Effects of progesterone on motility and prostaglandin levels in the distal guinea pig colon

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Xiao ZL, Biancani P, Behar J. Effects of progesterone on motility and prostaglandin levels in the distal guinea pig colon. Am J Physiol Gastrointest Liver Physiol 297: G886–G893, 2009. First published September 17, 2009; doi:10.1152/ajpgi.00184.2009.—Progesterone (P4) inhibits the gastrointestinal muscle contraction by downregulating Gq/11 proteins that mediate contraction, by upregulating Gs proteins that mediate relaxation, and by altering the pattern of cyclooxygenase (COX) enzymes and prostaglandins. We aimed to examine whether P4 treatment of guinea pigs in vivo affects basal colon motility [basal motility index (MI)] by altering the levels and actions of PGF2α, and PGE2. Guinea pigs were treated with intramuscular (IM) P4 for 4 days. The BASAL MI, the PGF2α-induced contraction, and PGE2-induced inhibition of contraction were examined in muscle strips and cells. The levels of PGF2α, and PGE2 were measured by radioimmunoassay. Treatment with P4 reduced the basal MI, the levels of PGF2α, and PGE2-induced contraction. P4 increased PGE2 levels, and PGE2 induced relaxation. Pretreatment with IM RU-486 (10 mg/kg per day), a P4 receptor antagonist, 1 h before P4 blocked the actions of P4. The PGF2α antagonist AI-1180 abolished basal MI and PGE2-induced contraction. N-ethylmaleimide, which blocks uncoupled membrane receptors, blocked Ach and VIP actions but had no effect on PGF2α and PGE2 effects. A COX-1 inhibitor decreased and a COX-2 inhibitor increased PGF2α levels; GTPyS increased and GDPBS decreased the levels of PGF2α. Gq/11 protein antibodies (Abs) reduced PGF2α levels, and Gs3 Abs blocked its motor actions. Gas Abs increased PGF2α, but decreased PGE2 levels. We concluded that P4 decreases basal MI by reducing PGF2α, levels caused by downregulation of Gq/11 and that PGE2-induced contraction was blocked by downregulating Gs. P4 also decreased the basal MI by increasing PGE2 levels, and PGE2 induced relaxation by upregulating Gq, proteins.

MATERIALS AND METHODS

Animals. Male guinea pigs (weighing 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. For the studies on the effects of P4, guinea pigs were selected at random and injected with P4 (2 mg/kg body wt) or equivalent volume of saline daily for 4 days. They were euthanized on the fifth day. After an overnight fast, the guinea pigs were sedated with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and euthanized by pentobarbital (30 mg/kg ip). Guinea pig colons were promptly removed, rinsed with ice-cold 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, and 5.4 mM glucose). The mucosa and serosa were carefully peeled off under a dissecting microscope. The circular muscle layer of the colon was further cleaned by gently removing the remaining connective tissue.

Isolation and permeabilization of colon muscle cells. Muscle cells were isolated, and in some experiments they were permeabilized using methods described previously (3, 5, 7, 9, 17, 33). The circular muscle layer was cut into 2-mm-wide strips and digested in HEPES buffer treated with P4 and during gestation that are presumably induced by rising P4 levels in the last two trimesters of pregnancy (1, 14, 15, 19, 29, 30). Moreover, the basal motility index (MI) of colon muscle strips from patients with STC is lower than that of muscle strips from control females without constipation (10). The reduction of basal MI abnormalities in patients with STC is associated with changes in cyclooxygenase (COX) enzymes and prostaglandin levels. In the muscle strips from these patients, the levels of COX-1 enzyme and of excitatory prostaglandins such as thromboxane A2 (TXA2) are reduced. In contrast, the levels of COX-2 enzymes and of inhibitory PGE2 levels are increased. Prostaglandins such as TXA2 and PGE2 contract muscle cells in the colon circular muscle layer generated by COX-1, and PGE2 relaxes muscle cells generated by COX-2. These COX enzymes and prostaglandin abnormalities are reproduced in normal muscle cells treated in vitro with P4 (10−5 M) for 6 h. However, it is not known whether changes in prostaglandins contribute to the genesis of the basal MI and whether these changes induced by P4 affect the contractility of the colon. The role of prostaglandins in the genesis of tonic and phasic contractions has been reported in different gastrointestinal muscle cells (4, 11).

