Progesterone receptor A mediates VIP inhibition of contraction

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Cheng L., Biancani P., Behar J. Progesterone receptor A mediates VIP inhibition of contraction. Am J Physiol Gastrointest Liver Physiol 298: G433–G439, 2010. First published December 17, 2009; doi:10.1152/ajpgi.00346.2009.—The slow transit time of the colon in females with constipation is due to impairment of agonist-induced contraction. The impairment is associated with downregulation of G proteins that mediate contraction and upregulation of Gs proteins that mediate relaxation. These changes are caused by overexpression of progesterone (P4) receptors in the colon, rendering its muscle cells sensitive to physiological P4 concentrations. Downregulation of Gq/11 is mediated by P4 receptor B (PR-B). We examined whether upregulation of Gs proteins increases the inhibition of contraction and whether the increase is mediated by the P4 receptor A (PR-A). These studies were conducted in colon-isolated colon muscle cells from human control and slow-transit constipation (STC) females and from guinea pigs. Muscle cell contraction was induced by CCK-8. Inhibition of contraction was induced by vasoactive intestinal polypeptide (VIP), and 8’bromo-c’AMP (8B-c’AMP) G protein levels were determined by Western blot. VIP-induced inhibition of contraction was greater in muscle cells from STC and P4-treated muscle cells. There were no differences in the inhibition induced by 8B-c’AMP between muscle cells from STC and P4-treated controls. The increased VIP-induced inhibition of muscle cells treated with P4 was blocked by pretreatment with PR-A antibodies and unaffected by PR-B antibodies. These antibodies had no effect on 8B-c’AMP induced-inhibition. The P4 upregulation of Gs proteins was blocked by PR-A antibodies and unaffected by PR-B antibodies. Similar results were obtained in muscle cells from guinea pig colons. We concluded that P4 upregulation of Gs proteins increases VIP-induced inhibition of contraction mediated by PR-A.

G proteins; vasoactive intestinal polypeptide

FEMALE PATIENTS WITH IDIOPATHIC constipation have prolonged transit times as determined by measurements with sitz markers or with radioisotopes using the γ camera (9, 35). These patients have an impaired muscle response to agonists that stimulate G protein-coupled receptors and impaired basal motility index, probably caused by abnormal prostaglandin levels. In contrast, the contraction of these muscle cells is normal when they are stimulated by G protein-independent agonists such as second messengers (diacylglycerol or inositol trisphosphate) and by KCl (35). Progesterone (P4) treatment reproduced these motility abnormalities in normal human and guinea pig muscle cells and strips from the colon and other gastrointestinal smooth muscle cells in vitro and in vivo (4, 7, 9, 25, 35). The decreased motility and slow transit of the gastrointestinal tract also occur in several pregnant animal species (1, 2, 11, 26, 27, 28, 29, 30). In addition, the G protein patterns occur in the myometrial muscle during pregnancy (1, 14, 15, 19, 34).

The impaired contraction of muscle cells from the colon of female patients with slow-transit constipation (STC) is due to the downregulation of G proteins that mediate contraction (Gq/11 and Gi3 proteins) (35). Treatment of muscle cells with P4 replicates these G protein patterns with downregulation of Gq/11 and Gi3 proteins (9, 35). The downregulation of Gq/11 appears to be responsible for the impaired contraction in response to CCK and ACh (7). These proteins play an important role in receptor internalization, intracellular trafficking, and receptor binding of agonists (30, 32), explaining the decreased binding of CCK-8 to CCK-1 receptors in muscle cells from the colon of females with STC or to normal colon muscle cells treated with P4 (35).

Moreover, muscle cells from the colon of females with STC also exhibit an upregulation of Gs proteins that mediate the relaxation of tonic muscles or inhibition of contraction of phasic muscles induced by inhibitory agonists that stimulate G protein-coupled receptors (9, 35). P4 treatment of normal muscle cells from normal colon also reproduces this upregulation of Gs proteins. However, whether the upregulation of Gs proteins translates in an increase in the relaxation or inhibition of contraction in muscle cells from females with STC or of normal muscle cells treated with P4 is not known.

