Nongenomic effects of progesterone on the contraction of muscle cells from the guinea pig colon

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Abstract

Progesterone (PG) affects mammalian cells by genomic and nongenomic mechanisms. The genomic actions of PG have been extensively reported and are due to the hormone actions responsible for nongenomic effects of PG affect smooth muscle, to establish whether these nongenomic effects are present in physiological conditions.

MATERIALS AND METHODS

Animals. Male guinea pigs (age 8–12 wk, 450–500 g body wt) were purchased from 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. In one experimental group, guinea pigs were treated with daily intramuscular injections of PG for 4 days. PG at a dose of 2 mg/kg body wt (inject volume 0.1 ml) was injected. This group was compared with a control that receive the same volume of normal saline intramuscularly (10). After an overnight fast, guinea pigs in all groups were anesthetized with an intramuscular injection of ketamine hydrochloride (30 mg/kg) followed by pentobarbital (30 mg/kg i.p.). The colon was removed and rinsed with ice-cold, oxygenated Krebs solution [composed of (in mM) 116.6 NaCl, 3.4 KCl, 21.9 NaHCO3, 1.2 NaH2PO4, 2.5 CaCl2, 1.2 MgCl2, and 5.4 glucose]. The colon tissue was placed in a dissecting pan containing the same solution continuously aerated with 95% O2-5% CO2. The mucosa and serosa as well as the longitudinal muscle layer were carefully peeled off under a dissecting microscope. The colon circular muscle layer was further cleaned by gently removing the remaining connective tissue.

Isolation and permeabilization of muscle cells. Single muscle cells were obtained by enzymatic digestion (9, 33, 34, 36, 37). Briefly, the muscle layer was 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. At the end of the digestion, the tissue was filtered through Nitex mesh 200 (Tetko; Elmsford, NY) and rinsed with 20 ml HEPES without collagenase and papain. The tissue remaining on the filter was collected. For the preparation of permeable muscle cells, the digested muscle tissue was rinsed again with "cytosolic buffer" (1, 31, 36, 37). The cell suspension was briefly treated with saponin (75 μg/ml) by centrifugation at 200 g for 3 min. Cells were then washed and

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resuspended in modified cytosolic buffer for further use. In some experiments, muscle cells were treated with PG for 10 min before agonists. In another experimental design, muscle cells were treated with PG for 60 min, washed out, remained untreated for 60 min, and then treated with PG again for 10 min before agonists.

Muscle cell contraction was induced in cell suspensions as described previously (31, 34, 36, 37). They were allowed to react with the agonists for 30 s, and 30 consecutive intact cells were measured using a phase-contrast microscope (Carl Zeiss; Oberkochen, Germany) with a television camera (Panasonic CCTV, model WV-CD51, Matsushita Communication; Osaka, Japan) connected to a Macintosh PowerPC computer. Muscle cell contraction was expressed as the mean percent shortening of cells with respect to the length of untreated cells.

Cytosolic Ca\(^{2+}\) measurements. Freshly isolated muscle cells were loaded with 2 × 10\(^{-6}\) M fura-2 AM and placed in a 3-ml chamber mounted on the stage of an inverted microscope (Carl Zeiss) (7). The agonist was applied directly to the cells using a pressure ejection ware.

Ca\(^{2+}\) concentrations were obtained from the ratios of fluorescence elicited by 340-nm excitation to 380-nm excitation using standard techniques. A pseudoisobestic image (i.e., an image insensitive to Ca\(^{2+}\) changes) was formed in computer memory from an accumulated sum of the images generated by 340-nm excitation and 380-nm excitation. This image was then threshold, and values below a selected level were considered outside the cell and called 0. For each ratiometric image, the outline of the cell was determined, and the generated mask was applied to the ratiometric image. This method allowed the simultaneous imaging of both rapid changes in Ca\(^{2+}\) and cell length. Our algorithm has been incorporated into the IonOptix software.

