Genistein or serotonin</td>
<td>Tyr. kinase (e.g., PYK2)</td>
<td>SMC contraction</td>
</tr>
<tr>
<td>Herbimycin A</td>
<td></td>
<td>SMC contraction</td>
</tr>
<tr>
<td>pp60^src^ antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rasGAP antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD 98059</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Legend**

- GEFs: Guanine exchange factors
- MAPKK: MAPK kinase
- MAPK (ERK1/2): Extracellular signal-regulated kinase
- Caldesmon phosphorylation: Phosphorylation of caldesmon
- SMC Contraction: Smooth muscle contraction

**Mechanism**

Mechanism was recently shown to be involved in the activation of ras and p^{44/42} MAPKs. In a Ca^{2+}-independent pathway, the requirement for Ca^{2+}-dependent stimulation of pp60^src^ and of ras may be circumvented by the protein kinase C (PKC)-mediated activation of raf, which subsequently causes a direct activation of p^{44/42} MAPKs (7). Ca^{2+}-independent contraction of differentiated smooth muscles by phenylephrine was shown recently to involve activation of p^{44/42} MAPKs (10). PKC-activator phorbol 12-myristate 13-acetate can stimulate MAPKs without activating ras in VSMC (39). The present studies were carried out under normal Ca^{2+} conditions and do not address the issue of the Ca^{2+}-independent pathway in MAPK activation. However, our earlier experiments have shown that ANG II-mediated contraction of the tonic smooth muscles uses both Ca^{2+}-dependent and Ca^{2+}-independent pathways. Furthermore, such contraction, in part, also utilizes the PKC pathway (16). In addition, whether ANG II-mediated influx of Ca^{2+} and PKC activation contribute to PYK2 and pp60^src^ stimulation in the tonic smooth muscles remains to be determined. It is possible that activation of extracellular-regulated MAPKs by ras in signal transduction mechanisms involving GPCRs may be tissue and stimulus specific.

Another novel finding of this investigation is that ANG II-induced contraction of the LES and IAS smooth muscles is accompanied by an increase in the levels of tyrosine phosphorylation of p^{120} rasGAP. Genistein, but not PD-98059, attenuated these increases in p^{120} rasGAP phosphorylations. These findings highlight the fact that genistein and PD-98059 work only at specific steps of the ras-related pathway. Data show also that p^{44/42} MAPK activation is downstream from p^{120} rasGAP. In addition, p^{120} rasGAP antibody causes specific inhibition of ANG II-induced contraction of LES and IAS SMC.

Tyrosine phosphorylation of p^{120} rasGAP may be an important step between pp60^src^ activation and ras activation (11, 15, 37, 47). Change of rasGDP (inactive form) to rasGTP (active form) is critical for ras activation in the ras-related pathway. Guanine exchange factors (GEFs) and GAPs are also known to play important roles in this regulation. GEFs serve as positive regulators for the conversion of rasGDP to rasGTP, whereas activation of p^{120} ras GTPase serves as a negative regulator by an increase in the rate of hydrolysis of rasGTP to rasGDP. Tyrosine phosphorylation of p^{120} rasGAP may lead to sustained activation of ras and then to MAPKs via multiple mechanisms, such as increased complex formation with other phosphoproteins, such as p^{60} and p^{190}, and rasGAP downregulation (11, 15, 37, 47).
It is known that in stimulated cells, the low intrinsic level of GTPase activity of ras is enhanced by p120 rasGAP, which may act as a vectorial manager (4). Both p120 rasGAP and p190 rhoGAP are substrates for phosphorylation by pp60c-src (30, 43). Our data are consistent with previous data demonstrating that pp60c-src antibody causes an attenuation of ANG II-mediated tyrosine phosphorylation of p120 rasGAP and contraction in rat VSMC (43). In addition, cells transformed with cytoplasmic (avian v-src) and receptor-like tyrosine kinases show the presence of an immune complex containing tyrosine-phospho-p120 rasGAP and p190 rhoGAP (15). Our data support the hypothesis that p120 rasGAP is a regulator of rasGTPase activity and that ras transmits signals from pp60c-src tyrosine kinases to serine/threonine kinases (p44/42 MAPKs) (27, 32, 43).

