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RhoA Kinase and Protein Kinase C Participate in Regulation of Rabbit Stomach Fundus Smooth Muscle Contraction

Paul H. Ratz
Eastern Virginia Medical School

Joel T. Meehl
Marquette University

Thomas Eddinger
Marquette University, thomas.eddinger@marquette.edu

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Rhoa Kinase and Protein Kinase C Participate In Regulation of Rabbit Stomach Fundus Smooth Muscle Contraction

Paul H. Ratz
Department of Physiological Sciences, Eastern Virginia Medical School
Norfolk, Virginia, VA

Joel T. Meehl
Department of Biological Science, Marquette University
Milwaukee, WI

Thomas J. Eddinger
Department of Biological Science, Marquette University
Milwaukee, WI

Abstract

1. The degree to which the RhoA kinase (ROK) blockers, Y-27632 (1 μM) and HA-1077 (10 μM), and the PKC blocker, GF-109203X (1 μM), reduced force produced by carbachol, a muscarinic receptor agonist, and phenylephrine, an α-adrenoceptor agonist, was examined in rabbit stomach fundus smooth muscle.
2. When examining the effect on cumulative carbachol concentration-response curves (CRCs), ROK and PKC blockers shifted the potency (EC_{50}) to the right but did not reduce the maximum response.

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3. In a single-dose carbachol protocol using moderate (~EC_{50}) and maximum carbachol concentrations, Y-27632 and HA-1077 reduced peak force, but GF-109203X had no effect. By contrast, all three agents inhibited the carbachol contractions of rabbit bladder (detrusor) smooth muscle.
4. Compared to carbachol, phenylephrine produced a weaker maximum response that was not inhibited by phentolamine, atropine nor capsaicin but was inhibited by Y-27632 , HA-1077 and GF-109203X.
5. In detrusor, classical down-regulation occurred, but in fundus, up-regulation of responsiveness occurred. This up-regulation in fundus may have been a post-receptor event, because a KCl-induced contraction produced after a carbachol CRC was stronger than one produced before the carbachol stimulus.
6. In conclusion, these data suggest that ROK plays a critical role in the regulation of rabbit fundus smooth muscle contraction, which is distinct from chicken gizzard smooth muscle, where ROK is reported to exist but to not play a role in muscarinic receptor-induced contraction. Additional unique findings are that PKC participates in phenylephrine- but not carbachol-induced contraction in fundus, that carbachol does not activate identical subcellular signalling systems in fundus and detrusor, and that fundus, unlike detrusor, responds to carbachol stimulation with post-receptor up-regulation of contraction.

Keywords: Signal transduction, stomach fundus, detrusor, smooth muscle, Ca^{2+}-sensitization, RhoA kinase inhibitors

Introduction

Several reports published just over a decade ago revealed that smooth muscle contractile proteins could be 'sensitized' to Ca^{2+} (Nishimura *et al*., 1988; Fujiwara *et al*., 1989; Kitazawa *et al*., 1989). In a review, Karaki (1989) described Ca^{2+} sensitization as a mechanism by which receptor agonists produce greater increases in force for a given increase in $[Ca^{2+}]_i$ than a stimulus such as KCl. This model remains valid, except that recent reports indicate that KCl can also modulate the degree of Ca^{2+} sensitivity (Yanagisawa & Okada, 1994; Ratz, 1995; Mita *et al*., 2002), although to a lesser degree (Ratz, 1999). The precise mechanisms that cause Ca^{2+} sensitization are still being investigated. However, in general terms, Ca^{2+} sensitization may be due to an alteration in the sensitivity of myosin light chain (MLC) phosphorylation to Ca^{2+}, principally through

inhibition of MLC phosphatase by RhoA kinase (ROK) and protein kinase C (PKC), and to thin filament regulation (Hori & Karaki, 1998; Somlyo & Somlyo, 2000).

Y-27632 and HA-1077 have recently proven invaluable in identification of the relative role that Ca^{2+} sensitization plays in regulation of contraction of intact smooth muscles of different organ systems. In permeabilized guinea-pig ileum, the ROK inhibitors, Y-27632 and HA-1077, inhibit GTPγS-induced Ca^{2+} sensitized contraction with IC_{50} values of, respectively, 1.7 μM and 2.7 μM (Sward *et al*., 2000). In arterial tissue, Y-27632 is reported to inhibit tonic contraction with an IC_{50} value of 0.7 μM (Uehata *et al*., 1997). These values are similar to K_i values reported for inhibition of ROK activity by these agents (Uehata *et al*., 1997; Davies *et al*., 2000; Sward *et al*., 2000), and a recent thorough study documenting the specificity of 28 commercially available kinase inhibitors indicates that Y-27632 and HA-1077 are highly selective for inhibition of ROK when used at appropriate concentrations (Davies *et al*., 2000).

To date, Y-27632 or HA-1077 is reported to inhibit vascular (Uehata *et al*., 1997), respiratory (Chiba *et al*., 1999), ileal (Sward *et al*., 2000), bladder (Jezior *et al*., 2001) and myometrial (Kupittayanant *et al*., 2001) smooth muscle contractions. However, the degree to which these agents inhibit, and thus, the extent by which ROK participates in contractions appears to differ in different smooth muscle types. For example, 10 μM Y-27632 only modestly reduces spontaneous human myometrial contractions (Kupittayanant *et al*., 2001), but abolishes tonic α-adrenoceptor-induced contraction of rabbit aorta (Uehata *et al*., 1997). Muscarinic receptor stimulation induces a biphasic contraction of ileum, bladder and chicken gizzard consisting of a rapid, peak contraction (phasic component) followed by a tonic component that is weaker than the peak response. ROK blockade by Y-27632 or HA-1077 abolishes the tonic component but leaves the phasic component nearly intact in ileum (Sward *et al*., 2000), while in bladder, both components are greatly diminished (Jezior *et al*., 2001). Interestingly, despite the finding that GTPγS induces ROK activation and Ca^{2+} sensitization in chicken gizzard smooth muscle, muscarinic receptor stimulation does not cause ROK-dependent Ca^{2+} sensitization in this muscle type (Anabuki *et al*., 2000). However, Y-27632 does reduce gastric antrum motility in

conscious rats (Tomomasa *et al*., 2000). The present study was designed to determine whether the selective ROK inhibitors, Y-27632 and HA-1077, inhibit contractions produced in isolated rabbit stomach fundus smooth muscle. For a comparison, the ability of GF-109203X, an inhibitor of conventional and novel PKC isotypes (Gailly *et al*., 1997), to inhibit fundus contraction was examined, and the effects of each agent on muscarinic receptor-induced contraction of rabbit bladder wall (detrusor) smooth muscle was tested.

Methods

Tissue preparation

Female New Zealand White rabbits (~2 kg) were killed by CO_2 asphyxiation or intravenous injection of a 191 mg kg^{-1} chloral hydrate/40 mg kg^{-1} pentobarbitone solution, as approved by the Marquette University Institutional Animal Care and Use committee. Stomach and bladder were removed, cleaned of adipose, loose connective tissue and mucosa of the stomach and transitional epithelium of the detrusor and stored in physiological salt solution (PSS) ((in mM) NaCl 140, KCl 4.7, Na_2HPO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, glucose 5.6, EDTA 0.02, 3-(*N*-morpholino)propanesulfonic acid 2.0, pH 7.4 at 37°C) at 4°C for up to 72 h until use.

