Tyrosine kinase-dependent modulation of calcium entry in rabbit colonic muscularis mucosae

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Hatakeyama, N., D. Mukhopadhyay, R. K. Goyal, and H. I. Akbarali. Tyrosine kinase-dependent modulation of calcium entry in rabbit colonic muscularis mucosae. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1780–C1789, 1996.—We studied the role of tyrosine kinase in the regulation of Ca^{2+} entry in single smooth muscle cells of the rabbit colonic muscularis mucosae using the whole cell patch-clamp technique. Step depolarization to +10 mV from a holding potential of − 60 mV produced inward currents that were abolished by 1 μM nifedipine, consistent with the activation of L-type Ca^{2+} channels. The tyrosine kinase inhibitors, genistein and tyrphostin B42, dose dependently inhibited these Ca^{2+} currents. The inactive analogue of tyrphostins, tyrphostin A1, did not affect the currents at concentrations of up to 100 μM. Conversely, the tyrosine phosphatase inhibitor, orthovanadate, enhanced peak Ca^{2+} currents by 30%. Spontaneous transient outward currents (STOCs) (50–600 pA) were elicited with high K^+ in the pipette and at 0-mV holding potential. STOCs were activated due to release of Ca^{2+} from intracellular stores, required the presence of extracellular Ca^{2+} concentration, and were insensitive to nifedipine. Genistein abolished STOCs; however, in its presence, outward currents activated by caffeine or carbachol were not affected. The refilling of the Ca^{2+} stores was studied by first depleting intracellular Ca^{2+} with carbachol in Ca^{2+}-free media followed by reperfusing with a Ca^{2+}-containing solution for 3–5 min. Under these conditions, a second application of carbachol evoked an outward current due to Ca^{2+} release. However, this effect was abolished when the refilling of the stores was carried out in the presence of genistein. Carbachol-evoked currents were not attenuated when the refilling was examined in the presence of orthovanadate. Epidermal growth factor (200 ng/ml) enhanced Ca^{2+} currents by 60% and markedly increased STOCs by over 200%. Western blot analysis, using an anti-phosphotyrosine antibody, showed a tyrosine phosphorylated protein of 60 kDa in control conditions. This was markedly increased after treatment with epidermal growth factor and carbachol. These results suggest that *1*) tyrosine kinase modulates the entry of Ca^{2+} through L-type channels and through nifedipine-resistant pathways involved in refilling of intracellular stores and *2*) stimulation of the kinase by agonists enhances Ca^{2+} entry in the smooth muscle cells of the rabbit colonic muscularis mucosae.

whole cell voltage clamp; tyrosine phosphatase; calcium stores; calcium release-activated current; calcium channels; epidermal growth factor

THE INVOLVEMENT OF TYROSINE kinase(s) in the signal transduction pathway leading to cell proliferation has been widely recognized. More recently, data have been presented that suggest tyrosine kinase may be involved in the signaling associated with receptor-mediated contraction of smooth muscle (7, 11). This evidence has been based on the use of specific tyrosine kinase inhibitors to modulate smooth muscle contractility and the correlation with biochemical studies demonstrating phosphorylation of tyrosine residues of various cellular proteins. DiSalvo et al. (7) showed that structurally unrelated tyrosine kinase inhibitors could reversibly suppress contraction of smooth muscle induced by carbachol or norepinephrine. In addition to the suppression of G protein-coupled contraction, tyrosine kinase inhibitors also suppress growth factor-induced contractions (11), which involve activation of receptor tyrosine kinase. Biochemical studies demonstrate that both types of receptor activation (i.e., G protein coupled and receptor tyrosine kinase) can lead to phosphorylation of tyrosine residues of various cellular proteins, including $pp60^{c\text{-}src}$, in a variety of cell types, including transfected mammalian cells (11). In concert with the actions of the tyrosine kinase inhibitors, vanadate and pervanadate, which are tyrosine phosphatase inhibitors and therefore enhancers of kinase activity, induce contraction of smooth muscle (17). The potential targets for the tyrosine kinase(s) may include direct phosphorylation of ion channels (10, 13, 32) and/or activation of other pathways such as the mitogen-activated protein (MAP) kinase.

The smooth muscle contraction that involves tyrosine kinase(s) requires extracellular Ca^{2+} (9, 11, 34), indicating that tyrosine kinase activation may modulate Ca^{2+} entry. Structurally unrelated tyrosine kinase inhibitors were shown to suppress Ca^{2+} currents in vascular smooth muscle (32).

Tyrosine kinase(s) has also been shown to modulate Ca^{2+} entry that is triggered by intracellular store depletion. In nonexcitable cells, such as platelets and epithelial cells, Ca^{2+} store depletion induced by agonists or by blocking Ca^{2+}-ATPase results in Ca^{2+} entry from the extracellular media. This pathway, termed "capacitative Ca^{2+} entry," has been shown to be suppressed by the tyrosine kinase inhibitors (5, 19, 26). Ca^{2+} entry after store depletion has also been observed in smooth muscle (21, 23, 25, 28, 33). However, little is known of the modulation of this Ca^{2+} entry pathway in smooth muscle.

In the present study, we examined the effects of tyrosine kinase inhibitors on Ca^{2+} entry through nifedipine-sensitive Ca^{2+} channels and through store depletion-activated pathways in the rabbit colonic muscularis mucosae. We measured the activity of Ca^{2+}-dependent K^+ channels (K_{Ca}) as a means of determining the effects of the tyrosine kinase inhibitors on Ca^{2+} release from intracellular stores and capacitative Ca^{2+} entry. Ca^{2+}-dependent K^+ currents have been used as reliable estimates of intracellular Ca^{2+} (16, 28). Numerous studies have shown that in smooth muscle, during whole cell voltage-clamp recordings, spontaneous tran-

sient outward currents (STOCs) are activated by release of Ca^{2+} from intracellular stores (1, 4). These stores may lie in close proximity to the plasma membrane, and random release results in activation of STOCs (22, 29). Thus release of Ca^{2+} from these subsarcolemma stores is not accompanied by smooth muscle contraction (4).

Our results show that, in the colonic muscularis mucosae, structurally unrelated tyrosine kinase inhibitors suppress *1*) voltage-dependent Ca^{2+} currents and *2*) Ca^{2+} entry through nifedipine-insensitive pathways.

METHODS

Cell dispersion. New Zealand White rabbits (1.5–2.0 kg) were anesthetized with ketamine (12.5 mg/kg) and then killed with pentobarbital sodium (32 mg/kg). The distal colon was excised and placed in Tyrode solution gassed with 95% O_2-5% CO_2. The colon was cut along the longitudinal axis, and the muscularis propria was dissected from the underlying muscularis mucosae and the mucosa under a stereo microscope. The muscularis mucosae was then peeled from the mucosa with a pair of fine forceps. Single cells were prepared in a manner similar to that for the esophageal muscularis mucosae (2). Briefly, the tissue was cut into small pieces and incubated in low-Ca^{2+} Tyrode solution containing collagenase (0.25 mg/ml), trypsin (0.3 mg/ml), and bovine serum albumin (1 mg/ml) for 20 min with gentle trituration with a wide-bore fire-polished pipette. The tissues were then transferred to an enzyme-free solution containing bovine serum albumin and again triturated gently until single cells were released. An aliquot of the cell suspension was transferred to the recording chamber on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). All experiments were carried within 4 h of cell dispersion at room temperature except where indicated.