The present studies were therefore aimed at determining the effects of the in vivo P4 treatment on basal MI of guinea pig colon and examining whether these actions are mediated by prostaglandins because of their role in the genesis of the colon motor functions.

WE HAVE SHOWN THAT COLON MUSCLE cells from female patients with slow-transit constipation (STC) have signal transduction abnormalities that are reproduced in normal human colon muscle cells treated in vitro with progesterone (P4) (10, 31). P4 inhibits the contraction of colon and of other gastrointestinal smooth muscle cells (5, 8, 12, 23, 26). These muscle cells have an impaired response to agonists that are G protein-coupled receptors such as CCK-8, ACh, and GTPyS (8, 28). The motility abnormalities of female patients with STC and of P4-treated normal muscle cells may be due to down-regulation of Gq/11 and upregulation of Gs proteins (10, 31). This conclusion is supported by findings that both types of colon muscle cells respond normally to receptor and G protein-independent agonists such as KCl and diacylglycerol. Similar G protein abnormalities have been reported in muscle cells...
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 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 NaH2PO4, 0.48 CaCl2, 5.0 MgSO4, and 1.0 EGTA, pH 7.2, 2% bovine serum albumin. The cells were maintained by equilibrating with 95% O2-5% CO2 at 31°C. The dispersed cells were exposed to saponin (75 g/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.

Studies of contraction and inhibition of contraction of dissociated muscle cells. Briefly, muscle squares were incubated at 31°C for 30 min in HEPES-buffered medium containing 150 U/ml collagenase (type II) and 0.01% soybean trypsin inhibitor (3, 7, 18). The partly digested tissues were washed with enzyme-free medium, and muscle cells were allowed to disperse spontaneously for 30 min. Muscle cells were harvested by filtration through 450-mm Nitex. Muscle contraction was measured as previously described in intact and permeable cells. Permeable cells were used to study the effect of antibodies against G proteins (Gαq, Gαi3, Gαi1/2, Gαs) and then fixed in acrolein at 1% final concentration (20). 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.

Inhibition of contraction. Inhibition of contraction was measured in permeable muscle cells by determining the effect of inhibitors on cell length using a method previously reported (3, 7, 18). 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 guinea pig colon. Individual cell lengths were measured by scanning micrometry using phase contrast microscopy. Relaxation was expressed as percent inhibition of DOG-induced contraction.

Measurement of phasic contractions in colon muscle strips. Strips were mounted in 1-ml muscle chambers as previously described in detail (6, 28). Briefly, circular muscle strips of the colon were obtained by removing the mucosa, longitudinal muscle layer, and serosa. They were initially stretched to 1.0 g of passive force and were equilibrated by continuous perfusion with oxygenated Krebs’s solu-
tion at 37°C. After 1-h perfusion, basal spontaneous phasic contractions gradually developed and stabilized after another 30-min period of equilibration. The strips were then treated with tetrodotoxin 10⁻⁵ M and after 30 min before any studies. Stable phasic contractions of control and treated muscle strips were measured with Grass isometric force transducers and amplifiers connected to a Biopac data acquisition system. The combined tonic and phasic activity was determined by calculating the MI measured over a 30-min period. It was calculated as

\[ \text{MI} = \frac{\text{A(g)}}{\text{D(s)}} \]

or area under the curve and expressed as mN/min (28).

Measurement of PGF₂α and PGE₂ content. PGF₂α and PGE₂ were measured using an Eicosanoid Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI) (10, 17). Muscle strips or cells were homogenized in eicosanoid homogenization buffer [0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA and 20 μg/ml indomethacin] at 4°C according to the manufacturer’s instructions. The homogenate was centrifuged at 15,000 g for 15 min at 4°C, and an aliquot of the supernatant was taken for protein measurement. The rest of the supernatant was used for PGF₂α purification using a specific Affinity Column. The resulting extracts were dissolved in enzyme immunoassay buffer (1.0 M phosphate buffer pH 7.4 containing 0.01% NaN₃, 0.037% EDTA, 0.1% BSA). The PGF₂α and PGE₂ concentration was quantified by using a PGF₂α Competitive Enzyme Immunoassay kit expressed as ng/mg protein.