Immediately before use each tissue was cut into longitudinal strips ~8 mm and ~10 mm long (fundus and detrusor, respectively) and ~2 mm wide. Strips were clamped on each end, with the clamps secured between hooks on a stationary metal rod and a metal rod hanging on an isometric force transducer (Harvard Apparatus, Holliston, MA, U.S.A.), in PSS bubbled with 95% O_2/5% CO_2 in water-jacketed muscle chambers (Radnoti Glass Technology, Monrovia, CA, U.S.A.) at 37°C. The length of each strip was varied by repositioning of the stationary metal rod.

Mechanical measurements

Strips were stretched to a passive tension of 1.75–2.25 g and 1.00–1.50 g (fundus and detrusor, respectively) and were allowed to equilibrate for 1 h. To contract tissues, PSS was then replaced with K^+-

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PSS, which had the same composition as PSS except that 109 mM KCl and 70 mM NaCl were substituted for 140 mM NaCl. After active force reached a maximum (3–7 min, chambers were flushed three times with PSS, muscle length was increased 0.5–2.0 mm depending on active force produced, and strips were given a 20 min incubation period before K^+-PSS was added again. If active force was not significantly different between the two contractions, muscle length was not changed. If force was significantly different, muscle length was adjusted and K^+-PSS was added a third time. The muscle length producing maximum force was designated L_o. At the end of the experiment, wet weight was recorded for each strip, and the force produced per cross-sectional area was calculated as $F/A\ (N/m^2)=(F\ (g) *9.807\times10^{-3}) / ((\text{wet weight (mg)}/L_o\ (mm)) * 9.52\times10^{-7})$. On average, when contracted with K+-PSS, fundus strips produced $\sim2\times10^5\ N/m^2$ while detrusor strips produced $\sim8\times10^4\ N/m^2$.

Cumulative concentration-response curve (CRC) protocol

For CRC studies, concentrations of agonist producing zero active force (10^{-9} M) to those producing maximum active force (10^{-5} M to 10^{-3} M) were added cumulatively to the muscle chamber. When force reached maximum after a dose was added (3–7 min), the next dose was added. After the curve was completed, chambers were flushed with PSS and incubated for 20 min, and an antagonist was added to chambers containing experimental muscle strips. Control tissues were not exposed to an antagonist, but were otherwise treated identically to experimental tissues. All antagonists were allowed at least 20 min incubation, except capsaicin, which was allowed 1 h incubation. After the incubation period, a second CRC was repeated on experimental and control strips.

Single dose (concentration) protocol

Concentrations of agonist producing maximum and half-maximum active force were computed from CRCs. These concentrations were then used for single-dose studies (i.e., tissues were exposed to a single concentration of agonist rather than to multiple cumulative concentrations). Maximum concentrations were:

3×10^{-6} M carbachol and 10^{-4} M phenylephrine, and half-maximum concentrations were: 1.2×10^{-7} M carbachol and 5.2×10^{-6} M phenylephrine for fundus and 3×10^{-6} M (Max) and 1.1×10^{-7} M (half-max) carbachol for the detrusor. After force reached a maximum (2–3.5 min), chambers were flushed and incubated in PSS for 30 min, and the activation was repeated a second time. After force reached a maximum, chambers were flushed, incubated in PSS for 10 min, and an antagonist was added to the chamber (for experimental strips). Muscle strips were incubated for an additional 20 min, and a third activation with contractile agonist was performed. Control strips were not exposed to an antagonist, but were otherwise treated identically.

Analysis of data

Voltage signals from force transducers were digitized by PowerLab 400 or 4SP hardware (ADInstruments, Castle Hill, Australia) visualized on a computer screen by the program Chart v3.6 or 4.0 (ADInstruments) as force (*g*) at 10 Hz and stored by software command to a hard disk for later analyses. Analyses were performed with the spreadsheet program Excel 2000 (Microsoft, Redmond, WA, U.S.A.). Active force from an agonist was normalized to maximum force from K^+-PSS (F/F_o) or to the first contraction produced by a contractile agonist (e.g., F_{Trial2}/F_{Trial1}). Best-fit CRCs characterized by the four parameters of a sigmoidal curve (maximum, minimum, slope, and the concentration producing half-maximum active force (EC_{50})) were determined with the program GraphPad (GraphPad Software, Inc., San Diego, CA, U.S.A.). Figures were constructed using SigmaPlot v5.0 (SPSS, Chicago, IL, U.S.A.).

Statistics

Statistical comparisons were carried out with the program Statistica (StatSoft, Tulsa, OK, U.S.A.). For CRC studies, maximum force (F/F_o) and log EC_{50} (M) were compared. For single dose studies, forces (F/F_o) of the second (Trial 2) and third (Trial 3) contractions were compared. A one-way repeated measures ANOVA with a between grouping factor was used to test the null hypothesis that any difference of changes between trials in experimental groups to changes between trials in the control group was due to chance. The

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null hypothesis was rejected at $P<0.05$. If the null hypothesis was rejected, planned comparison contrast analyses were performed to determine which specific experimental groups differed from the control group. The null hypothesis in these tests was also that differences were due to chance, and this hypothesis was rejected at $P<0.05$. A paired T-test was used to test for significant differences between maximum force produced from CRCs and maximum force produced from a single dose activation as well as the difference between force from the KCl contractions before and after carbachol activations. Data are expressed as mean±s.e.mean. The value of *n* is equal to the number of rabbits from which strips were obtained.

Drugs

Agonists used were carbachol (Acros Organics, NJ, U.S.A.) and phenylephrine (PE) (Sigma, St. Louis, MO, U.S.A.). Antagonists were 10^{-7} M atropine (Sigma), 10^{-6} M capsaicin (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.), 10^{-6} M GF-109203X (GF) or GF-109203X-HCl (both which gave similar results, Calbiochem, La Jolla, CA, U.S.A.), 10^{-5} M HA-1077 (HA) (ICN), 10^{-6} M phentolamine (Sigma), and 3×10^{-6} M Y-27632 (Y) (Calbiochem). All drugs were reconstituted in distilled water at the desired dose, except capsaicin which was reconstituted in 100% ETOH.

Results

Effects of antagonists on carbachol-induced cumulative concentration-response curves (CRCs) in fundus

Original experimental traces of typical CRCs of tissue strips to carbachol are presented in Figure 1a,b,d,e). The maximum force values produced during the first and second carbachol-induced CRCs were not different, although the average maximum response of the second response (2.02±0.10 F/F_o) was slightly larger than the first (1.74±0.09 F/F_o; Figure 1c). Likewise, the potencies (EC_{50}) of carbachol produced during the first (−6.60±0.09) and second (−6.49±0.07) CRCs were not different (Figure 1c). Analysis of variance showed significant differences between the first and second trial without (control) and with antagonists added. As expected, 10^{-7} M

atropine produced a large rightward shift in the carbachol CRC without a reduction in the maximum response (Figures 1f and and2a),2a), indicating that the carbachol-induced contraction in this tissue is due to activation of muscarinic receptors.