\(^{[3]}\)HPG incorporation studies. Muscle cells were assigned as two groups. Group 1 was the control group; cells were pretreated with buffer for 10 min before \(^{[3]}\)HPG (10\(^{-9}\) M) for 10, 30, and 60 min. Group 2 was composed of cells pretreated with unlabeled PG (10\(^{-7}\) M) for 10 min, washed out for 60 min, and then with \(^{[3]}\)HPG (10\(^{-9}\) M) for 10, 30, and 60 min. Cells were then washed with buffer thoroughly and homogenized with Dounce grinder. The plasma membrane and cytosol were separated by centrifugation at 500 × g for 5 min. The resulted cytosol was concentrated by centrifugation through a 100,000 × g ultrafilter. The radioactivity in the cytosol was counted by a scintillation β-counter.

PG-3-\(^{125}\)labeled BSA binding studies. Radioiodination of PG-3-BSA was achieved by means of chloramine-T (4, 5). Intact colonic circular muscle cells were pretreated with either buffer or PG for 10 min and then incubated with 10\(^{-9}\) M of PG-3-\(^{125}\)labeled BSA (PG-3-\(^{125}\)I[BSA]) for 180 min at 0°C. Separation of the bound form is achieved using a vacuum-filtering manifold (Millipore). Radioactivity remaining on the filters was counted in a γ-scintillation counter.

Chemicals. PG-3-\(^{125}\)I[BSA] and \(^{[3]}\)HPG were obtained from NEN Life Science Products (Boston, MA). Type F collagenase, papain, PG, and other reagents were purchased from Sigma Chemical (St. Louis, MO).

Data analysis. One- and two-factor repeated ANOVA and Student’s t-test were used for statistical analysis. P < 0.05 was considered to be statistically significant.
RESULTS

The average resting length of intact circular muscle cells isolated from the control guinea pig colon was 90.2 ± 2.6 μm (mean ± SE, n = 4) and was the same as that reported by others (10, 24). After cells were incubated with PG in vitro for 10 min, cell length did not change (91.2 ± 1.6 μm, mean ± SE, n = 4) and was the same as that in cells of guinea pigs treated with an in vivo PG injection (91.5 ± 2.3 μm, mean ± SE, n = 4).

Pretreatment with PG (10⁻⁷ M) for 10 min reduced the contraction induced by CCK (from 19.3 ± 2.8% to 4.9 ± 1.8%, P < 0.01 by Student’s t-test, n = 4) and by NKA (from 21.8 ± 2.3% to 8.2 ± 2.7%, P < 0.01 by Student t-test’s, n = 4). CCK-8 and NKA-induced contractions are exclusively dependent on Ca²⁺ release from intracellular stores (7, 35) (Fig. 1). Similarly, inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and caffeine-induced contractions, which contract muscle cells by directly inducing a release of Ca²⁺ intracellular stores, were inhibited by PG treatment for 10 min in vitro. Ins(1,4,5)P₃- and caffeine-induced contractions were reduced from 20.0 ± 0.3% and 19.8 ± 0.6% to 8.3 ± 3.1% and 3.2 ± 3.9%, respectively (P < 0.01 and P < 0.001 by Student’s t-test, n = 4).

In contrast, ACh- and KCl-induced contractions utilize extracellular Ca²⁺ influx (12, 23, 25, 29) and were not impaired (Fig. 2). These findings are different from those found in pregnant guinea pigs, where ACh-induced contraction was impaired (Ref. 11 and unpublished data), suggesting that a different mechanism of action of PG may be involved.

To determine whether an injection of PG in guinea pigs has similar effects on these agonists, guinea pigs received intramuscular injections of PG (2 mg·kg⁻¹·day⁻¹ for 4 days). As shown in Fig. 3, this treatment did not block calcium release from intracellular stores because muscle cells from these guinea pigs exhibited a normal contraction in response to Ins(1,4,5)P₃ and thapsigargin. These findings indicate that the inhibitory effect of PG on intracellular calcium release is time dependent and short lasting. In Fig. 3, KCl-induced contraction is shown as a normal control because it is mediated exclusively by calcium influx (10).