Solutions. The low-Ca^{2+} Tyrode solution contained (in mM) 137 NaCl, 2.7 KCl, 0.01 $CaCl_2$, 0.88 $MgCl_2$, 0.36 NaH_2PO_4, 12 $NaHCO_3$, and 5.5 glucose. The solution was equilibrated with 95% O_2-5% CO_2. For the electrophysiological recordings, an *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)- buffered solution (physiological salt solution) was used (in mM: 135 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 5 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, and 5.5 glucose). When the inward current through Ca^{2+} channels was measured, using Ba^{2+} as charge carrier, $CaCl_2$ was replaced with 2 mM $BaCl_2$. HEPES-buffered solutions were equilibrated with 100% O_2, and the pH was adjusted to 7.4 with NaOH (1 N).

The recording pipettes for the measurement of inward currents were filled with (in mM) 100 cesium aspartate, 30 CsCl, 2 $MgCl_2$, 5 HEPES, 10 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 ATP (sodium salt), and 0.1 GTP. The pH of this solution was adjusted to 7.2 with CsOH. When outward K^+ currents were examined, the pipette solution used contained (in mM) 100 potassium aspartate, 30 KCl, 5 HEPES, 2 $MgCl_2$, 2 ATP (sodium salt), and 0.1 GTP, and the pH was adjusted to 7.2 with KOH.

Collagenase was obtained from Yakult (Tokyo, Japan), and reagents were from Fluka Chemical (Ronkonkoma, NY) and Sigma Chemical (St. Louis, MO). Genistein and cyclopiazonic acid were purchased from Research Biochemicals International (Natick, MA). Tyrphostin B42 and A1 were purchased from Calbiochem (La Jolla, CA).

Electrical recordings. The gigaseal patch-clamp technique was used in the whole cell configuration. The voltage-clamp amplifier was an Axopatch 200A (Axon Instruments). Microelectrodes (World Precision Instruments, 1.00 mm diameter) were prepared on Flaming/Brown horizontal puller (Sutter Instruments). The resistance of these filled patch pipettes was 3–5 MΩ. The pipettes were fire polished with a microforge (MF-83; Narishige, Tokyo, Japan) before use. Membrane currents were monitored on a storage oscilloscope (2522A; BK Instruments) and stored on a microcomputer (Deskpro 33M; Compaq), using an analog-to-digital conversion board (Digidata 1200; Axon Instruments) controlled by pCLAMP 6.0 software (Axon Instruments). The average series resistance in acceptable experiments was <10 MΩ (n > 30). The access resistance remained stable for the duration of the experiment.

Data are reported as means ± SE.

Western blots. Tissue strips (0.5 × 1.0 cm) were placed in Tyrode solution and treated with either epidermal growth factor (EGF; 200 ng/ml) or carbachol (10 μM) for 2 min before freezing in liquid nitrogen. The tissues were then crushed with a homogenizer and solubilized in 50 μl of 2× Laemmli's sample buffer, denatured by heating to 97°C for 10 min and separated by 7.5% polyacrylamide gel electrophoresis. The gel was then transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA). The membrane was then probed with anti-phosphotyrosine antibody PY-10 (ICN, Irvine, CA). Target proteins of this antibody were then visualized with the use of enhanced chemiluminescence, according to the protocol of the manufacturer (Amersham).

RESULTS

Effects of tyrosine kinase inhibitors on Ca^{2+} currents. The average capacitance of single cells from the colonic muscularis mucosae was 73 ± 3 pF (n = 68). In conventional whole cell recordings with Cs^+ in the pipette, inward Ca^{2+} currents were obtained when cells were depolarized positive to −20 mV, from a holding potential of −70 mV. Peak Ca^{2+} currents measured −225 ± 21 pA (n = 15) at +10-mV test potentials and were increased to −356 ± 28 pA (n = 20) when the Ca^{2+} in the bathing solution was replaced with 2 mM Ba^{2+}. The effects of three tyrosine kinase inhibitors, genistein, tyrphostin B42, and its inactive analogue tyrphostin A1, were studied on Ba^{2+} currents (I_{Ba}). The cells were held at −60 mV and depolarized to +10 mV for 300 ms at a rate of 0.2 Hz. Figure 1*A* shows the effect of genistein on I_{Ba}. Genistein decreased the peak current by 70% and did not alter the threshold or the apparent reversal potential of the currents (data not shown). The effects of genistein on I_{Ba} were reversible. Figure 1*B* shows the time course of genistein's effect. The onset of inhibition was rapid (within 2 min) and could be washed out completely within 4–6 min. Genistein and tyrphostin B42 dose dependently inhibited I_{Ba}. Peak I_{Ba} was measured at +10-mV test potential, and the inhibition produced by each agent was examined and plotted (Fig. 1*C*). The inhibition amounted to 62% at 50 μM and was not significantly increased when the concentration of either genistein or tyrphostin B42 was increased to 100 μM (n = 6–8 cells). The effects of genistein on the inward currents were also examined at 32°C (n = 4). At this higher temperature, the effects of genistein only differed at 10 μM, in which the inhibition was enhanced to 34% compared with 17% at room temperature. On the other hand, tyrphostin A1, an inactive analogue of the tyrphostins, was ineffective in