Thus it appears that P4 causes two quite different effects on G proteins, downregulation of G proteins that mediate contraction and upregulation of G proteins that mediate inhibition of contraction. P4 activates two subnuclear receptors or transcription factors, raising the possibility that each of these two P4 actions may be mediated by a different P4 receptor. This hypothesis is supported by several studies performed in a variety of normal and dysplastic cells showing that P4 receptor A (PR-A) and PR-B have different functions (8, 16, 23, 32). We have shown that PR-B is responsible for downregulating Gq/11 because P4 receptor transfection of normal colon muscle cells increases their sensitivity to P4-reduced Gq/11 protein expression and impaired contraction (8). Overexpression of the PR-B, however, does not affect the P4 actions on Gs proteins that mediate relaxation.

It is therefore conceivable that 1) the PR-A mediates the upregulation of Gs proteins and 2) because G proteins contribute to normal receptor binding and recycling, upregulation of Gs proteins may result in increased response to inhibitory neurotransmitters such as vasoactive intestinal polypeptide (VIP) that stimulate Gs protein-coupled receptors. The increased response to inhibitory neurotransmitters could cause greater inhibition of contraction in phasic muscle cells and conceivably prolong colonic transit time in females with STC and in animals treated with P4. The following studies were performed in colon muscle cells from the colon of normal females and from STC and from the guinea pig colon.
Gassed with 100% O2 during the tissue digestion. At the end of the digestion process, the tissue was filtered through Nitex mesh 200 (Tetko, Elmsford, NY) and rinsed with 20 ml of HEPES. The tissue remaining on the filter was collected and incubated in HEPES buffer at 35°C for 15 min before the experiment. The modified cytosolic buffer was prepared with cytosolic buffer plus 1.5 mM ATP, 5 mM creatine phosphate, 10 U/ml of creatine phosphokinase, and 10 μM antimycin A.

**Studies of contraction of dissociated muscle cells.** Muscle contraction was measured as previously described in intact and permeable cells (3, 9, 18, 33, 34, 35). Permeable cells were used to study the effect of antibodies against G proteins (Gq/11, Gi3, Gi1/2, Gs) and then fixed in acrolein at 1% final concentration (23). The cell length was measured with a phase-contrast microscope (Carl Zeiss, Jena, Germany) and a closed circuit television camera (Panasonic, Secaucus, NJ) connected to a Macintosh Computer with NIH Image software. The average length of 30 cells, measured in the absence of agonists, was taken as the control length and compared with length measured after addition of agonists. Shortening was defined as the percent decrease in the average length of 30 cells after treatment with agonists compared with the control length. Contraction was measured after cells were exposed to buffer alone (controls) or treated with P4 for 6 h alone or pretreated with PR-A or PR-B antibodies for 1 h before treatment with P4. Muscle contraction was induced by CCK-8 10^{-5} M treatment for 30 s.

Inhibition of contraction of dissociated muscle cells was measured in intact and permeable muscle cells by determining the effect of inhibitors on cell length using a method previously reported (3, 5, 6). Single muscle cells were initially incubated with VIP 10^{-6} M for 60 s followed by 10^{-6} M L-a-1,2-dioctanoyl glycerol (DOG) for 30 s, after which the cells were fixed with 1% acrolein. DOG (10^{-6} M) causes maximal contraction in intact and permeable smooth muscle cells from the colon of guinea pigs. Cell lengths were measured by scanning micrometry using phase contrast microscopy. Inhibition of contraction was expressed as percent inhibition of DOG-induced contraction. The experimental conditions of these studies were buffer alone or P4 treatment for 6 h alone or after treatment with PR antibodies for 1 h.