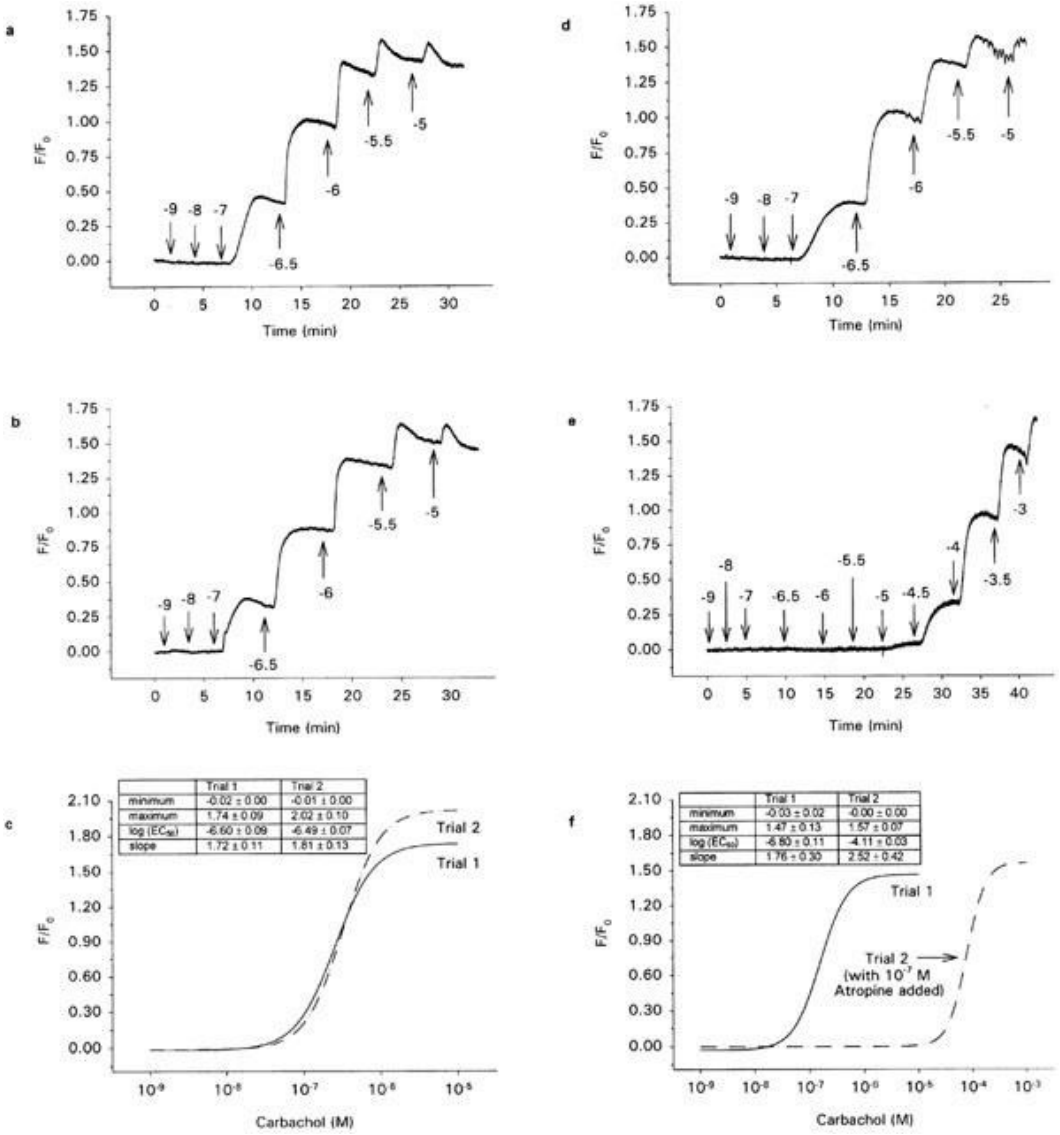

Figure 1 Typical force tracings produced by fundus muscle strips in response to the cumulative addition of carbachol in the absence (a, b and d) and presence (e) of 10^{-7} M atropine. Arrows and numbers indicate when carbachol (concentration shown in −log units) was added. (a) and (b) are tracings from one tissue, and (d) and (e) are tracings from another tissue. After a first carbachol concentration-response curve (CRC) was generated (a and d), tissues were washed for 30 min to cause relaxation, and either no drug

(control) or a drug was added for at least 20 min (not shown). A second carbachol CRC was then generated (b and e). Data from Trial 1 and Trial 2 CRCs were fitted to a sigmoidal curve characterized by the four parameters shown in the inserts of (c, n=13) and (f, n=4). A similar protocol was used to examine the effects of GF-109203X, HA-1077 and Y-27632 (see Figure 2.

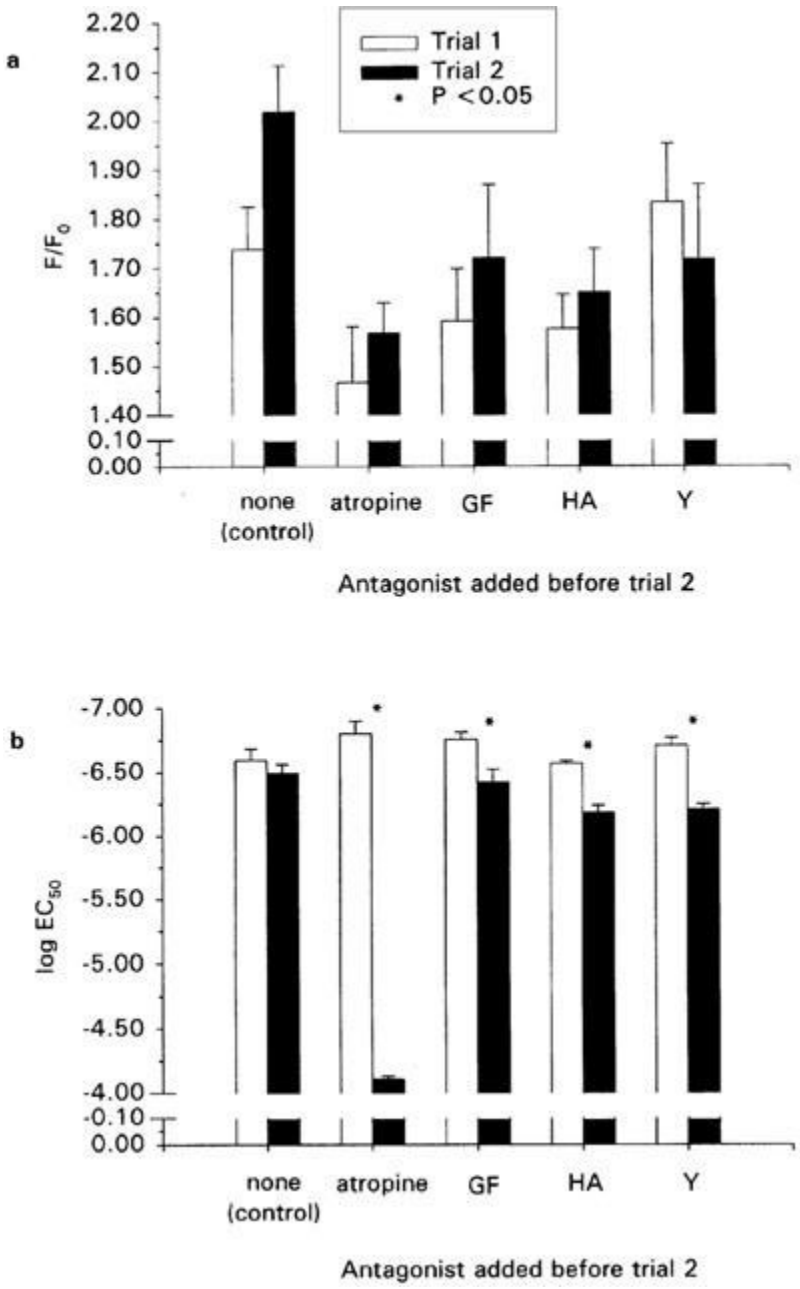

Figure 2 Carbachol cumulative response curves. Comparisons of maximum force values (a) and EC_{50} values (b) produced by Trial 1 and Trial 2 carbachol CRC in fundus muscle strips (see Figure 1 legend for protocol). Tissues were

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either not treated (control) or treated with an antagonist for at least 20 min before Trial 2. Data are means+s.e.mean. Control, n=13; 10^{-7} M atropine, n=4; 10^{-6} M GF-109203X (GF), n=7; 10^{-5} M HA1077 (HA), n=4; 3×10^{-6} M Y-27632 (Y), n=4.