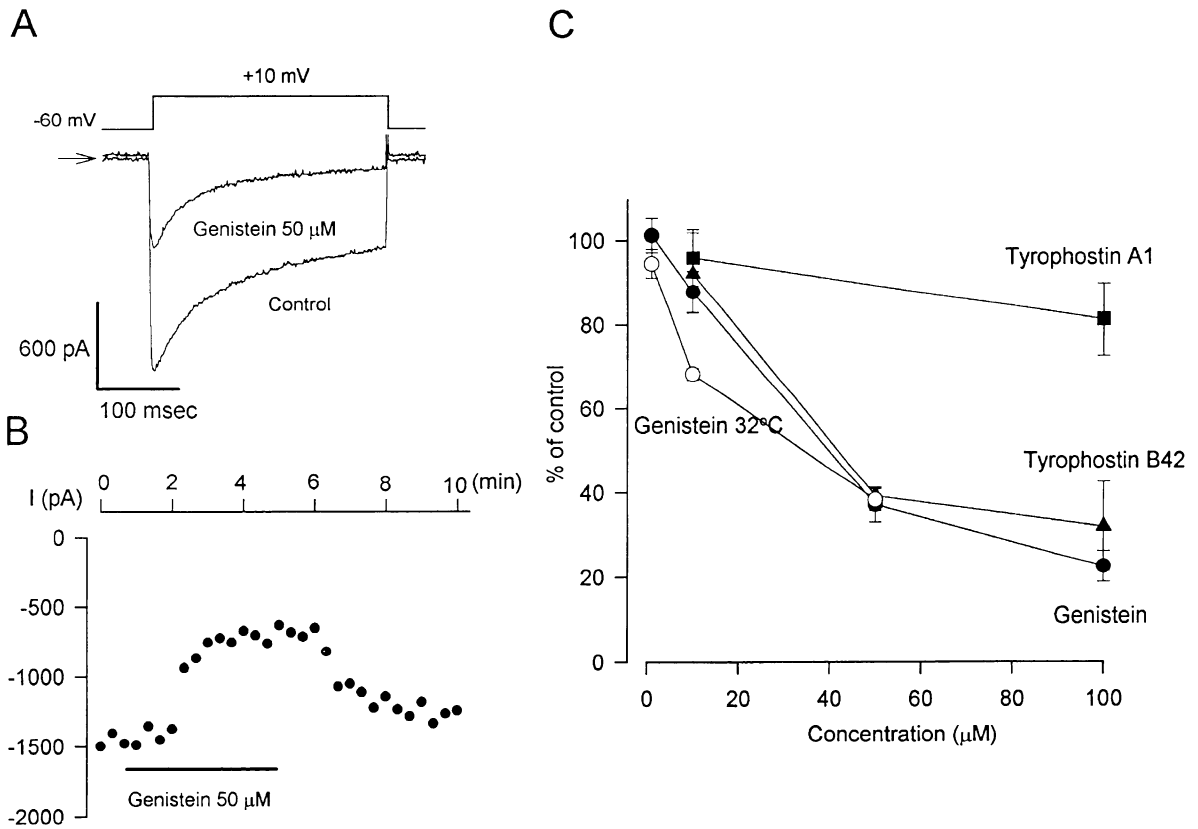

Fig. 1. Effect of tyrosine kinase inhibitors on Ca^{2+} currents. *A*: membrane current recording of inward current in control and in presence of genistein (50 μM). Currents were activated by step depolarization, according to protocol shown. Ba^{2+} (2 mM) was used as a charge carrier. Arrow, zero current level. Currents were recorded at 32°C. *B*: time course of effects of genistein on inward current on same cell as in *A*. Amplitude of peak current was measured at +10-mV test potential every 20 s. Bath application of genistein (50 μM) resulted in inhibition of current within 2 min of perfusion. This effect was reversible. *C*: dose-response relationship of tyrosine kinase inhibitors on peak inward currents. Peak currents were obtained at +10-mV test potential. Percent inhibition for each concentration was measured with respect to current in absence of inhibitor (n = 3–8 cells for each concentration).

blocking the inward currents. At concentrations of 100 μM, the inhibition by tyrphostin A1 was ~10%.

To further determine the role of tyrosine kinase in the modulation of Ca^{2+} currents, we examined the effect of the tyrosine phosphatase inhibitor, sodium orthovanadate (Na_3VO_4; Ref. 8). Figure 2 shows that peak I_{Ba} were enhanced by Na_3VO_4 (300 μM). The inward currents increased from −317 ± 32 to −451 ± 42 pA at a test potential of +10 mV (n = 13), resulting in ~30% enhancement. The current-voltage relationship of the I_{Ba} showed that Na_3VO_4 did not alter either the threshold or the apparent reversal potential.

The suppression of I_{Ba} by structurally unrelated tyrosine kinase inhibitors and enhancement by the tyrosine phosphatase inhibitor suggest that Ca^{2+} channels are likely to be modulated by tyrosine kinase(s). EGF has intrinsic tyrosine kinase activity, and it activates cellular tyrosine kinase. We therefore examined the effects of EGF on I_{Ba}. Figure 3 shows that EGF (200 ng/ml) increased I_{Ba} from −247 ± 32 to −396 ± 43 pA (n = 11), representing an increase of ~60%. The effects of EGF were not observed in the presence of genistein.

Effects of tyrosine kinase inhibitors on STOCs. STOCs were obtained in cells dialyzed with K^+-containing solutions. Holding the cell at 0 mV elicited STOCs, as has been reported in a large number of vascular and visceral smooth muscle cells. These outward currents were blocked by tetraethylammonium and were absent in cells dialyzed with Cs^+, which is indicative of K^+ channel activation. The amplitude of STOCs in the colonic muscularis mucosae ranged from 50 to 600 pA. Figure 4 shows the dependence of STOCs on external Ca^{2+}. STOCs were abolished when the bathing solution contained high EGTA (Fig. 4*A*) or when cells were perfused with nominally free Ca^{2+} (Fig. 4*B*), suggesting the requirement for extracellular Ca^{2+} in the generation of STOCs. To determine whether Ca^{2+} entry was due to voltage-dependent Ca^{2+} channels, we examined the effects of nifedipine on the ability to decrease STOCs. Up to 1 μM nifedipine did not significantly change the amplitude or frequency of STOCs (Fig. 4*C*; n = 5). Similar results were obtained in the presence of verapamil (10 μM; data not shown). STOCs in the colonic muscularis mucosae cells are also dependent on Ca^{2+} stores. Thus bath-applied caffeine (10 mM) induced a large outward current with subsequent block of STOCs. In addition, carbachol also activated a similar pattern in that it induced an outward current and abolished STOCs (Fig. 5*A*). STOCs did not return in the

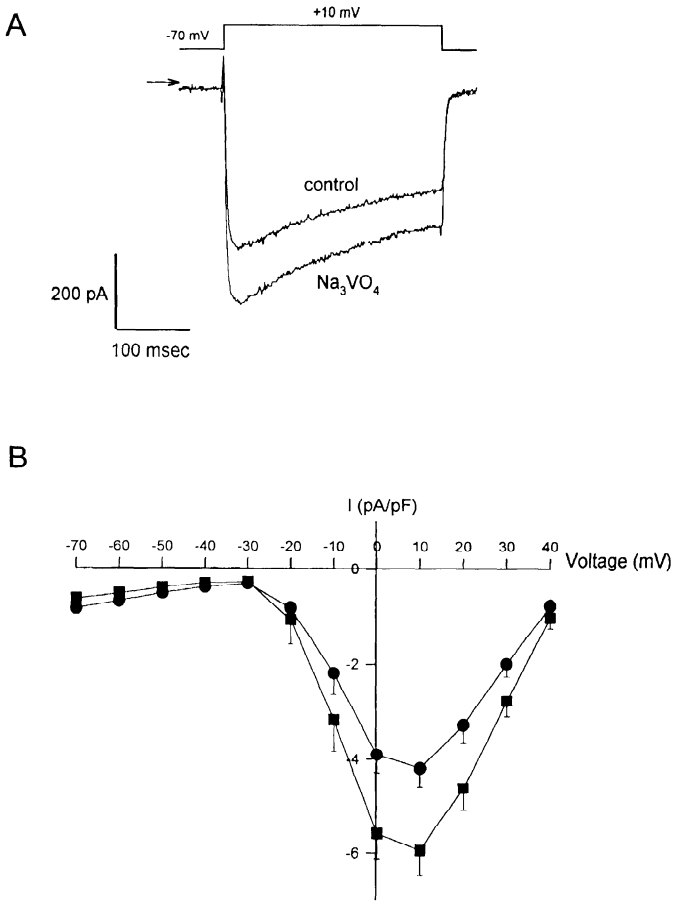

Fig. 2. Effect of sodium orthovanadate (Na_3VO_4) on Ca^{2+} currents. *A*: traces show activation of current, using Ba^{2+} as a charge carrier (using protocol shown). Currents are shown for control and in presence of Na_3VO_4 (0.6 mM). *B*: current-voltage relationship for control (●) and in presence of Na_3VO_4 (■); $n = 13$.