To support these findings, measurements of cytosolic Ca²⁺ were obtained in muscle cells loaded with fura-2 AM using a calcium image system. Muscle cells were pretreated with PG for 10 or 60 min before NKA stimulation. In normal muscle cells, Ca²⁺ release from intracellular stores induced by NKA was ~4.2 ± 0.6 × 10⁻⁷ M (peak value, n = 3; Fig. 4). It was abolished when cells were pretreated in vitro with PG for 10 min (1.4 ± 0.1 × 10⁻⁷ M, P < 0.002, n = 3; Fig. 5A). In contrast, after muscle cells were pretreated with PG for 60 min (n = 3; Fig. 5B), NKA-induced calcium release from intracellular stores was not different from controls (4.5 ± 0.9 × 10⁻⁷ M).

To explain the discrepancy between the effects of PG after 10 and 60 min, we examined the duration of the short-term (nongenomic) effects of PG. Normal muscle cells from guinea pig colonic circular muscle were incubated with PG for up to 360 min, and aliquots of cells were stimulated by thapsigargin at different time points (Fig. 6). PG treatment completely blocked thapsigargin-induced contraction at 10 min and partially blocked it at 30 min. By 60 min, cells had fully recovered from the effects of PG because the contraction induced by thapsigargin returned to normal amplitude (n = 4). These data further confirm the short acting of effects of PG on intracellular calcium release.

To determine whether these nongenomic effects were specific for PG, we examined the effects of other steroid hormones such as aldosterone and testosterone on thapsigargin-induced contractions. As shown in Fig. 7, pretreatment with aldosterone (10⁻⁷ M) for 10 min also blocked the thapsigargin-induced contraction (n = 3; Fig. 7A). A similar inhibitory effect was observed after pretreatment with testosterone (10⁻⁶ M, n = 3; Fig. 7B). Similarly to PG (as shown in Fig. 6), pretreatment...
with aldosterone or testosterone for 10 min abolished thapsigargin-induced contraction. Their inhibitory effects to thapsigargin-induced contraction declined after 30 min and disappeared after 60 min.

To investigate whether muscle cells were desensitized after exposure to PG, they were initially incubated with PG for 10 min, washed out, and after 60 min retreated with PG for 10 min followed by thapsigargin stimulation (Fig. 8). In the control group (PG incubation for 10 min only), PG treatment blocked thapsigargin induced contraction (from 19.7 ± 2.3% of control to 2.9 ± 0.9%, P < 0.001 by Student’s t-test, n = 3). However, a second dose of PG (60 min later) failed to block the thapsigargin-induced contraction in muscle cells pretreated with PG (n = 3), suggesting that PG-induced inhibition appeared to last <1 h.

To determine the mechanisms responsible for these findings, PG incorporation by the cytosol (Fig. 9) was examined using radiolabeled PG. Muscle cells were assigned to two groups. In the control group, cells were pretreated with buffer for 10 min before [3H]PG (10−7 M) for 10, 30, and 60 min. In the
Fig. 6. Effect of PG (10^{-7} M) on thapsigargin-induced contraction of colonic circular muscle cells from guinea pigs over a period of 360 min. The inhibitory effect of PG on thapsigargin-induced contraction was time dependent. Thapsigargin-induced contraction was abolished after 10 min but fully recovered after 60 min (n = 4).

Fig. 7. Effects of steroid hormones on thapsigargin-induced contraction of colonic circular muscle cells from guinea pigs over a period of 360 min. Like PG, the inhibitory effect of aldosterone (A) and testosterone (B) on thapsigargin-induced contraction was also time dependent. Thapsigargin-induced contraction was abolished by these hormones after 10 min of incubation and fully recovered after 60 min (n = 3).

Fig. 8. Normal colonic circular muscle cell desensitization to the effects of PG. Muscle cells were initially incubated with PG for 10 min, washed out, and, after 60 min, retreated with PG for an additional 10 min and then treated with thapsigargin. PG pretreatment for 10 min blocked thapsigargin-induced contraction (*P < 0.001 vs. control). A second dose of PG failed to inhibit the thapsigargin-induced contraction in muscle cells pretreated with PG (n = 3).