**Materials and Methods**

**Human patients.** This study was approved by the IRB of the Rhode Island Hospital. Females with STC, age 18–45 yr, with a history of constipation of 2 yr or longer were included in this study (9, 34). Constipation was defined by the Rome 2 criteria (3 bowel movements or less a week). The patients required the use of laxatives to have bowel movements. Patients with constipation-predominant irritable bowel syndrome as defined by the Rome 2 criteria were excluded (21). Patients with neurological and collagen disease, pseudo-obstruction, or a history of using constipating (opiates, calcium blockers) or psychotropic drugs (tricyclics or selective serotonin reuptake inhibitors) were also excluded. Unless they had a hysterectomy, premenopausal women were required to have a negative pregnancy test and abstain from contraceptives or sex hormones for at least 2 wk before the studies were performed. Patients with STC had a prolonged gastrointestinal transit time measured with radio-opaque markers on a regular diet and without probiotic drugs or laxatives (5 days or longer). They had a normal colonoscopy and no evidence of pelvic dysinnegria by digital exam and rectal motility. Normal female patients had normal bowel patterns and no history of constipation, gastroparesis, diabetes, or neurological or collagen disorders (e.g., scleroderma). They underwent segmental colectomy (descending-sigmoid colon) because of adenocarcinoma of the colon. Patients were not taking calcium blockers or anticholinergic, tricyclic, or selective serotonin reuptake inhibitor drugs. Clinical data of patients are included in Table 1.

**Animal type.** Male guinea pigs (weight 450–500 g) were purchased from the Charles River Laboratory (Wilmington, MA). The animal Welfare Committee of Rhode Island Hospital approved their use. Animals were housed in thermoregulated rooms with free access to food and water. After an overnight fast, the guinea pigs were sedated with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and euthanized by sodium pentobarbital (30 mg/kg ip). Guinea pig colons were promptly removed, rinsed with ice-cold, modified oxygenated Krebs solution, and placed in a dissecting pan containing the same solution continuously aerated with 95% O2-5% CO2. The colon was kept in ice-cold modified oxygenated Krebs’s solution (116.6 mM NaCl, 3.4 mM KCl, 21.9 mM NaHCO3, 1.2 mM NaH2PO4, 2.5 mM CaCl2, 1.2 mM MgCl2, 5.4 mM glucose).

**Isolation and permeabilization of colon muscle cells.** Muscle cells were isolated, and, in some experiments, they were permeabilized using methods described previously (3, 9, 10, 12, 17, 20, 22, 34, 37). For these studies, the circular muscle layer of the descending-sigmoid colon from humans and guinea pigs was dissected from the mucosa, submucosa, and longitudinal and serosal layers under a dissecting microscope. It was then cut into 2-mm-wide strips and digested in HEPES buffer containing 0.5 mg/ml type F collagenase and 2 mg/ml papain (activity of 13.9 U/mg protein) for 20 min at 35°C in a shaking water bath. The buffer was gently gassed with 100% O2 during the tissue digestion. At the end of the digestive process, the tissue was filtered through Nitex mesh 200 (Tetko, Elmsford, NY) and rinsed with 20 ml of HEPES. The tissue remaining on the filter was collected and incubated in HEPES buffer at 35°C for 15 min to allow free dispersion of cells.

For preparation of permeable cells, the partly digested muscle cells were washed with “cytosolic buffer,” a medium with the following composition (in mM): 20 NaCl, 100 KCl, 25 NaHCO3, 0.96 mmol/L NaH2PO4, 0.48 CaCl2, 5.0 MgSO4, and 1.0 EGTA, pH 7.2, 2% bovine serum albumin. It was maintained by equilibrating with 95% O2-5% CO2 at 31°C. The dispersed cells were exposed to saponin (75 μg/ml) and then centrifuged at 200 g for 3 min. Cells were washed once with modified cytosolic buffer by centrifugation and resuspended in modified cytosolic buffer and equilibrated at 31°C for 15 min before the experiment. The modified cytosolic buffer was prepared with cytosolic buffer plus 1.5 mM ATP, 5 mM creatine phosphate, 10 U/ml of creatine phosphokinase, and 10 μM antimycin A.