The inhibitor of conventional and novel isoforms of PKC ($PKC_{c,n}$), GF-109203X, and the inhibitors of ROK, HA-1077 and Y-27632 had no effect on the maximum contraction produced by carbachol (Figure 2a). However, all three agents produced a slight but significant reduction in the potency of contraction (Figure 2b).

Effects of antagonists on contractions produced by single doses of carbachol in fundus

The maximum force values from the second and third contractions (Trial 2 and Trial 3, respectively) were not significantly different when normalized to the maximum force value produced by the first contraction (Trial 1; control in Figure 3a). This was found also to be the case when the $PKC_{n,c}$ inhibitor, GF-109203X, was added to tissues 20 min before the third stimulation with carbachol (i.e., Trial 3 contraction was not different than Trial 2 contraction; GF in Figure 3a). However, the ROK inhibitors, HA-1077 and Y-27632, both inhibited maximum force when added for 20 min before tissues were contracted with carbachol a third time (HA and Y in Figure 3a). The degree of inhibition by HA-1077 and Y-27632 was even more pronounced when tissues were contracted with a lower (EC_{50}) concentration of carbachol (Figure 3b). However, GF-109203X had no effect on maximum contraction produced by this lower carbachol concentration (Figure 3b).

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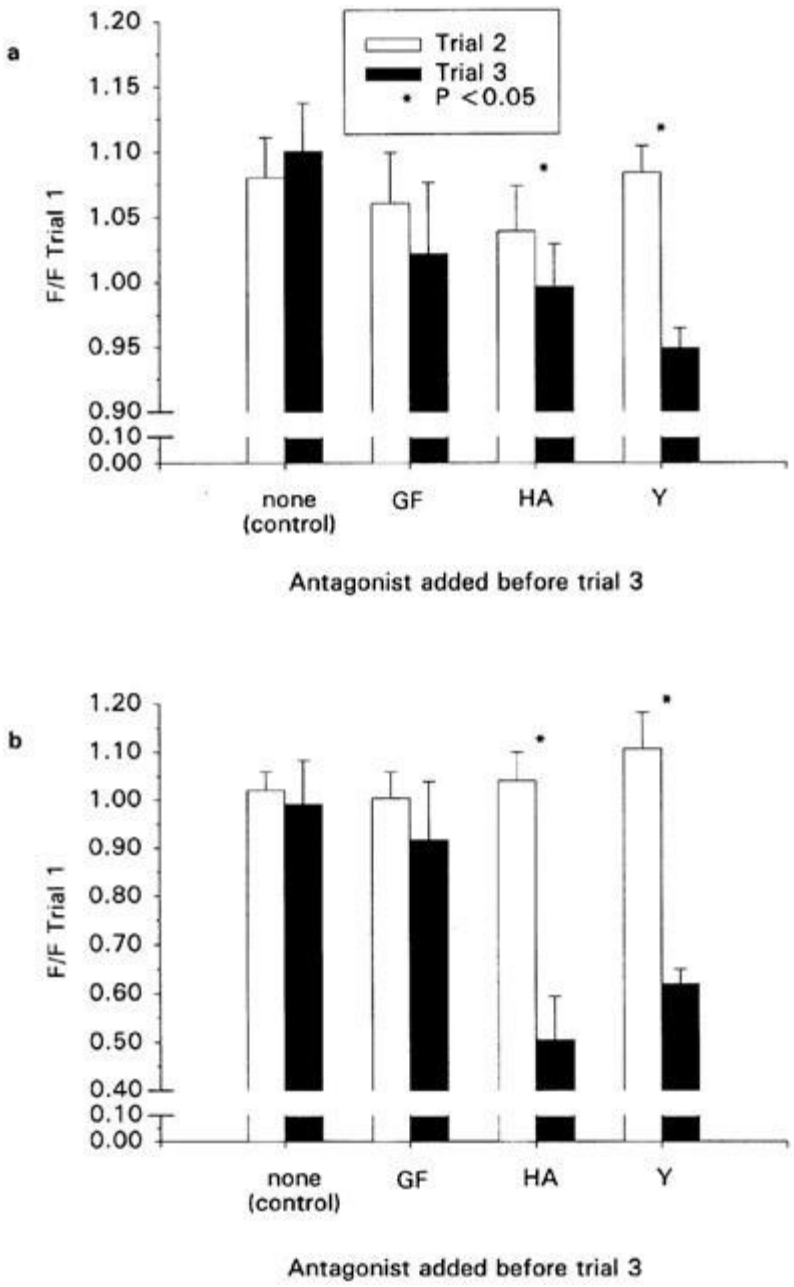

Figure 3 Carbachol single dose response. Comparisons of maximum force values (a) and half-maximum force values (b) produced by Trial 2 and Trial 3 single-dose carbachol stimulus in fundus muscle strips. Tissues were either not treated (control) or treated with an antagonist for 20 min before Trial 3. Data are means+s.e.mean. Control, n=9; 10^{-6} M GF-109203X (GF), n=4; 10^{-5} M HA-1077 (HA), n=5; 3×10^{-6} M Y-27632 (Y), n=4.

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Effects of antagonists on contractions produced by single doses of carbachol in detrusor

When contracted using the single-dose protocol, detrusor, like fundus, produced two identical maximum contractions (i.e., the second and third contractions were not different from each other when normalized to the first contraction; control in Figure 4). This was true when using carbachol concentrations that produced maximum (Figure 4a) and EC_{50} (Figure 4b) responses. Also like fundus, detrusor smooth muscle was inhibited by both ROK inhibitors (HA-1077 and Y-27632), although detrusor was inhibited to a greater degree than was fundus. In detrusor, Y-27632 produced ~50% inhibition of maximum carbachol-induced contraction (Figure 4a), while producing only ~15% inhibition in fundus (Figure 3a). Likewise, Y-27632 produced over 80% inhibition of the contraction produced by an EC_{50} concentration of carbachol in detrusor (Figure 4b), but produced less than 50% inhibition in fundus (Figure 3b).