Fig. 9. Incorporation of radiolabeled PG by the cytosol of normal colonic circular muscle cells. Muscle cells were assigned to two groups. In the control group, cells were pretreated with buffer for 10 min before the addition of [3H]PG (10^{-9} M) for 10, 30, and 60 min; in the experimental group, cells were pretreated with unlabeled PG (10^{-7} M) for 10 min, washed out for 60 min, and then treated with [3H]PG (10^{-9} M) for 10, 30, and 60 min. The plasma membrane and cytosol were separated by homogenization and centrifugation. Pretreatment with unlabeled PG significantly reduced radiolabeled PG incorporation in the cytosol (P < 0.001 and P < 0.05 vs. control by Student’s t-test; n = 3). These findings suggest muscle cell unresponsiveness to PG inhibition of its transport system into cells or postentry events.

To further support these findings, saturation studies were performed using PG-3-[125I]BSA. Intact muscle cells were

Fig. 10. Incorporation of radiolabeled PG by the cytosol of normal colonic circular muscle cells. Muscle cells were assigned to two groups. In the control group, cells were pretreated with buffer for 10 min before the addition of [3H]PG (10^{-9} M) for 10, 30, and 60 min; in the experimental group, cells were pretreated with unlabeled PG (10^{-7} M) for 10 min, washed out for 60 min, and then treated with [3H]PG (10^{-9} M) for 10, 30, and 60 min. The plasma membrane and cytosol were separated by homogenization and centrifugation. The radioactivity in the cytosol was counted with a β-counter. Pretreatment with unlabeled PG significantly reduced the incorporation of radiolabeled PG in the cytosol (P < 0.001 and **P < 0.05 vs. control by Student’s t-test; n = 3).
pretreated with either buffer or PG (10⁻⁷ M) for 10 min before PG-3-[^125I]BSA (10⁻⁹ M) for 180 min (Fig. 10). Pretreatment of muscle cells with PG significantly decreased the entry of PG-3-[^125I]BSA into the cytosol (P < 0.001 by ANOVA), suggesting that PG transport systems were partially saturated by unlabeled PG pretreatment.

DISCUSSION

The present study shows that in dissociated colonic muscle cells, PG induces nongenomic effects within 10 min after exposure. PG selectively inhibited the contraction induced by CCK-8, NKA, Ins(1,4,5)P₃, thapsigargin, and caffeine that is mediated by Ca²⁺ release from intracellular stores (6, 7, 35, 36). The contraction induced by these agents is inhibited because PG blocks Ca²⁺ release from intracellular stores, through both Ins(1,4,5)P₃-dependent and ryanidine-dependent (caffeine) calcium stores. In colonic circular smooth muscle cells of guinea pigs, CCK-8-induced contraction is initiated by binding of CCK-8 to its receptors and coupling of Go₃q, followed by activation of phospholipase C-β₁, hydrolysis of phosphatidylinositol bisphosphate, and production of Ins(1,4,5)P₃, which causes calcium release and cell contraction (20). However, NKA-induced contraction of human sigmoid circular muscle is also mediated by the activation of Go₃q protein and calcium release from intracellular stores (7, 8).

The mechanisms of PG-induced inhibition of calcium release from intracellular stores are not known. Because PG is rapidly transported into the cytosol, it is conceivable that it may transiently bind and block calcium release channels in the endoplasmic reticulum (13). This effect, however, is transient, because it disappears within 60 min.

Others (2, 14, 19, 28) have suggested that PG impairs muscle contraction by blocking the influx of extracellular calcium in myometrial, vascular, and intestinal muscle cells. Our data, however, indicate that the extracellular influx of calcium is intact because PG did not affect contraction induced by ACh, which may be mediated by both extracellular calcium influx and calcium release from intracellular sotres (23, 29), and KCl-induced contraction, which is mediated by extracellular calcium influx through voltage-dependent calcium channels.