continued presence of carbachol; however, STOC activity was restored within 2 min of washout. Because carbachol is known to release intracellular Ca^{2+} via generation of inositol 1,4,5-trisphosphate (IP_3), we examined the effect of carbachol in the presence of heparin, a blocker of IP_3 receptors on the sarcoplasmic reticulum. Figure 5*B* shows that when 2 mg/ml heparin were dialyzed into the cells via the patch pipette, carbachol failed to induce an outward current; however, caffeine readily activated outward currents. This effect was observed in all 11 cells examined in this fashion. Interestingly, heparin did not appear to significantly affect the amplitude of STOCs, which ranged from 200–600 pA ($n = 11$). Application of carbachol, however, resulted in a decrease in the amplitude of STOCs by ~20%. This may reflect a direct effect of muscarinic suppression of Ca^{2+}-activated K^+ channels (6). Further studies will be required to identify the cellular mediators of spontaneous Ca^{2+} release and STOC generation. However, these results together suggest that STOCs are dependent on intracellular Ca^{2+} release from stores that are replenished by extracellular Ca^{2+}. A Ca^{2+} entry pathway other than voltage-dependent Ca^{2+} channel may be involved.

To determine whether such a pathway is modulated by tyrosine kinase, we examined the effect of genistein and Na_3VO_4 on STOCs. Figure 6*A* shows that genistein (0.1 mM) completely inhibited spontaneous outward currents. This effect of genistein was observed in over 30 cells. On the other hand, Na_3VO_4 markedly enhanced STOC activity (Fig. 6*B*). Both the amplitude and frequency appeared to be increased. To determine the dose relationship of this effect, we measured the amount of charge transferred (current × time) over a 2-min period before addition of Na_3VO_4 and for 2 min in its presence. The percentage of increase by Na_3VO_4 at 0.2, 0.3, and 0.6 mM is shown in Fig. 6*C*.

There are three possible explanations for the inhibition of STOCs by genistein. *1*) Genistein could be directly blocking Ca^{2+}-activated K^+ channels, *2*) genistein could be affecting the release of Ca^{2+} from the stores, and *3*) genistein may be blocking the Ca^{2+} entry required for refilling the stores.

To determine if genistein was affecting Ca^{2+}-activated K^+ channels or the stores, we examined the effect of caffeine in the presence of genistein. Figure 7*A* shows that, in the presence of genistein, although STOCs were completely inhibited, caffeine-induced outward currents due to release of intracellular Ca^{2+} were not abolished. The activation of the outward current by caffeine also suggests that Ca^{2+}-activated K^+ channels were not inhibited by genistein. The amount of charge

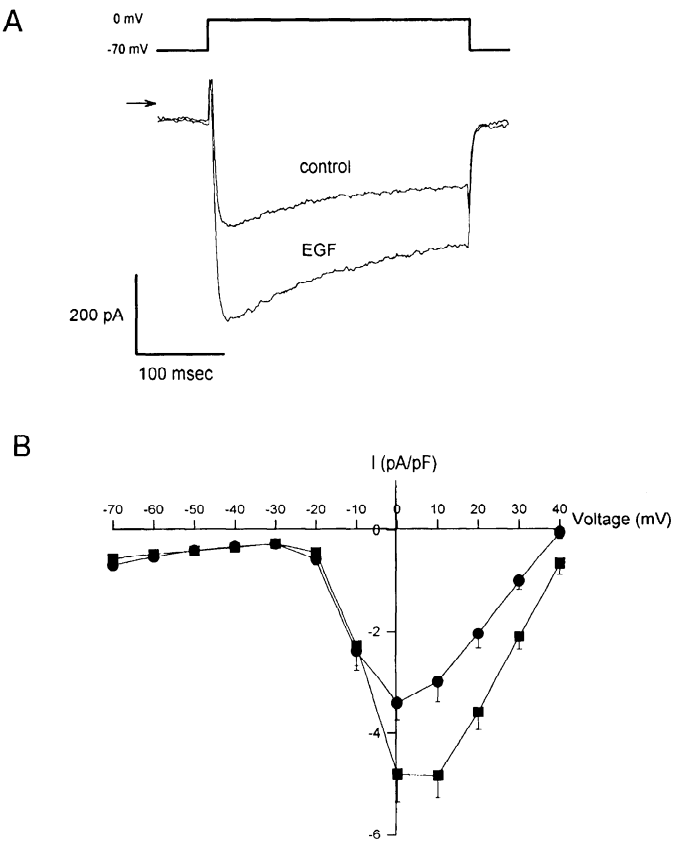

Fig. 3. Effect of epidermal growth factor (EGF) on Ca^{2+} currents. *A*: traces show enhancement of current by EGF (200 ng/ml); voltage protocol is also shown. *B*: current-voltage relationship in absence (●) and presence (■) of EGF (200 ng/ml); $n = 11$.