Chemicals. P₄, PGF₂α, GTPγS, GDPβS, COX enzyme inhibitors, 8’bromo-c’AMP (8B-cAMP), cysteine alkylating agent N-ethylmaleimide (NEM) and pertussis toxin (PTX) and other reagents were purchased from Sigma Chemical (St. Louis, MO); AL-8810 (11⁻fluoro analog of PGF₂α from Cayman Laboratories, Ann Arbor, MI) (16); G protein subunit antibodies were purchased from CytoSignal (Irvine, CA).

Statistics. Statistical analysis was performed using one- and two-factorial repeated ANOVA comparing values between controls and experimental and progesterone-treated guinea pigs or muscle cells treated with inhibitor or antibodies. *P value of <0.05 was considered significant. Previous studies using similar treatments had shown that significance could be achieved using three to four samples of controls and experimental treated.

RESULTS

Effect of P₄ on basal colonic motility (basal MI) and prostaglandins. P₄ treatment [2 mg/kg intramuscularly (IM)] daily for 4 days markedly reduced the basal MI of the phasic contractions of the descending sigmoid colon of guinea pigs compared with that of muscle strips from saline-treated guinea pigs (Fig. 1).

We then evaluated two prostaglandins, PGF₂α, that contracts and PGE₂ that inhibits the phasic contractions of colon circular muscle strips. P₄ reduced the contraction induced by PGF₂α and by ACh (Fig. 2A). P₄ does not affect the contraction of

Fig. 3. A: treatment with P₄ in vivo decreased the levels of PGF₂α (*P < 0.01 by ANOVA) and was blocked by pretreatment with RU-486 (RU) (#P < 0.001 by ANOVA, mg/kg body wt). B: P₄ increased the levels of PGE₂ (**P < 0.01 by ANOVA) and was blocked by pretreatment with RU-486 (##P < 0.01 by ANOVA). Values are means ± SE of n = 4.

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Fig. 4. Effects of selective and nonselective (indomethacin) inhibitors on basal MI (A) and on PGF₂α levels (B) in cells from normal animals. SC560, a cyclooxygenase (COX)-1 inhibitor (10⁻⁵ M), decreased the basal MI (**P < 0.01 by ANOVA) and the levels of PGF₂α (#P < 0.01 by ANOVA). In contrast, NS398 (10⁻⁵ M), a COX-2 inhibitor, increased the basal MI (##P < 0.01 by ANOVA) and the levels of PGF₂α (#P < 0.05 by ANOVA). Indomethacin (10⁻³ M) lowered the basal MI and the PGF₂α levels (&P < 0.01 by ANOVA). Values are means ± SE of n = 4.
agonists that are G protein independent such as KCl (data are not shown). In contrast, P4 increased the inhibition of muscle contraction induced by PGE2 (Fig. 2B) but had no effect on the inhibition of contraction induced by 8B-cAMP, a second messenger that is receptor and G protein independent (Fig. 2C). These findings are in agreement with previous studies showing that P4 blocks excitatory agonists that act on G protein-coupled receptors.

We next investigated the effect of P4 on PGF2α, and PGE2 muscle levels. To examine whether these effects were specific, P4 was antagonized by pretreating guinea pigs with the P4 receptor antagonist RU-486 (10 mg/kg body wt per day) or saline for 4 days. Guinea pigs were treated with RU-486 or equivalent volumes of saline injections 1 h before the injection of P4. Consistent with previous studies, the IM administration of P4 alone decreased PGF2α (Fig. 3A) and increased PGE2 levels (Fig. 3B). Pretreatment with RU-486 blocked the changes in the levels of both prostaglandins induced by P4.

We next investigated the role of PGF2α in the genesis of the basal MI of the colon by examining the effects of COX enzyme inhibitors on the basal MI and levels of PGF2α. The levels of prostaglandins were determined in muscle strips treated with selective and nonselective inhibitors of COX enzymes. We have previously shown that COX-1 enzyme generates excitatory prostaglandins such as TxA2 and PGF2α and that COX-2 enzyme generates PGE2 (10). The COX-1 enzyme inhibitor SC560 (10−6 M) reduced the basal MI (Fig. 4A) and caused a concomitant reduction in the levels of PGF2α (Fig. 4B). In contrast, the COX-2 inhibitor NS398 (10−6 M) increased the basal MI (Fig. 4A) and the levels of PGF2α (Fig. 4B). The nonselective COX inhibitor indomethacin reduced both basal MI and lowered PGF2α levels (Fig. 4, A and B). Moreover, we also compared the percent increase in PGE2 levels in cells treated with P4 and COX-2 inhibitor NS398. No significant differences were found between P4-induced increase (31.4 ± 3%) and NS398-induced increased (33.8 ± 4%) in PGE2 levels.