**Table 1. Clinical data of controls and patients with STC**

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>STC (32–47)</th>
<th>Controls (44–56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bowel movements/week</td>
<td>&lt;3/week</td>
<td>1–2/day</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>mild</td>
<td>absent</td>
</tr>
<tr>
<td>GI transit time</td>
<td>&gt;5 days</td>
<td>—</td>
</tr>
<tr>
<td>Laxative therapy</td>
<td>poor response</td>
<td>none</td>
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STC, slow-transit constipation.
**Western blot analysis.** Cell lysates prepared in M-PER mammalian protein extraction buffer (cat. no. 78503; Pierce, Rockford, IL). The supernatants obtained after centrifuging the samples at 12,000 g for 15 min at 4°C were used for Western blot analysis (9, 35). Protein concentration was measured by colorimetric analysis (Bio-Rad, Melville, NY) according to the method of Cheng et al. (8). Proteins were fractionated by SDS-PAGE, transferred to PVDF transfer membrane (PerkinElmer Life and Analytical Sciences, Boston, MA), and nonspecific binding sites were blocked with Superblock-TBS (Pierce). The membranes were then incubated with primary antibody overnight at 4°C with gentle agitation. The immunoreactivity was detected using horseradish peroxidase-conjugated IgG (Pierce), the enhanced chemiluminescence kit (Amersham International, Buckinghamshire, UK), and film autoradiography. Immunoreactivity was quantified using Kodak Digital Science Image Station.

**Chemicals.** P4, VIP, 8′bromo-c′AMP (8B-c′AMP), (16), and G protein subunit antibodies were purchased from CytoSignal (Irvine, CA), and antibodies against PR-A and PR-B were from Cayman Chemical (Ann Arbor, MI) (13).

**Statistics.** Statistical analysis was performed using one- and two-factorial repeated ANOVA comparing values between controls and P4-treated muscle cells with or without antibodies. A P value of <0.05 was considered significant. Previous studies using similar treatments had shown that significance could be achieved using three to four controls and experimental samples.

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**RESULTS**

Before these studies, we determined the levels of the protein expression of the PR-A in the tissues from females with STC and controls that were being studied. The Western blot analysis showed that the optical density (OD) of the PR-A/β-actin ratio was higher in STC than in control females (Fig. 1).

We next examined whether the inhibition of contraction induced by VIP, an agonist that stimulates a G protein-coupled receptor, was increased in female patients with STC who have an overexpression of P4 receptors and whether this P4 effect is mediated by the P4 subnuclear PR-A. We then examined the effects of VIP-induced inhibition of contraction in muscle cells from control and STC females. Figure 2A shows that a higher inhibition of contraction induced by VIP (10^-6 M) occurred in STC than in control females. There was also a higher inhibition of contraction induced by VIP in muscle cells treated with P4 than in control muscle cells exposed to buffer alone (Fig. 2B). In contrast, there were no statistically significant differences in the inhibition of contraction induced by 8B-c′AMP between muscle cells from STC or muscle cells treated with P4 compared with control muscle cells (Fig. 2, A and B).

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**Fig. 2.** Inhibition of contraction induced by vasoactive intestinal polypeptide (VIP) (10^-6 M) and 8′bromo-c′AMP (8B-c′AMP) (10^-4 M) in muscle cells from the colon of control females and females with STC. A: inhibition of contraction induced by VIP was greater in muscle cells from STC than from controls (P < 0.01 by ANOVA). There are no significant differences in the 8B-c′AMP-induced inhibition of contraction between the two groups of female subjects. B: effect of P4 (10^-5 M) on the inhibition of contraction induced by VIP and 8B-c′AMP in muscle cells from normal human colons. P4 increased VIP-induced inhibition of contraction (**P < 0.01 by ANOVA**) but had no effect on the inhibitory actions of 8B-c′AMP. Values are means ± SE of n = 4.

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**Fig. 3.** Effect of VIP (10^-6 M) on c′AMP levels in muscle cells from the colon of females with STC and in muscle cells from normal human colons treated with P4. A: VIP increased the mean basal levels of c′AMP in cells from control and females with STC. These c′AMP levels induced by VIP are higher in females with STC than in control females (**P < 0.005 by ANOVA**). B: levels of c′AMP levels induced by VIP are higher in females with STC than in control females (**P < 0.005 by ANOVA**). Values are means ± SE of n = 3.
We also examined the effect of VIP on the levels of c’AMP in control muscle cells and in muscle cells from females with STC. VIP relaxes and inhibits contraction of muscle cells mostly by increasing the levels of c’AMP (18). Figure 3A shows that VIP induced a greater increase in the c’AMP levels in muscle cells from females with STC compared with control muscle cells. Likewise, the levels of c’AMP induced by VIP were greater in muscle cells treated with P4 compared with controls (Fig. 3B).