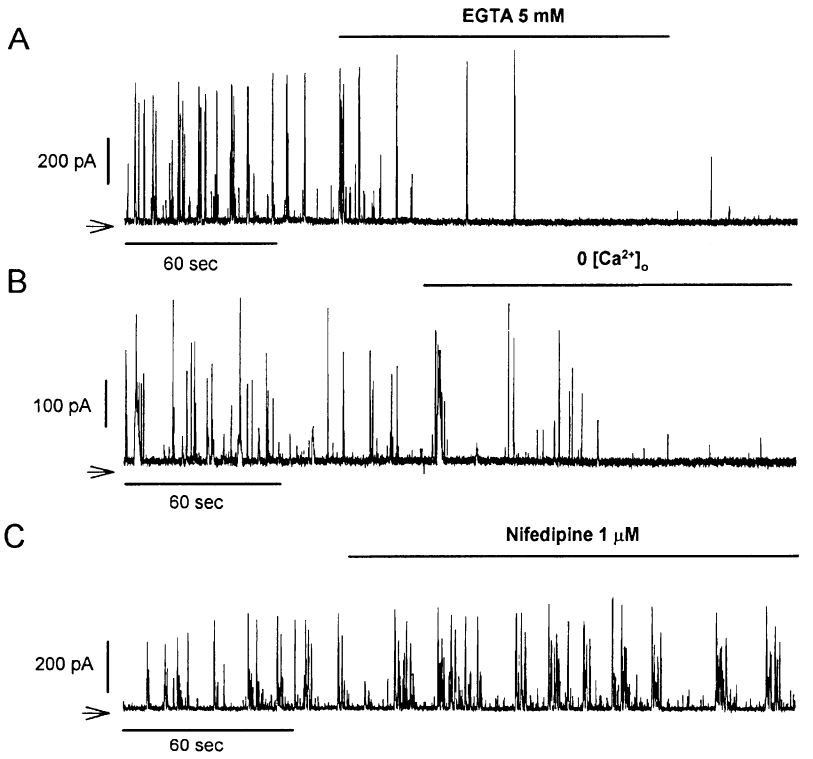

Fig. 4. Spontaneous transient outward currents (STOCs) in rabbit colonic muscularis mucosae is dependent on extracellular Ca^{2+}. *A*: inhibition of STOCs by bath-applied EGTA (5 mM). STOCs were obtained at holding potential of 0 mV. Pipette solution contained high-K^+ solution. *B*: inhibition of STOCs by bath application of nominally Ca^{2+}-free Tyrode solution. $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration. *C*: bath-applied nifedipine (1 μM) did not significantly alter amplitude or frequency of STOCs.

transferred in response to caffeine in the presence and the absence of genistein was 0.79 and 0.70 nC, respectively, in the cell shown in Fig. 7. Reproducible responses to caffeine could be produced in all cells in the presence of genistein (n = 5). As shown in Fig. 7*B*, cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase, completely abolished STOCs and in its presence caffeine failed to evoke an outward current. These results indicate that genistein does not deplete intracellular Ca^{2+} or block Ca^{2+}-activated K^+ currents.

To further test the hypothesis that genistein may be affecting Ca^{2+} entry, the colonic muscularis mucosae cells were first perfused with nominally Ca^{2+}-free solution in the presence of 100 μM genistein, which resulted in complete suppression of STOCs. Under these conditions, an outward current to carbachol could be evoked owing to release of intracellular stores. The cells were then perfused with Ca^{2+}-containing solution in the continued presence of genistein. Application of carbachol after 4 min in the presence of genistein failed to

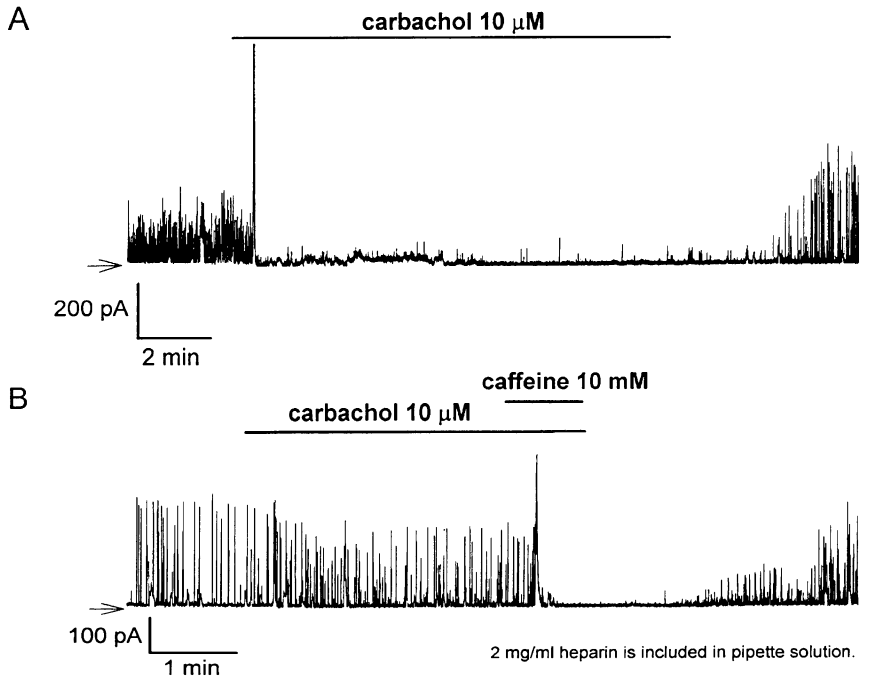

Fig. 5. Intracellular Ca^{2+} release by carbachol and caffeine abolishes STOCs. *A*: currents were recorded at 0-mV holding potential. Bath application of carbachol (10 μM) activated an outward current and subsequently abolished STOCs. STOC activity returned after 2 min of washout of carbachol. *B*: effect of intracellular heparin on Ca^{2+} release. Heparin (2 mg/ml) was included in pipette. Bath application of carbachol reduced amplitude of STOCs but did not evoke any current; however, caffeine (10 mM) induced an outward current and subsequently abolished STOCs.