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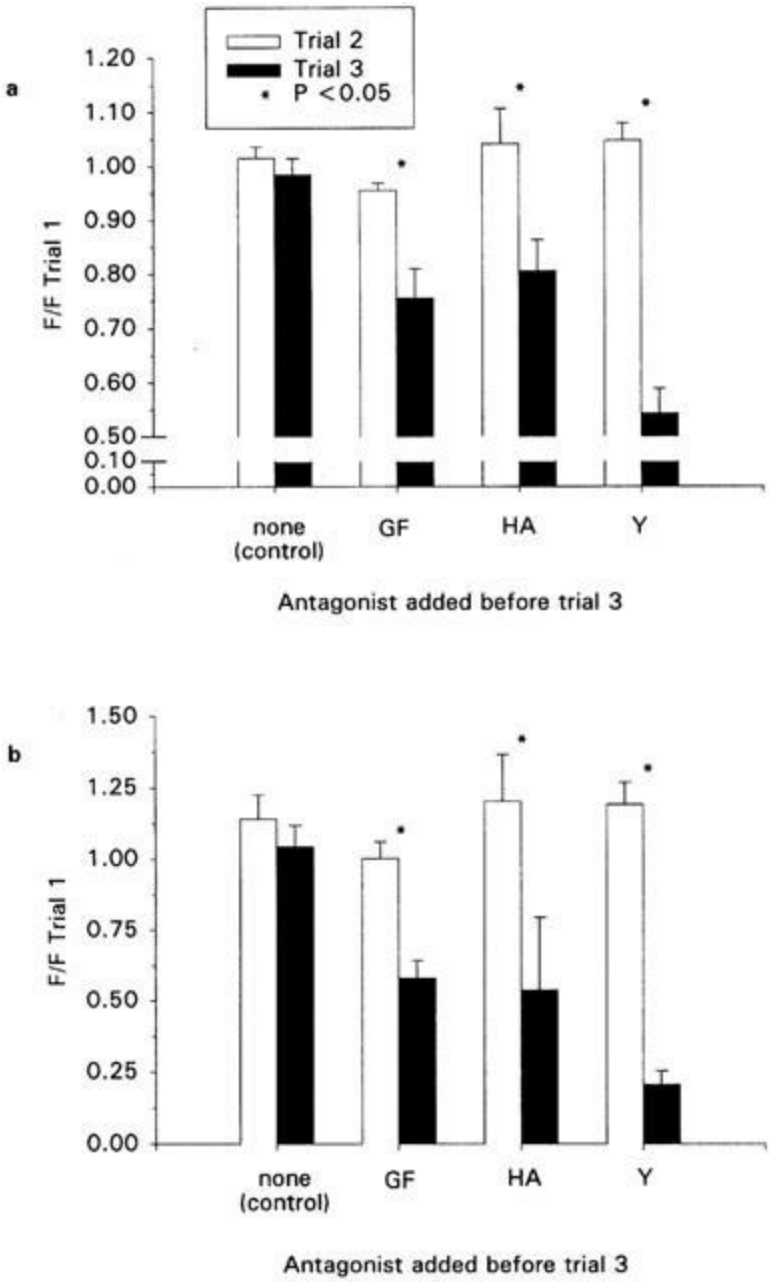

Figure 4 Comparisons of maximum force values (a) and half-maximum force values (b) produced by Trial 2 and Trial 3 single-dose carbachol stimulus in detrusor muscle strips. Tissues were either not treated (control) or treated with an antagonist for 20 min before Trial 3. Data are means+s.e.mean. Control, n=7; 10^{-6} M GF-109203X (GF), n=4; 10^{-5} M HA-1077 (HA), n=6; 3×10^{-6} M Y-27632 (Y), n=5.

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Unlike fundus, in the detrusor, the $PKC_{c,n}$ inhibitor, GF-109203X, inhibited contractions produced by both a maximum (Figure 4a) and EC_{50} (Figure 4b) concentration of carbachol.

Effects of antagonists on phenylephrine-induced cumulative concentration-response curves (CRCs) in fundus

Although muscarinic receptor activation is the primary autonomic receptor responsible for fundus smooth muscle contraction, α-adrenoceptor activation may also participate (Bulbring & Tomita, 1987). Thus, we examined the effects of inhibitors of ROK and $PKC_{c,n}$ to determine the relative contribution of these enzymes to contractions produced by the α-adrenoceptor agonist, phenylephrine. Original experimental traces of a typical contractile response show that cumulative addition of phenylephrine does cause contraction of fundus (Figure 5a). However, this tissue was not highly responsive to phenylephrine (insert, Figure 5c), given that the maximum contraction was, on average, ~0.3-fold that produced by KCl, and the potency was 7 μM, a value equal to that in ovarian artery, but over 10 fold less potent than thoracic aorta (Oriowo *et al*., 1987). The α-adrenoceptor blocker, phentolamine, produced a marked rightward shift in the phenylephrine CRC without producing a reduction in the maximum response (Figure 5a *vs* b; 5c and phentolamine in Figure 6).

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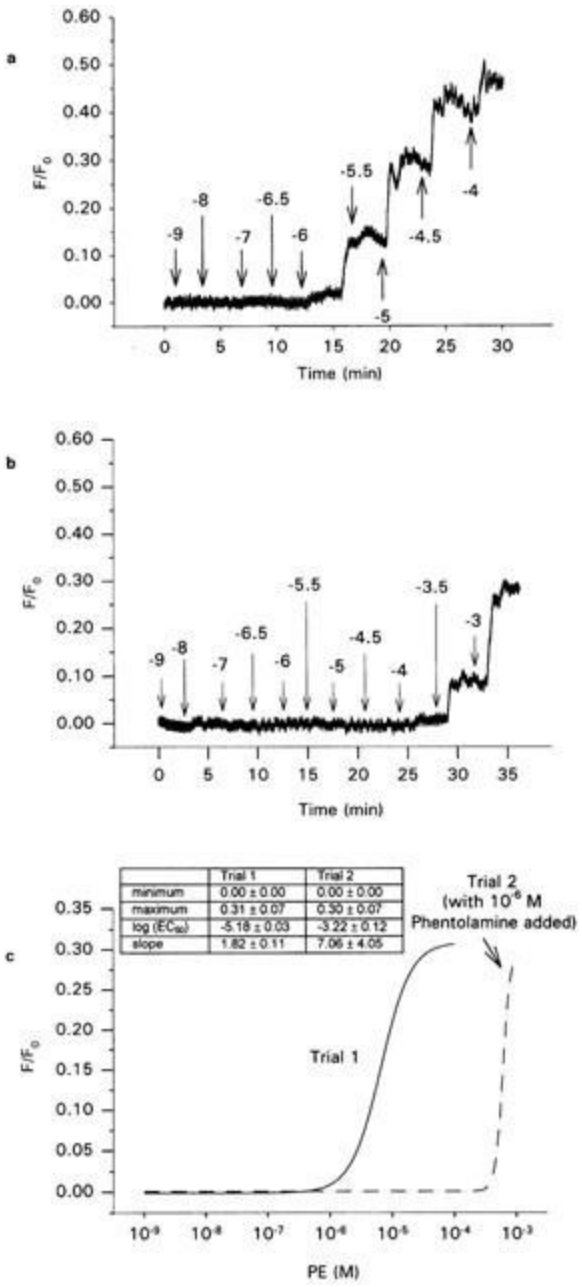

Figure 5 Typical force tracings produced by fundus muscle strips in response to the cumulative addition of phenylephrine in the absence (a) and presence (b) of 10^{-6} M phentolamine. Arrows and numbers indicate when phenylephrine (concentration shown in −log units) was added. After a first phenylephrine concentration-response curve (CRC) was generated (a), tissues were washed for 30 min to cause relaxation, and either no drug (control) or phentolamine was added for at least 20 min (not shown). A second phenylephrine CRC was then generated (b). Data from Trial 1 and Trial 2 CRCs were fitted to a sigmoidal curve characterized by the four parameters

shown in the insert of (c, *n*=4). A similar protocol was used to examine the effects of atropine, capsaicin, GF-109203X, HA-1077 and Y-27632 (see Figure 6).