We next examined whether the increased inhibition of contraction and c’AMP levels induced by VIP in muscle cells treated with P4 were mediated by a specific P4 receptor. Figure 4A shows that pretreating these muscle cells from normal human colon with antibodies against PR-A blocked increased inhibition of contraction induced by VIP. In contrast, antibodies against PR-B had no effect. Neither antibody, however, had any effect on the inhibition of contraction induced by 8B-c’AMP (Fig. 4B).

Similar results were obtained for VIP-induced c’AMP levels induced by VIP in muscle cells pretreated with P4. VIP-induced increase in c’AMP levels were higher in P4-treated muscle cells compared with buffer-treated muscle cells. This increase induced by P4 was blocked by pretreating these muscle cells with antibodies against PR-A (Fig. 5). Antibodies against PR-B had no effect on the P4-induced increase in c’AMP levels caused by VIP stimulation.

We have shown that the likely effects of P4 on muscle contraction and relaxation (inhibition of contraction in phasic muscles) is the result of P4 actions on G proteins, resulting in downregulation of Gq/11 and upregulation of Gs proteins. We, therefore, examined whether PR-A and PR-B differentially mediate these effects. Western blot analysis shows that P4 upregulates Gs proteins that were blocked by pretreating these cells with antibodies against PR-A (Fig. 6). Pretreatment of these muscle cells with antibodies against PR-B had no effect on P4-induced upregulation of Gs proteins.

Antibodies against PR-A had no effect on P4-induced downregulation of Gq/11 proteins by Western blot analysis (Fig. 7). In contrast, P4 reduced the OD/β-actin ratio in buffer-treated cells (controls) after P4 treatment. The downregulation of Gq/11 induced by P4 was unaffected by pretreatment with P4.
PR-A antibody, but it was blocked by PR-B antibody, maintaining similar levels to those of controls.

We then examined the CCK-8-induced contraction because it is mediated by Gq/11 proteins. The CCK-8-induced percentage of shortening in buffer-treated muscle cells of 24.2 ± 3.5% was reduced to 12.4 ± 1.9% after 6 h treatment with P4 (10^{-5} M, P < 0.001 by ANOVA). P4 reduction of the percentage of shortening induced by CCK-8 in buffer-treated cells was blocked by pretreating the cells with antibodies against PR-B (CCK8-induced contraction was 24.2 ± 3.5% after buffer and 22.1 ± 2.9% after PR-B antibody treatment). In contrast, pretreatment with PR-A antibody had no effect on the P4 inhibition of the CCK-8-induced shortening of 8.4 ± 3.1%.

Similar studies were undertaken in muscle cells from the colon of guinea pigs. Figure 8 shows that the VIP inhibition of contraction was greater in P4-treated muscle cells than in control muscle cells (Fig. 8A). These actions of P4 were antagonized by pretreating these muscle cells with antibodies against PR-A. Antibodies against PR-B had no effect on the actions of P4. In contrast, neither P4 nor antibodies against PR-A had any effect on 8B-c’AMP-induced inhibition of contraction (Fig. 8B).

Further studies were performed in muscle cells from the guinea pig colon to examine the effects of P4 on c’AMP levels induced by VIP. P4 increased the levels of c’AMP induced by VIP compared with control muscle cells treated with buffer (Fig. 9). This action was antagonized by pretreating muscle cells with antibodies against PR-A. Antibodies against PR-B had no effect on the P4-enhanced actions on the VIP-induced increased levels of c’AMP.

Moreover, the effects of P4 on G proteins in muscle cells from the guinea pig colon were similar to those found in human colon muscle cells. P4 upregulated the levels of Gs proteins. This effect was blocked by antibodies against PR-A, but it was unaffected by PR-B (Fig. 10). In contrast, antibodies against PR-B blocked the downregulation of Gq/11 induced by P4. It was not antagonized by pretreating muscle cells with antibodies against PR-A (Fig. 11).