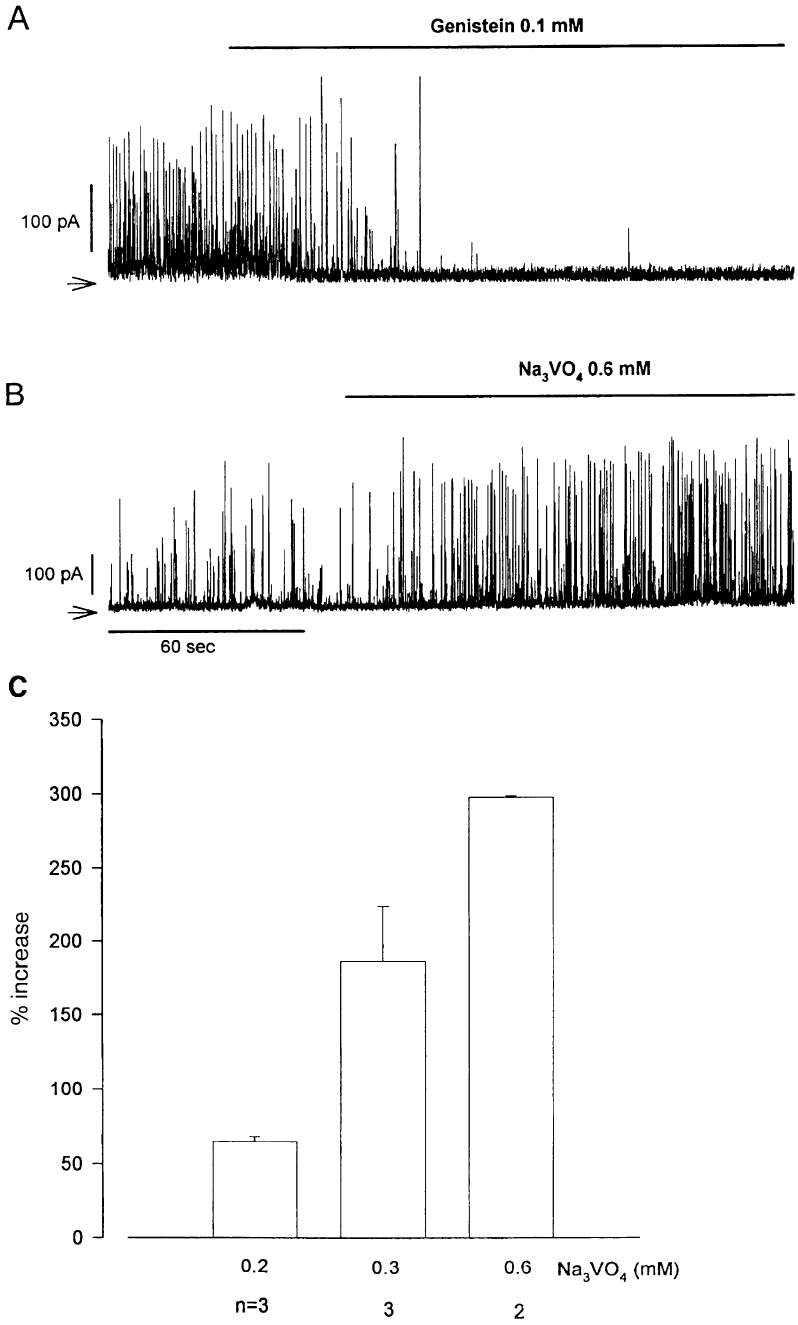

Fig. 6. Effect of genistein and Na_3VO_4 on STOCs. *A*: bath application of genistein (0.1 mM) completely abolished STOCs. Holding potential was 0 mV. Arrowhead, zero current level. *B*: tyrosine phosphatase inhibitor, Na_3VO_4 (0.6 mM), enhanced both frequency and amplitude of STOCs. *C*: dose-dependent enhancement of STOCs by Na_3VO_4. Amount of charge due to STOCs (current × time) was measured 2 min before and 2 min in continued presence of Na_3VO_4.

elicit an outward current, suggesting that filling of the Ca^{2+} stores was inhibited. After a 4-min washout of genistein, carbachol-induced responses were restored, suggesting that there was no desensitization of the muscarinic receptor. Figure 8 shows an example, using the above protocol. As shown in this cell, carbachol responses were abolished in the presence of genistein after depletion of intracellular stores. Similar results were obtained in six cells.

We next tested the effects of Na_3VO_4. As shown in Fig. 9, the same protocol that was used for genistein did not attenuate the responses to carbachol. The amount of charge carried in response to carbachol was 0.17 nC in the absence of extracellular Ca^{2+} and 0.16 nC after Ca^{2+} was readmitted, in the continued presence of Na_3VO_4.

The effects of EGF were also examined on the STOCs. Figure 10 shows an example of an increase in the amplitude and frequency by EGF (200 ng/ml). The charge (current × time) in the presence of EGF increased by 246 ± 45% (n = 6).

Tyrosine phosphorylated proteins. Proteins phosphorylated on tyrosine residues after treatment with EGF or carbachol for 2 min were visualized by immunoblotting with anti-phosphotyrosine antibody PY-10 (Fig 11). These include proteins phosphorylated by receptor autophosphorylation, the various substrates of the receptor protein kinase, and further downstream kinases such as MAP kinase. In control tissue, a constituent of ~60 kDa was readily observed with increased immunostaining in treated samples, reflecting an increase in phosphotyrosine content. The extent of phos-

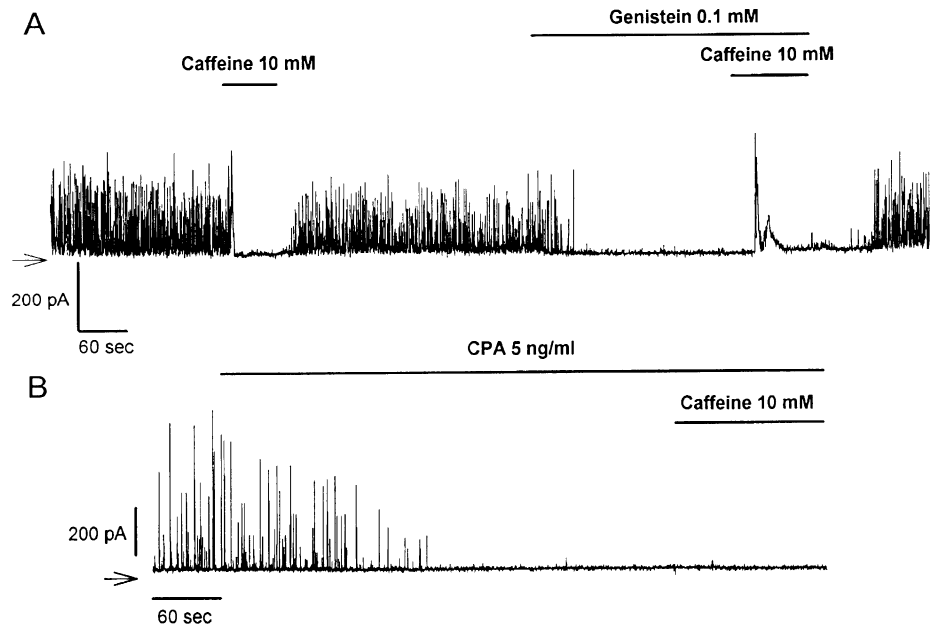

Fig. 7. Release of intracellular Ca^{2+} in presence of genistein. STOCs were obtained at 0-mV holding potential. *A*: application of caffeine (10 mM) produced an outward current and abolished STOCs, which recovered after washout. Genistein (0.1 mM) abolished STOCs, and, in its presence, caffeine (10 mM) evoked an outward current due to intracellular Ca^{2+} release. After washout, STOCs recovered. *B*: STOCs were inhibited by bath-applied cyclopiazonic acid (CPA, 5 ng/ml), a Ca^{2+}-ATPase inhibitor. Bath-applied caffeine failed to evoke a current in presence of CPA.

phorylation was estimated with densitometry. Phosphorylation of the 60-kDa protein increased compared with control by 3.3- and 1.8-fold after treatment with EGF and carbachol, respectively. In the presence of EGF, other components were detected around 180 kDa (presumably reflecting the autophosphorylation of the receptor) and 90 kDa. Additionally, both EGF- and carbachol-treated tissues showed species of 110, 44, and 42 kDa; the latter two components are most likely to be MAP kinases.

DISCUSSION

In this study, we have identified a tyrosine kinase-dependent modulation of Ca^{2+} currents and refilling of intracellular Ca^{2+} stores in the smooth muscle cells of the rabbit colonic muscularis mucosae.