To further investigate the role of PGF2α in the genesis of the basal MI, we examined the effect of PGF2α receptor antagonist Al-8810 (16) on PGF2α (10−10–10−6 M)-induced contraction and on the basal MI. Al-8810 (10−6 M) blocked the contraction in dissociated muscle cells induced by increasing concentrations of PGF2α (Fig. 5A) and almost abolished the basal MI of colonic muscle strips (Fig. 5B). These data taken together
suggest that PGF$_{2\alpha}$ contributes to the genesis of basal colonic motility.

**Effect of the cysteine alkylating agent NEM on basal MI and agonist-induced contraction.** We then examined the cellular source of PGF$_{2\alpha}$ and PGF$_2$. Colon muscle strips and dissociated muscle cells were treated with NEM that inactivates membrane receptors when unoccupied by their endogenous ligands (13). NEM (10$^{-4}$ M) had no effect on the basal MI of muscle strips pretreated with TTX (10$^{-5}$ M). It also did not impair the PGF$_{2\alpha}$-induced contraction in muscle strips (Fig. 6B) or in muscle cells (Fig. 6C). However, NEM blocked the contraction induced by ACh in muscle strips (Fig. 6B) and in cells (Fig. 6C).

NEM also blocked the VIP inhibition of contraction in dissociated muscle cells (17) but did not antagonize the actions of PGF$_2$ and 8B-cAMP, a receptor- and G protein-independent second messenger (Fig. 7). These results suggest that endogenous factors, among them PGF$_{2\alpha}$ and PGF$_2$, contribute to the genesis of the basal MI of the colon muscle strips. Thus prostaglandins by occupying their receptors protect them against the actions of NEM. These findings also suggest that colon muscle cells are the source of these prostaglandins that act as autacoids or paracrine factors. Taking all these findings together, they suggest that PGF$_{2\alpha}$ and PGF$_2$ may contribute to genesis of the colon basal MI.

**P4 effects on PGF$_{2\alpha}$ and PGF$_2$ levels mediated by G proteins.** Because P4 alters the pattern of G proteins in smooth muscle cells, we examined in permeable muscle cells the role of G proteins in P4-induced changes in the levels of prostaglandins. Muscle cells were stimulated with GTP$_\gamma$S, a direct G protein activator (18), and with GDP$_\beta$S, a nonspecific G protein blocker. GTP$_\gamma$S increased (Fig. 8A) and GDP$_\beta$S reduced (Fig. 8B) the levels of PGF$_{2\alpha}$ within 15 min (Fig. 8, A and B). Pretreatment of muscle cells with P4 (10$^{-5}$ M) for 6 h prevented the GTP$_\gamma$S increase in PGF$_{2\alpha}$ levels. In contrast, GDP$_\beta$S lowered PGF$_{2\alpha}$ levels and did not decrease them further in muscle cells pretreated with P4 for 6 h. These results suggest that P4 modulates the levels of PGF$_{2\alpha}$ through its known effects on G protein patterns (10, 30). Like the results obtained with GDP$_\beta$S, antibodies against Gq/11 proteins (1: 400 titer) did not reduce the levels of PGF$_{2\alpha}$ further in cells pretreated with P4 for 6 h.

We next examined whether specific G proteins mediate the effects of P4 on the levels of PGF$_{2\alpha}$ and PGF$_2$. Permeable muscle cells were pretreated with antibodies (Abs) (1:400 titer) against specific G proteins G$_{q/11}$, G$_{\alpha_3}$, G$_{\alpha_{11/2}}$, and G$_{\alpha_s}$ for 60 min before their exposure to prostaglandins. G$_{q/11}$ Abs reduced and G$_{\alpha_s}$ Abs increased the basal levels of PGF$_{2\alpha}$ (Fig. 9A). On the other hand, Abs against G$_{\alpha_s}$ proteins decreased and G$_{q/11}$ increased the levels of PGF$_2$ (Fig. 9B). Abs against the proteins of G$_{\alpha_s}$ had no effect on either prostaglandin.