These short-term, nongenomic action-induced changes in signal transduction are different from the genomic effects observed in colonic myocytes from pregnant or PG-treated guinea pigs. Muscle cells from these animals contract poorly in response to agonists that are receptor-G protein (CCK-8, ACh, and PGE₂) or G protein dependent (GTPγS or AlF₄⁻) (10, 11). Moreover, inhibition of calcium release from intracellular stores has not been reported in colonic myocytes treated with PG in vivo. Our findings indicate that these nongenomic effects are absent in PG-treated guinea pigs, even though nongenomic effects are brought about by relatively low concentrations of PG (10⁻⁹–10⁻⁷ M) that are similar to those found in the plasma in physiological conditions (17). Discrepancies between the effects of PG during physiological conditions and brief in vitro exposures to PG have not been previously reported. It is conceivable that the absence of nongenomic changes in colonic muscle cells in PG-treated guinea pigs may be the result of muscle desensitization to a continuous exposure to PG.

The finding that PG inhibits calcium release from intracellular stores without altering calcium influx is further supported by calcium-imaging studies using fura-2 AM. In PG-treated muscle cells, CCK and NKA did not induce a release of calcium from intracellular stores in normal and calcium-free media. Calcium release from intracellular stores induced by CCK-8 was inhibited by a brief exposure with PG. In contrast to opossum intestinal muscle cells (2), these results suggest that PG does not alter calcium influx because it did not impair the contraction induced by KCl or by agonists that can stimulate both calcium release and calcium influx (ACh and PGE₂).

Moreover, colonic muscle cells obtained from PG-treated animals appear to lack these nongenomic effects, confirming our observations that nongenomic actions of PG are transient even though the genomic effects remain (10). These cells respond normally to agents that release calcium from intracellular stores such as Ins(1,4,5)P₃ and thapsigargin (Fig. 3). Discrepancies between the actions of PG in short in vitro experiments (10 min) and in physiological settings have not been reported.

Additional experiments performed to explain these discrepancies indicate that the nongenomic actions are transient because cells recovered fully within 60 min and remained desensitized to additional doses of PG. Nongenomic effects cannot be reproduced in muscle cells that have been previously pretreated in vitro with this hormone. Cells incubated with PG for 10 min recover fully after 1 h because they responded normally to thapsigargin. These cells, however, were unable to respond to a second dose of PG. More important, these genomic effects could be induced in muscle cells obtained from animals that had been treated with intramuscular PG (2 mg/kg) for 4 days even 24 h after the last dose. The mechanism whereby muscle cells are desensitized to additional doses of PG is not known.

The 60-min transport of 10⁻⁷ M PG plus[^3H]PG (10⁻⁹ M) across the plasma membrane 1 h after a 10-min exposure to PG was reduced to <5 × 10⁻⁸ M compared with 1.5 × 10⁻⁷ M when the cells were pretreated with buffer alone (Fig. 10). These findings suggest that the cells are unresponsive to additional doses of PG because the transport system may be saturated. It is conceivable that cytosolic concentrations of PG lower than 10⁻⁸ M may be unable to induce nongenomic
The mechanism of muscle cell desensitization to additional doses of PG was examined by performing transport studies with radioabeled PG into the cytosol over a 60-min period. PG, a steroid hormone, is believed to enter into the cell due to its lipophylic properties and is transported toward its nuclear receptors by chaperones like heat shock protein (HSP)70 and 90. Our findings revealed that pretreatment with cold PG (10^{-7} M) decreased the transport of [^3H]PG to 5 \times 10^{-8} compared with 1.5 \times 10^{-7} M:mg protein^{-1}\cdot60\text{ min}^{-1} into the cytosol when muscle cells were pretreated with buffer. Pretreatment with cold PG also inhibited the maximal transport of radioabeled PG into the cytosol by 50%, as shown in Fig. 10. These results suggest that the transport system may be partially saturated, preventing the development of additional nongenomic effects by PG concentrations that remain within physiological range from 10^{-9} to 10^{-7} M. Moreover, muscle cells from guinea pigs treated in vivo with intramuscular injections of PG remained resistant to the development of nongenomic actions even after 24 h when treated with PG in vitro for 10 min. These findings also support the view that the transport system in patients with pigment and cholesterol stones.

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