Ca^{2+} currents. The effects of the inhibitors are likely to be because of specific inhibition of tyrosine kinase for the following reasons. *1*) The inhibitions were observed at concentrations that do not block other kinases. Significantly higher concentrations (>400 μM) are required to inhibit appreciably serine/threonine kinase such as protein kinase C (3). *2*) Inhibition of the current was observed by structurally different kinase inhibitors. Moreover, the inactive analogue of tyrphostin, tyrphostin A1, was without effect on the currents. *3*) The tyrosine phosphatase inhibitor, Na_3VO_4, markedly enhanced Ca^{2+} currents. Vanadate, an insulin-mimetic agent, has been shown to increase tyrosine kinase activity by inhibition of phosphotyrosine phosphatase (8) and to produce contractions of smooth muscle that are dependent on extracellular Ca^{2+} (17).

These results suggest that there may be basal tyrosine kinase activity in the rabbit colonic muscularis mucosae that modulates Ca^{2+} channels. Consistent with this hypothesis is data that show high levels of $pp60^{c\text{-}src}$ in smooth muscle (7). We have also identified a 60-kDa phosphotyrosine in basal conditions using the anti-phosphotyrosine antibody. This activity was markedly enhanced by EGF and carbachol, suggesting the

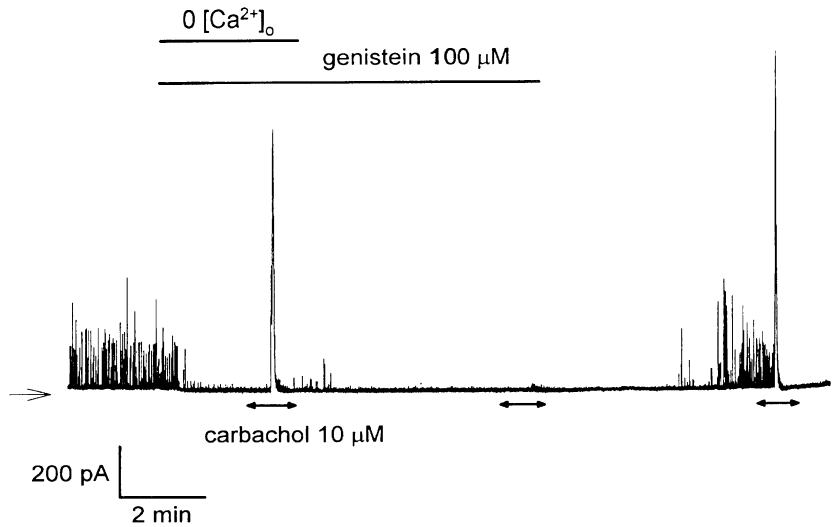

Fig. 8. Genistein blocks refilling of Ca^{2+} stores. STOCs were blocked in presence of genistein and in nominally Ca^{2+}-free solution. Carbachol evoked an outward current in 0 Ca^{2+} solution. Cell was then perfused with normal Tyrode solution (2 mM Ca^{2+}) in continued presence of genistein. After 4 min, carbachol was applied, which failed to evoke any currents. After a further 4 min of washout of genistein, in normal Tyrode solution, bath applied carbachol resulted in a transient outward current.

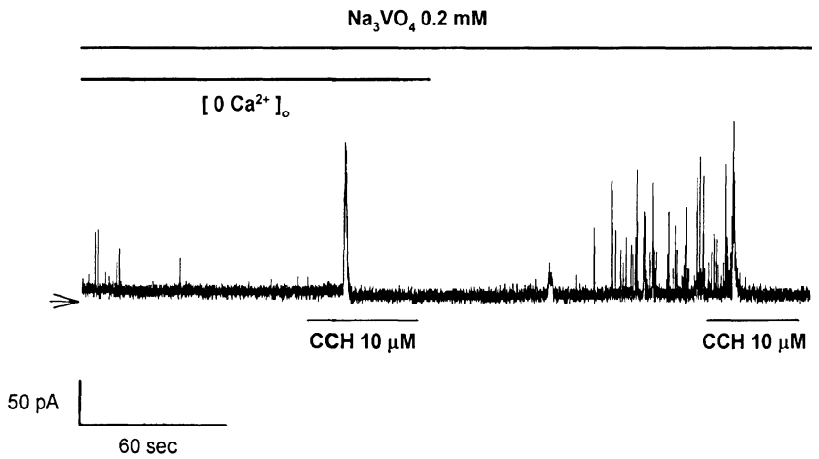

Fig. 9. Effect of Na_3VO_4 (0.2 mM) on refilling of Ca^{2+} stores. Similar protocol was used as in Fig. 8. In presence of Na_3VO_4, carbachol (CCH) responses were not attenuated. Note enhancement of STOCs in presence of Na_3VO_4.

involvement of this protein in the signaling cascade. The 60-kDa protein most likely represents the gene product of cellular *src* (Mukhopadhyay and Akbarali, unpublished observations).

Refilling of Ca^{2+} stores. STOCs are due to K_{Ca} and are activated by release of Ca^{2+} from intracellular stores that may be in close proximity to the plasmalemma K^+ channels (22). A constant recycling of Ca^{2+} is required to maintain the activation of these K^+ currents (28), since both the absence of extracellular Ca^{2+} and/or the depletion of intracellular stores abolishes STOCs. Because nifedipine or verapamil did not block STOCs, Ca^{2+} entry through a non-L-type channel may be involved in activation and refilling of Ca^{2+} stores. It is unlikely that nonselective cation channels could be the route for Ca^{2+} entry, since these channels generally have greater permeability for Na^+ than Ca^{2+} in physiological solutions and reverse at 0 mV, the potential at which we recorded STOCs. STOCs were abolished after intracellular Ca^{2+} release by carbachol and required washout for STOC activity to return. Because the amplitude of STOCs was also reduced by carbachol in the presence of heparin (Fig. 5*B*), it is likely that carbachol may additionally have direct blocking effects on the Ca^{2+}-activated K^+ channels as described in canine colonic smooth muscle (6). However, this effect only amounted to 10–20% of the STOC amplitude, suggesting that other mechanisms, including maintained depletion of intracellular stores, may account for the effects of carbachol. Nevertheless, washout of carbachol resulted in complete restoration of STOC activity.

The inhibition of the STOCs by genistein is not due to effects on the K^+ channels or the intracellular stores, since caffeine and carbachol were both effective in releasing intracellular Ca^{2+} and activating K_{Ca} in the presence of the inhibitor. Our data show that it is the refilling of the subsarcolemma store as well as the "deep sarcoplasmic reticulum" that is most likely modulated by tyrosine kinase. Vostal et al. (30) have proposed that, in platelets, the state of the Ca^{2+} store is modulated by a tyrosine phosphatase such that depletion of the store results in inhibition of the phosphatase, leading to tyrosine phosphorylation of a 130-kDa protein that regulates Ca^{2+} entry. It is conceivable that a similar phenomenon may be operative in smooth muscle cells, since Na_3VO_4 markedly enhanced STOCs. This effect could result from an increase in the Ca^{2+} content of the subsarcolemma sarcoplasmic reticulum and therefore an increase in the extrusion to the plasma membrane. It is possible that vanadate could release Ca^{2+} from the stores, which in turn would trigger further Ca^{2+} entry. However, because neither STOCs nor Ca^{2+} release by carbachol was blocked by Na_3VO_4, it is unlikely that direct release by the phosphatase inhibitor could account for the increase in STOC activity.