Similar results were obtained in guinea pig colons when muscle cell contraction was induced by CCK-8 pretreated with buffer or P4 for 6 h. P4 lowered the percentage of muscle shortening induced by CCK (control 21.7 ± 4.2% vs. 12.7 ± 2.9% after P4, P < 0.001, n = 3). Antibodies against PR-B (CCK8-induced contraction was 24.2 ± 3.5% after buffer and 22.1 ± 2.9% after PR-B antibody treatment). In contrast, pretreatment with PR-A antibody had no effect on the P4 inhibition of the CCK-8-induced shortening of 8.4 ± 3.1%.
Antibodies against PR-A had no effect (percentage of shortening after PR-A/H11001 P4/H11001 CCK-8 was 14.1/H11006 2.0% was compared with 20.9/H11006 3.8% after PR-B antibody/H11001 P4/H11001 CCK-8, P/H11021 0.001, n/H11005 3).

**DISCUSSION**

These studies show that muscle cells from females with STC exhibit an increased inhibition of contraction induced by VIP compared with controls. In contrast, there were no differences in the inhibition of contraction caused by 8B-c'AMP. P4 treatment of normal muscle cells from human and guinea pig colons reproduced these findings. VIP acts on membrane receptors that couple with Gs proteins, whereas 8B-c'AMP, a second messenger, bypasses G proteins, inhibiting muscle contraction by stimulating PKA, one of the inhibitory pathways (18). The increased inhibition of contraction in muscle cells from STC or in muscle cells treated with P4 is associated with upregulation of Gs proteins and increased c'AMP levels. Moreover, pretreatment with antibodies against PR-A blocked the increased inhibition of contraction and the higher levels of c'AMP induced by VIP in muscle cells treated with P4. In contrast, antibodies against PR-B had no effect on the VIP-induced inhibition of contraction. We had previously shown that PR-B mediates the P4-induced downregulation of Gq/11 and the impaired contraction induced by CCK-8 (8).

The increased inhibition of contraction in muscle cells from STC and P4-treated muscle cells is likely to be attributable to upregulation of Gs proteins. It is possible that the upregulation of these G proteins increases receptor affinity to their respective ligands. Stimulation or inactivation of G proteins abolishes the binding of radio-labeled ligands but has no effect on receptor antagonists (33, 35). Downregulation of Gq/11 induced by P4 results in significant decreases in CCK binding to CCK-1 receptors (34). Thus the levels and functional integrity of G proteins appear to play an important role in the complex process of internalization and recycling of receptors. Normal recycling of receptors to the plasma membrane determines the number of receptors available for ligand binding and cell response. It is therefore possible that the upregulation of Gs proteins increases the binding of VIP to its receptors, resulting in greater inhibition of contraction. Binding studies will be needed using radio-labeled VIP to demonstrate this hypothesis.

P4 stimulates two subnuclear receptors, PR-A and PR-B, that act as transcription factors (13). These two receptors mediate different effects in normal and in dysplastic cells. The
presence of these two receptors in muscle cells explains the finding that P4 induces the downregulation of one group of G proteins (Gq/11 and Gi3) and upregulation of Gs proteins. These findings therefore support the growing evidence of the functional differentiation of these two receptors. Receptor A may mediate the upregulation of Gs proteins, resulting in increased inhibition of contraction by inhibitory neurotransmitters that act on Gs protein-coupled receptors, whereas down-regulation of Gq/11 and Gi3 reduces binding of excitatory ligands that act on G protein-coupled receptors, resulting in decreased contraction induced by CCK and ACh.

In summary, muscle cells from the colon of female patients with STC have an increased response to inhibitors like VIP that stimulate G protein-coupled receptors compared with muscle cells from control females. Whether this increased inhibition of contraction by inhibitory neurotransmitters like VIP contributes to the longer transit time in the colon that occurs in these patients with STC will require further studies in experimental animals treated in vivo with P4. It is conceivable that the inhibition of contraction in the peristaltic reflex in these patients may still be normal. Other neurotransmitters like nitric oxide that do not activate G protein-coupled receptors may play a more important role in the inhibition of contraction than VIP in muscle cells from the colon.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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