ANG II-mediated activation of p44/42 MAPKs plays an important role in ANG II-induced contraction of tonic smooth muscles of LES and IAS. The suggestion is based on the following observations. First, ANG II causes increases in the basal levels of phospho-p44/42 MAPKs in these smooth muscle tissues. Second, tyrosine kinase inhibitor genistein (2) and a specific inhibitor of MEK 1/2, PD-98059 (12), attenuate ANG II-induced increases in phospho-p44/42 MAPKs. Third, an increase in phospho-p44/42 MAPKs levels is associated with ANG II-induced contraction of the LES and IAS SMC. The exact role of pp60c-src and p44/42 MAPKs in ANG II-induced contraction of adjoining phasic smooth muscles of the esophagus and rectum are difficult to ascertain, because ANG II does not produce a reproducible contraction of these tissues in most of the species examined (40). Our data with PD-98059 are similar to those obtained in other smooth muscles and SMC (10, 48). Investigations of the cause and effect relationship between smooth muscle contraction and activation of p44/42 MAPKs have been carried out only recently (10, 52).

Although phosphorylation of myosin light chain (20 kDa) (MLC20-P) is the major determinant of smooth muscle contraction after ANG II treatment in different smooth muscles, phosphorylation of other contractile proteins such as caldesmon may also play an important role under certain conditions (5, 9, 22, 23, 31, 34). Caldesmon is an effector of p44/42 MAPKs and inhibits the ATPase activity of the actomyosin complex in smooth muscles (31). Phosphorylation of caldesmon may promote smooth muscle contraction by relieving inhibition of the ATPase activity (10, 18, 31). Our data support this hypothesis because ANG II causes significant increase in caldesmon phosphorylation. In addition, genistein and PD-98059 cause significant attenuation of LES and IAS SMC contraction as well as that of caldesmon phosphorylation. Two types of caldesmon, low-molecular-mass caldesmon (l-caldesmon; 87 kDa) (29), and high-molecular-mass caldesmon (h-caldesmon; 140–150 kDa) (53), have been shown to be present in the smooth muscles. Our data suggest that activation of p44/42 MAPKs causes an increase in phosphorylation of l- as well as h-caldesmon in ANG II-induced contraction of LES and IAS smooth muscles.

The present studies focus primarily on the role of the ras-related MAPK pathway in ANG II-induced contraction of LES and IAS smooth muscle. However, multiple intracellular pathways such as phospholipase C, phospholipase D, protein kinase C, Ca2+-calmodulin-myosin light-chain kinase, MAPKs other than p44/42, rho kinase, and changes in ion channel activation may also contribute to the smooth muscle contractions by ANG II. The contribution of these pathways and their interactions with ras-related activation of MAPK after ANG II-induced contraction of the LES and IAS smooth muscles is not known. It also remains to be determined whether the proposed pathway for the partial contraction of ANG II is applicable to other smooth muscle contractile agents such as muscarinic agonists. In different smooth muscles, there appears to be an overlap of signal transduction pathways involved in contraction by muscarinic receptor activation (e.g., by betahanechol chloride and carbachol) and ANG II (3, 13, 19, 49).

In summary, data suggest an important role of pp60c-src tyrosine kinase in the activation of ras and p44/42 MAPKs in ANG II-induced contraction of LES and IAS smooth muscles. In addition, LES and IAS smooth muscles are found to have significant levels of phosphorylated p44/42 MAPKs, p120 rasGAP, and caldesmon in the basal state. Our findings support the notion that the ras/pp60c-src/MAPK pathway is involved in the agonist-stimulated as well as in the basal tone of the smooth muscles. Identification of intracellular mechanisms in the contraction of LES and IAS smooth muscles in the basal state and after contractile neuropeh- moral agonists will provide important information on the regulation and modulation of these tonic smooth muscles. This information may be vital in the understanding of the pathophysiology and therapeutic rationale for gastroesophageal and anorectal motility disorders.

We thank Dr. John J. Gartland for editing the manuscript. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-35385 and by an institutional grant from Thomas Jefferson University.

REFERENCES


46. Timmermans PBMWM, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RD, Wexler RR, Saye JAM, and