Moreover, G$_{\alpha_3}$ Abs blocked the contraction induced by PGF$_{2\alpha}$, whereas Abs against G$_{q/11}$, G$_{\alpha_s}$, and G$_{\alpha_{11/2}}$ proteins had no effect (Fig. 10A). In contrast, Abs against Gs protein significantly reduced the PGF$_{2\alpha}$-induced inhibition of contraction (10B). These results suggest that PGF$_{2\alpha}$ generation is mediated by G$_{q/11}$ protein, whereas its receptor appears to couple to G$_{\alpha_3}$ proteins. Gs proteins appear to increase whereas Gq/11 proteins inhibit the levels of PGF$_2$.

The role of these G proteins in PGF$_{2\alpha}$ generation and in mediating induced contraction was supported by additional
studies with PTX in muscle strips. PTX (100 µg/ml) treatment for 1 h had no effect on the genesis of PGF2α (Fig. 11C) but significantly reduced the basal MI (Fig. 11A) and blocked the contraction induced by PGF2α (Fig. 11B). PTX inhibits the functions of Gq proteins.

DISCUSSION

The present studies show that P4 reduces the basal MI in guinea pigs in vivo by altering the normal levels of prostaglandin that induced contraction (PGF2α) and relaxation (PGE2). P4 decreases PGF2α levels because of downregulation of Gq/11 proteins and increases PGE2 levels by upregulating Go proteins. P4 also reduces the basal MI by impairing the contraction induced by PGF2α through downregulation of Go3 proteins, and, by upregulating Go3 proteins, it may increase the relaxation induced by PGE2. Furthermore, consistent with previous observations, treatment with P4 in vivo had no effect on receptor- and G protein-independent agonists. P4 treatment had no effect on KCl that contracts muscle cells by increasing calcium influx through calcium channels and had no effect on muscle relaxation induced by 8B-cAMP, a second messenger that relaxes colon muscle cells through receptor- and G protein-independent mechanisms by stimulating protein kinase A.

**Fig. 9.** The effects of G protein antibodies (Abs) on levels of PGF2α and PGE2 in permeable colon muscle cells from normal animals. Left: Abs against Gq/11 decreased (*P < 0.01 by ANOVA) and Abs against Go increased the basal levels of PGF2α (**P < 0.01 by ANOVA). Right: Abs against Gs lowered (#P < 0.005 by ANOVA) and Go11 Abs increased (##P < 0.01 by ANOVA) the levels of PGE2. Values are means ± SE of n = 3.

**Fig. 10.** A: effect of G protein Abs on contraction induced by PGF2α (10⁻⁶ M) in normal permeable colon muscle cells. Abs against Gs blocked the contraction induced by PGF2α (10⁻⁶ M) (*P < 0.01 by ANOVA), whereas Abs against Gq/11, Go1/2, and Go3 had no effect. Values are means ± SE of n = 4. B: Go3 protein Ab treatment in normal permeable cells almost completely abolished the inhibition of contraction induced by PGE2 (10⁻⁶ M) compared with that induced in control muscle cells (##P < 0.005 by ANOVA). Values are means ± SE of n = 3.

**Fig. 11.** Effect of pertussis toxin (PTX) (100 µg/ml) on muscle contraction induced by PGF2α (10⁻⁶ M) and basal levels of PGF2α in intact colon muscle cells. A: PTX reduced the basal MI (*P < 0.01 by ANOVA). B: it also decreased the contraction induced by PGF2α (10⁻⁶ M) (**P < 0.01 by ANOVA). C: PTX had no effect on the PGF2α levels. Values are means ± SE of n = 4.
are present in the colon muscle cells. Second, NEM, a nonspecific inhibitor of COX enzymes, acting as autacoids or paracrine factors. First, they

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levels, whereas GDP

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i3 proteins because its antibody and PTX, which inhibits

affects the colon transit time by reducing the basal MI and neurotransmitter-induced motility.

GRANTS

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REFERENCES