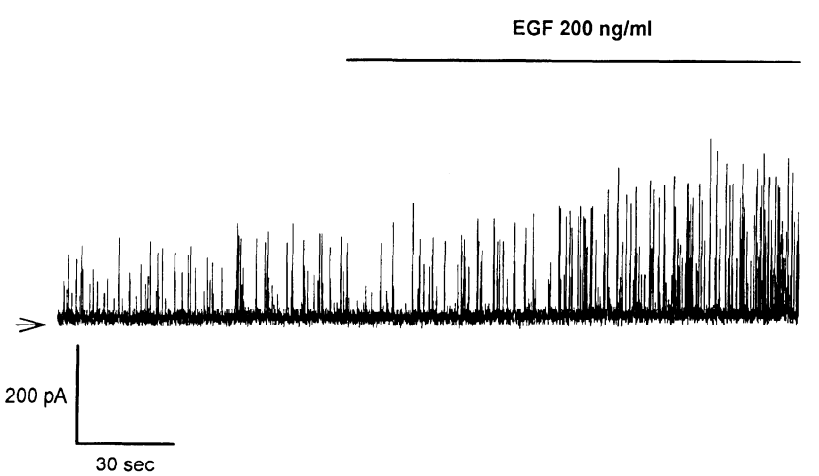

Fig. 10. Effect of EGF on STOCs. Holding potential is 0 mV. EGF enhanced both amplitude and frequency of STOCs.

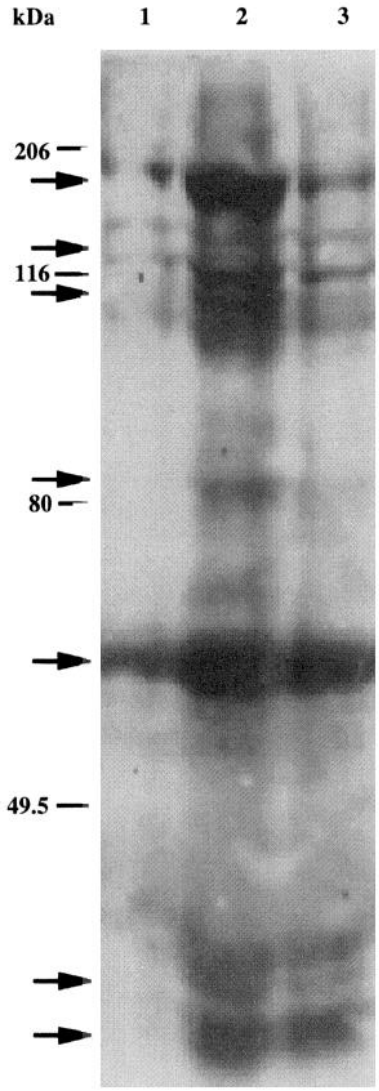

Fig. 11. Western blot analysis of protein tyrosine phosphorylation in rabbit colonic muscularis mucosae. Tissue strips were quick frozen from normal Tyrode (*lane 1*) or after 2-min treatment with EGF (200 ng/ml, *lane 2*) or carbachol (10 μM, *lane 3*) and processed for electrophoresis and Western blot as described in METHODS. Position of molecular mass markers (kDa) is shown at *left* of blot. Proteins specifically phosphorylated on tyrosine residues are shown by arrows. Apparent molecular masses were estimated from standard calibration curves.

Recent studies have identified store-depleted Ca^{2+} entry in nonexcitable cells as a route for Ca^{2+} entry. Currents through the Ca^{2+} release-activated channels (I_{crac}) have been recorded from mast cells, lymphocytes, and endothelial cells (12, 27, 35). Noise analyses indicate unitary conductance of these channels to be ~10 fS in physiological solutions. Thus, under the present recording conditions, signal-to-noise ratio precludes measurement of I_{crac}. Further studies will be required to identify these currents in smooth muscle. Nevertheless, measurement of K_{Ca} provides a reliable indication of the state of the Ca^{2+} stores in smooth muscle and has been extensively studied to identify the role of intracellular Ca^{2+}. When refilling was monitored after complete depletion of the intracellular stores by carbachol in the absence of extracellular Ca^{2+}, genistein prevented activation of the current due to inhibition of the refilling process. Tyrosine kinase modulation of the Ca^{2+} entry after store depletion has been shown in platelets and in epithelial cells (5, 26). Bischof et al. (5) recently showed that, in intestinal epithelial cells, store depletion by carbachol was followed by a genistein-sensitive Ca^{2+} entry. It is noteworthy that a phosphatase has been suggested to be involved in the activation of I_{crac} (24).

Tyrosine kinase has been implicated in the modulation of ion channels and neurotransmitter function (15, 31). This effect could be direct, as shown by Huang et al. (13), on the cloned delayed rectifier K^+ channels and/or could involve more downstream kinases such as protein kinase C and MAP kinase. We have previously shown the involvement of tyrosine kinase in the muscarinic suppression of ATP-sensitive K^+ channel in the rabbit esophageal muscularis mucosae (10). It has also recently been shown that genistein can activate cystic fibrosis transmembrane conductance regulator Cl^- channels in epithelial cells (14), suggesting that tyrosine kinase may mediate inhibition of the current. Similarly, Minami et al. (20) observed activation of the nonselective cation channel by genistein in vascular smooth muscle.

The effects of EGF merit separate comment. In addition to its mitogenic activity, EGF contracts both vascular and visceral smooth muscle (see Ref. 11 for review). EGF has intrinsic tyrosine kinase activity, resulting in autophosphorylation of the kinase domain after receptor activation; this leads to association with various proteins and activation of a signaling cascade, including phospholipase C, phophatidylinositol-3-kinase, GTPase-activating protein, and raf-1, increased phosphatidylinositol turnover, Ca^{2+} mobilization, and cellular tyrosine kinase activity. Consistent with this effect was the finding that EGF increased $pp60^{c\text{-}src}$ and other proteins in the rabbit colonic muscularis mucosae, most notably at 42 and 44 kDa, which presumably reflects activation of MAP kinase. EGF also increased Ca^{2+} currents and STOC activity, an effect similar to the tyrosine phosphatase inhibitor, Na_3VO_4.

The colonic muscularis mucosae lies in close proximity to the lamina propria, where inflammatory mediators, cytokines, and growth factors may be released during chronic inflammation, which may result in significant thickening associated with Crohn's disease (18). In addition to the proliferation of smooth muscle, tyrosine kinase activated by these agents may be importantly involved in the regulation of Ca^{2+} entry in the colonic muscularis mucosae.

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