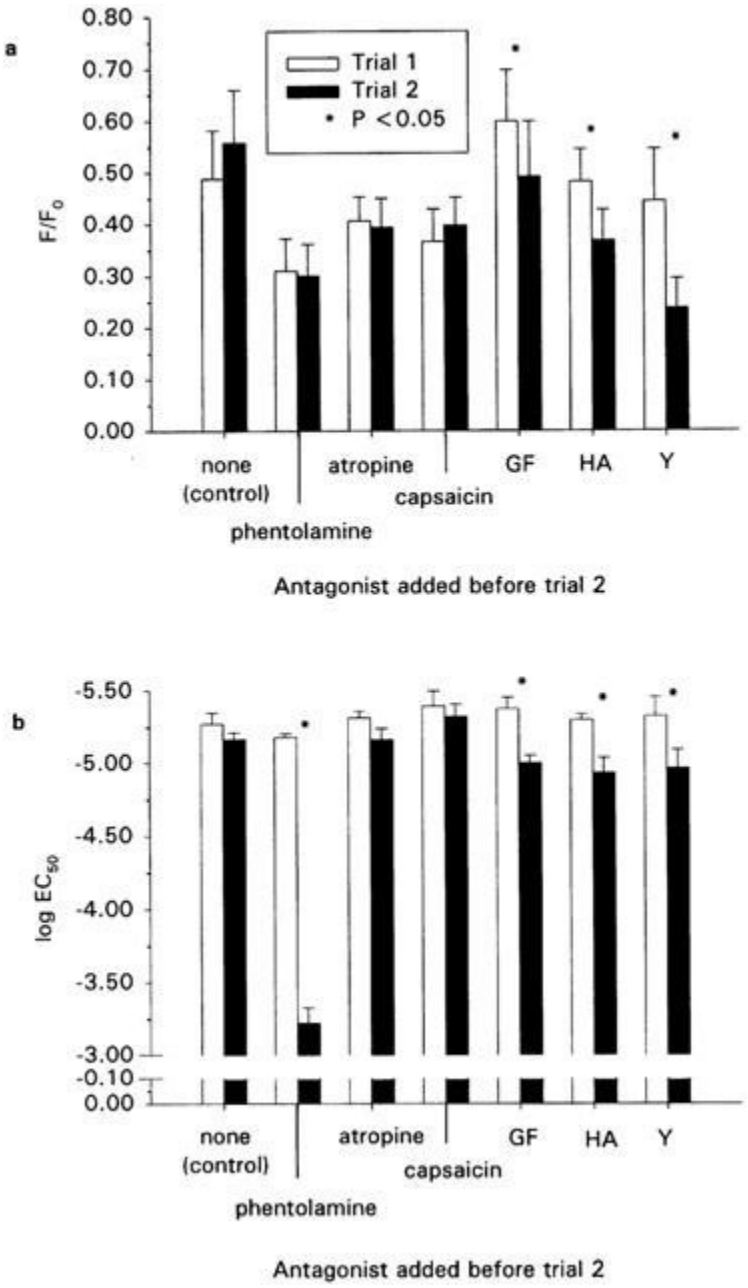

Figure 6 PE cumulative response curves. Comparisons of maximum force values (a) and EC_{50} values (b) produced by Trial 1 and Trial 2 phenylephrine CRC in fundus muscle strips (see Figure 5 legend for protocol). Tissues were either not treated (control) or treated with an antagonist for at least 20 min (60 min for capsaicin) before Trial 2. Data are means+s.e.mean. Control,

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n=15; 10^{-6} M phentolamine, *n*=4; 10^{-7} M atropine, *n*=4; 10^{-6} M capsaicin, *n*=4; 10^{-6} M GF-109203X (GF), *n*=8; 10^{-5} M HA-1077 (HA), *n*=7; 3×10^{-6} M Y-27632 (Y), *n*=7.

The muscarinic receptor blocker, atropine, and the vallinoid receptor agonist and sensory nerve toxin, capsaicin, had no effect on phenylephrine CRCs in rabbit fundus (Figure 6). These data support the contention that phenylephrine caused contraction by stimulation of α-adrenoceptors, and not by indirectly causing release of motor or sensory nerve neurotransmitters. The $PKC_{c,n}$ blocker, GF-109203X and the ROK blockers, HA-1077 and Y-27632 reduced the maximum response (Figure 6a) and produced a small but significant reduction in the potency (Figure 6b) of phenylephrine contractions.

Effects of antagonists on contractions produced by single doses of phenylephrine in fundus

As seen with carbachol stimulation in both fundus and detrusor when using the single-dose protocol, phenylephrine produced three identical maximum contractions when stimulated with maximum and EC_{50} concentrations, such that second (Trial 2) and third (Trial 3) contractions, when normalized to the first (Trial 1) contraction, were not different (control, Figure 7).

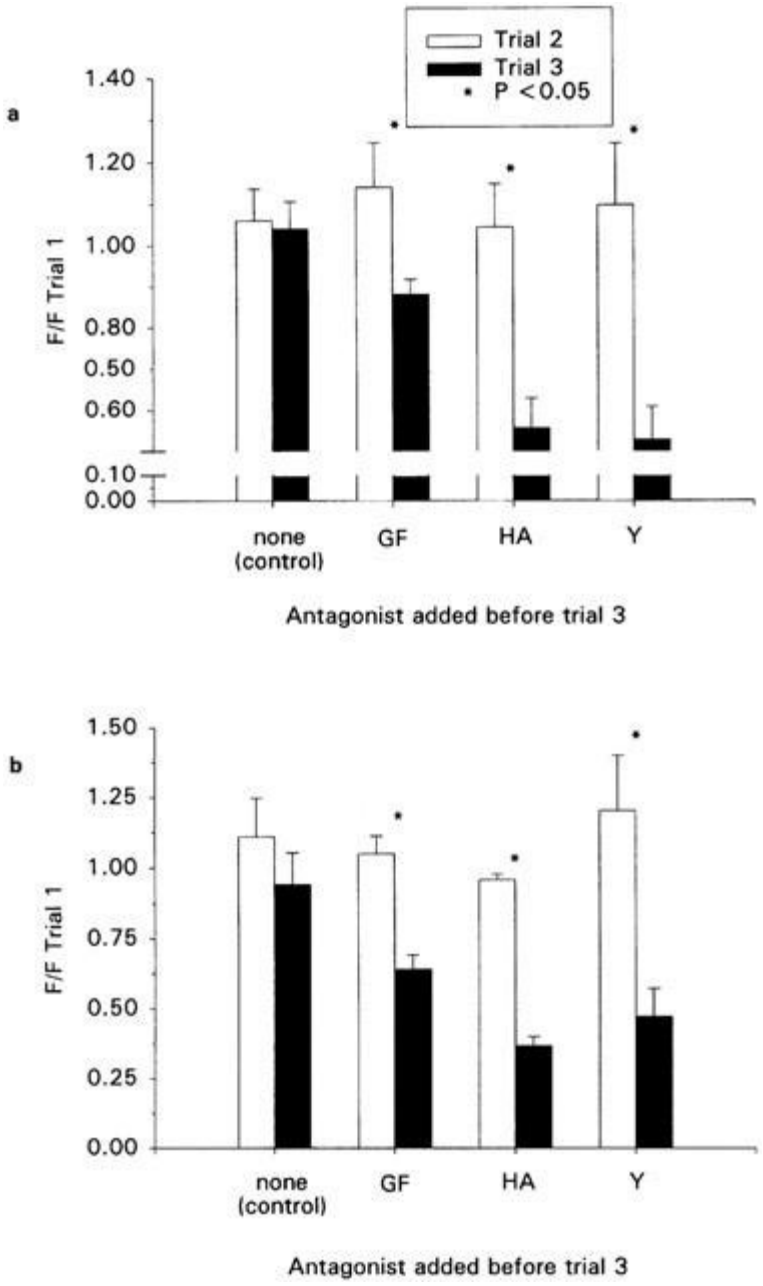

Figure 7 PE single dose responses. Comparisons of maximum force values (a) and half-maximum force values (b) produced by Trial 2 and Trial 3 single-dose phenylephrine stimulus in fundus muscle strips. Tissues were either not treated (control) or treated with an antagonist for 20 min before Trial 3. Data are means+s.e.mean. Control, n=9; 10^{-6} M GF-109203X (GF), n=6; 10^{-5} M HA-1077 (HA), n=4; 3×10^{-6} M Y-27632 (Y), n=4.

Unlike that seen with carbachol stimulation in fundus, but like that seen with carbachol stimulation in detrusor, the $PKC_{c,n}$ inhibitor,

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GF-109203X, inhibited contractions produced by both a maximum (Figure 7a) and EC_{50} (Figure 7b) concentration of phenylephrine in fundus. Also, as seen with carbachol stimulation in both fundus and detrusor, the ROK blockers HA-1077 and Y-27632, inhibited contractions produced by both a maximum (Figure 7a) and EC_{50} (Figure 7b) concentration of phenylephrine in fundus.

Analysis of tachyphylaxis of carbachol-induced contractions

A well-known phenomenon linked to receptor stimulation is tachyphylaxis, a reduction in contractile responsiveness resulting from the continued occupation of a particular receptor by an agonistic ligand that is often linked to receptor (Benovic *et al*., 1988) or post-receptor (Ratz, 1995) down-regulation. Because we used two distinct activation protocols in this study that differed in the duration of receptor stimulation, and we examined multiple stimulations, we could use these data to compare the degree of tachyphylaxis caused by exposure to carbachol in fundus and detrusor. Moreover, because tissues were activated with carbachol, a receptor stimulus, as well as KCl, an agent that bypasses receptors, we could obtain information about tachyphylaxis potentially caused by post-receptor down-regulation (Ratz, 1995; 1999; Ratz *et al*., 1995; 1996).

Detrusor displayed classical tachyphylaxis of response, in that the maximum contraction produced by the maximum concentration of carbachol during a carbachol CRC was significantly weaker than the maximum concentration produced by an equivalent carbachol concentration added as a single-dose stimulus (Figure 8a, detrusor). Interestingly, fundus displayed the opposite response. The single-dose maximum stimulus produced a weaker maximum contraction than did the equivalent concentration of carbachol when employed using a CRC (Figure 8a, fundus), implying that in fundus, exposure to carbachol causes up-regulation rather than down-regulation of responsiveness. This up-regulation may be due to a post-receptor phenomenon because, in fundus, KCl produced a significantly greater contraction after compared to before carbachol exposure, while KCl produced the same degree of contraction before and after exposure to carbachol in detrusor (Figure 8b).

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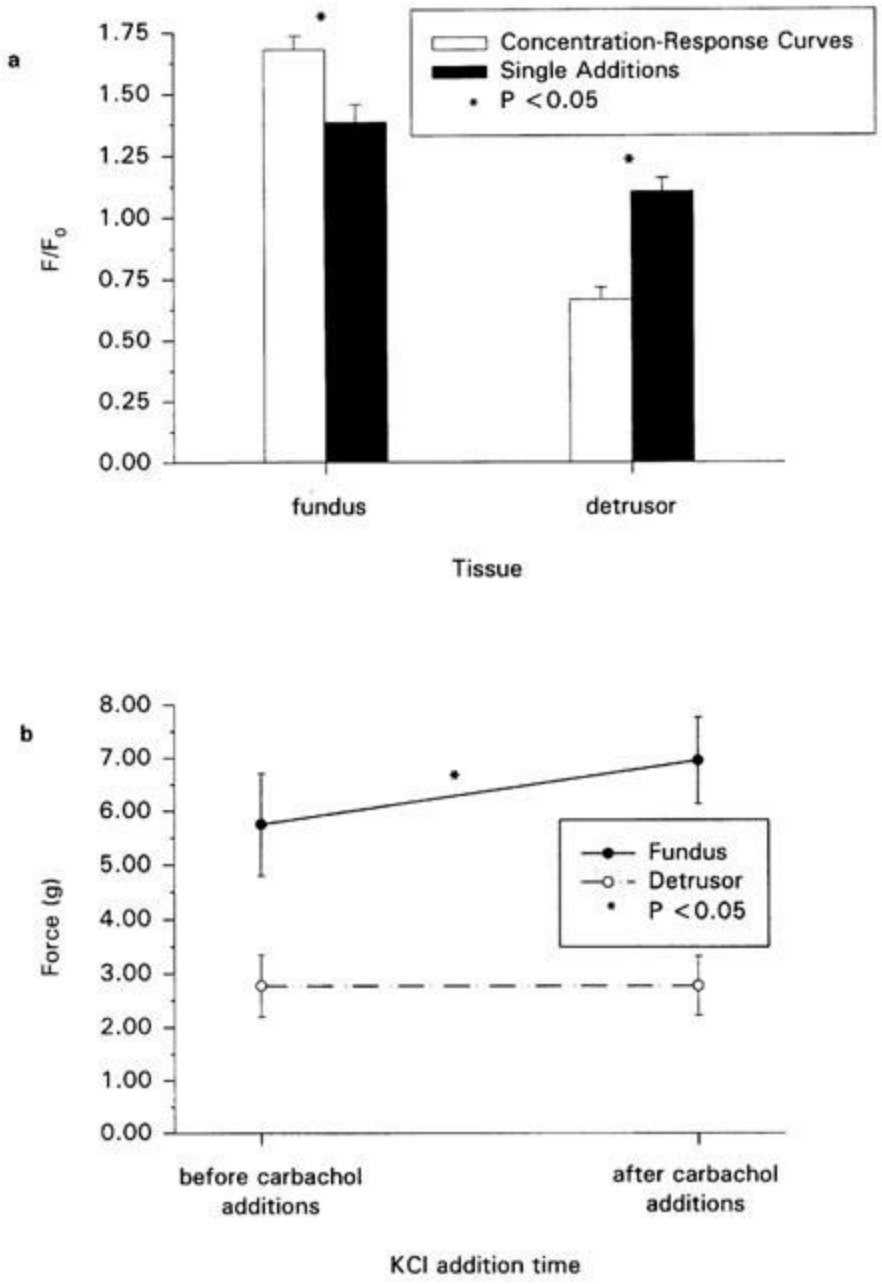

Figure 8 Comparison of the maximum force responses produced in fundus and detrusor strips when contracted by (a) the maximum concentration of carbachol using the two different protocols in this study, concentration-response curve (CRC) and single-dose stimulation, and (b) 109 mM KCl before and after stimulation with three single-dose activations of 3×10^{-6} M carbachol. In (a), the maximum force value was derived from the first CRC, and the maximum force produced in response to the first single-dose carbachol at 3×10^{-6} M was used. Data in (a) are average values+s.e.mean, n=14 for fundus data from CRCs, n=8 for fundus single-dose stimulus, n=4

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from detrusor CRCs, *n*=7 from detrusor single-dose stimulus. Data in (b) are average±s.e.mean. Fundus *n*=9, detrusor *n*=7.

Discussion

This study used selective kinase inhibitors to identify the degree of participation of ROK- and PKC-induced Ca^{2+}-sensitization in muscarinic and α-adrenoceptor stimulated contraction of isolated strips of rabbit stomach fundus. The data support the hypothesis that both ROK and PKC participated in fundus contraction. However, ROK participated in muscarinic receptor-induced contraction and α-adrenoceptor-induced contraction, while PKC participated only in α-adrenoceptor-induced contraction. By comparison, muscarinic receptor-induced contraction of bladder wall (detrusor) smooth muscle involved ROK and PKC. This study also revealed that muscarinic receptor stimulation caused up-regulation of fundus contraction that appeared to be caused by post-receptor mechanisms.

It is now known that ROK and conventional and novel PKC isoforms ($PKC_{c,n}$) play prominent roles in regulation of smooth muscle contraction (Somlyo & Somlyo, 2000; Eto *et al*., 2001; Pfitzer, 2001), but the relative contribution that each makes in regulation of force in the many diverse smooth muscle types is only now being revealed. The gut represents a complex organ system comprised of multiple types of smooth muscle. It is evident that ROK participates in regulation of ileal smooth muscle contraction, but whether ROK plays a role in regulation of vertebrate stomach smooth muscle remains to be determined. Our data using the selective ROK inhibitors, Y-27632 and HA-1077 (Uehata *et al*., 1997; Davies *et al*., 2000; Sward *et al*., 2000), support the hypothesis that ROK participates in development of maximum contraction of rabbit stomach fundus produced by both the muscarinic receptor agonist, carbachol, and the α-adrenoceptor agonist, phenylephrine. These data provide *in vitro* support of the *in situ* finding that Y-27632 reduces gastric motility in rat (Tomomasa *et al*., 2000). Moreover, our data demonstrate that stimulation of muscarinic receptors does not produce activation of identical subcellular signalling systems in chicken gizzard and rabbit stomach fundus smooth muscles, because Y-27632 had no effect on chicken gizzard contraction (Anabuki *et al*., 2000), but produced significant inhibition of rabbit fundus smooth muscle in the present study.

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Both ROK and $PKC_{c,n}$ may participate in regulation of smooth muscle contraction by causing an increase in the Ca^{2+} sensitivity of contraction through inhibition of myosin light chain phosphatase. However, the targets of these enzymes are different in that ROK acts by phosphorylating the large regulatory subunit of myosin phosphatase (MYPT) and by directly phosphorylating serine-19 of the 20 kDa myosin light chains (Hartshorne *et al*., 1998; Woodsome *et al*., 2001), whereas $PKC_{c,n}$ acts by phosphorylating and activating an inhibitor of myosin phosphatase, CPI-17 (Eto *et al*., 2001; Woodsome *et al*., 2001). Our data showed that α-adrenoceptor stimulation produced a weak contraction of rabbit stomach smooth muscle that was inhibited by ROK inhibitors and by GF-109203X, an inhibitor of $PKC_{c,n}$ (Gailly *et al*., 1997; Eto *et al*., 2001). Thus, although muscarinic receptor stimulation of rabbit fundus smooth muscle does not appear to cause contraction through activation of $PKC_{c,n}$, α-adrenoceptor activation does. Moreover, we found that GF-109203X inhibited detrusor smooth muscle contractions produced by muscarinic receptor stimulation, indicating that the signal transduction events leading from muscarinic receptor stimulation to contraction differ in detrusor and fundus.

Tachyphylaxis is a general phenomenon describing down-regulation of a cell signalling system, that when applied to smooth muscle contraction, refers to reductions in contractile activity (Furchgott, 1955). Stimulation of receptors can lead to tachyphylaxis, which may reflect receptor down-regulation (Benovic *et al*., 1988) or post-receptor down-regulation (Ratz, 1995). In the present study, exposure of detrusor smooth muscle to multiple concentrations of carbachol in a continuous fashion to establish a cumulative concentration-response curve (CRC) led to tachyphylaxis. That is, a maximum concentration of carbachol produced a stronger contraction when tissues were exposed only briefly to that single concentration than when tissues were subjected to a cumulative CRC. Surprisingly, fundus produced the opposite response; brief stimulation with a single concentration produced a weaker response than that produced by a cumulative CRC. The finding that KCl-induced contractions were greater in fundus smooth muscle after, as compared to before, the tissue had been exposed to carbachol suggests that carbachol induced up-regulation of post-receptor signalling systems leading to contraction. These data together support the notion that the

fundamental signalling system linking muscarinic receptor stimulation with contraction of fundus and detrusor is different. The implication of these results is that these differences may be utilized to design therapeutic treatments of disorders involving gastrointestinal smooth muscle that may be free of adverse effects related to actions on other smooth muscle systems.

Precisely how prolonged or repeated stimulation of muscarinic receptors lead to up-regulation of fundus was not identified in the present study. However, it is tempting to speculate that either Ca^{2+} signalling or Ca^{2+} sensitivity were involved. For example, the sacoplasmic reticulum of smooth muscle regulates the degree of Ca^{2+} that can be made available to the cytosol (Lee *et al*., 2002), and up-regulation of force produced by fundus may have been due to enhanced loading of sarcoplasmic reticular Ca^{2+} stores. Up-regulation of force may also have been due to enhanced activity of an enzyme activated by muscarinic receptors that is responsible for Ca^{2+} mobilization, such as phospholipase C (Schmidt *et al*., 1998). An alternative hypothesis is that up-regulation was due to enhancement in the sensitivity of contractile proteins to Ca^{2+}. Several enzymes in addition to ROK and PKC, including ILK (Deng *et al*., 2001), ZIP-like kinase (MacDonald *et al*., 2001; Niiro & Ikebe, 2001), MAPKAP kinase-2 (Komatsu & Hosoya, 1996), and PAK (van eyk *et al*., 1998), have recently been identified that directly or indirectly elevate the degree of myosin light chain phosphorylation independently of an increase in $[Ca^{2+}]_i$. However, whether any of these enzymes were involved in up-regulation of fundus remains to be determined. Moreover, because it has been proposed that ZIP-like kinase and possibly other kinases may be required downstream from ROK in order for ROK to regulate smooth muscle force (MacDonald *et al*., 2001), the present study cannot rule out the participation of additional kinases in regulation of fundus contraction.

In conclusion, these data suggest that ROK plays a critical role in the regulation of carbachol- and phenylephrine-induced contractions in rabbit fundus smooth muscle, and that PKC participates in phenylephrine- but not carbachol-induced contraction. These results are in contrast to that seen in chicken gizzard smooth muscle, in which ROK is reported to exist but not to play a role in muscarinic receptor-induced contraction. By contrast, both ROK and PKC appear to

participate in regulation of carbachol-induced contraction of rabbit detrusor. Moreover, carbachol appeared to produce a long-lasting up-regulation of contraction of fundus, but not detrusor, that may have involved post-receptor signalling events.

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Abbreviations

CRC	concentration-response curve
EC_{50}	concentration producing half-maximum contraction
$PKC_{c,n}$	conventional and novel isoforms of protein kinase C
ROK	RhoA kinase

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