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Effects of inhibitors for tyrosine kinase and non-selective cation channel on capacitative Ca^{2+} entry in rat ileal smooth muscle

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Abstract

The effects of tyrosine kinase inhibitors and non-selective cation channel blockers on capacitative Ca^{2+} entry were examined in the presence of methoxyverapamil in rat ileal smooth muscles. In Ca^{2+}-free solution, carbachol or caffeine produced a rapid contraction mediated by Ca^{2+} release from the stores (Ca^{2+}-release response), and then led to Ca^{2+} depletion of the stores. Subsequently, reintroduction of Ca^{2+} caused a transient contraction due to capacitative Ca^{2+} entry. Tyrosine kinase inhibitors, genistein and tyrphostin 47 but not herbimycin A, suppressed the responses to Ca^{2+}-reintroduction much greater than Ca^{2+}-release responses to carbachol or caffeine. Similar inhibitory effects on the responses to Ca^{2+}-reintroduction were obtained with daidzein and tyrphostin A1, respective inactive analogue of genistein and tyrphostins. After continuous depletion of the stores with thapsigargin, Ca^{2+}-reintroduction produced a sustained contraction, which was inhibited by these agents to different extents, but not by herbimycin A. In β-escin-treated skinned muscles, genistein slightly reduced Ca^{2+}-induced contraction. In fura-2-loaded tissues, SK&F 96365 inhibited contractile and $[Ca^{2+}]_i$ responses to Ca^{2+}-reintroduction but minimally affected Ca^{2+}-release responses. Tetrandrine suppressed both responses to Ca^{2+}-reintroduction and to Ca^{2+}-release. These results suggest that genistein and tyrphostin 47 inhibit capacitative Ca^{2+} entry through an inhibition of Ca^{2+} entry channels rather than tyrosine kinase. SK&F 96365, but not tetrandrine, seems to selectively inhibit the contractile responses to capacitative Ca^{2+} entry in rat ileal smooth muscles.

Keywords: Ca^{2+} entry, capacitative; Ca^{2+} store, internal; Fura-2; Cation channel, non-selective; Tyrosine kinase

1. Introduction

Intracellular Ca^{2+} stores play a crucial role in regulating cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in smooth muscle cells (Bolton, 1979). Stored Ca^{2+} is released by inositol 1,4,5-trisphosphate (IP_3; Berridge, 1993) and by Ca^{2+} itself (Iino, 1989), and increased $[Ca^{2+}]_i$ is then sequestered into the Ca^{2+} stores due to the activation of Ca^{2+}-pump ATPase (Van Breemen and Saida, 1989). Regarding the regulation of $[Ca^{2+}]_i$ in connection with the Ca^{2+} stores, recent attention is particularly directed to capacitative Ca^{2+} entry activated by depletion of the Ca^{2+} stores (Putney and Bird, 1993). The regulatory mechanisms of capacitative Ca^{2+} entry proposed include diffusible small molecules, IP_3 metabolites, and direct coupling of IP_3 receptors or small GTP binding protein with the putative Ca^{2+} entry channels, but still remain to be elucidated (reviewed by Parekh and Penner, 1997).

Tyrosine kinase is well known to be associated with the cellular receptors for several growth factors and is related to the signal transduction pathways leading to cell growth and proliferation (Ullrich and Schlessinger, 1990). In smooth muscle cells, it has been reported that there is a high activity of tyrosine kinase (Di Salvo et al., 1989) which is involved in the signaling associated with receptor-mediated contractions (Laniyonu et al., 1994; Di Salvo et al., 1997). Recently, protein tyrosine phosphorylation has been shown to be accompanied by capacitative Ca^{2+} entry evoked by thapsigargin or thrombin in platelets, and a regulatory role for tyrosine kinase in capacitative Ca^{2+} entry is hypothesized (Vostal et al., 1991). This hypothesis has been further supported pharmacologically by the use of

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a tyrosine kinase inhibitor, genistein, which suppresses capacitative Ca^{2+} entry in fibroblasts (Lee et al., 1993), neutrophils (Montero et al., 1994), endothelial cells (Sharma and Davis, 1996) and platelets (Sargeant et al., 1993). Also in smooth muscles, genistein is shown to inhibit contractions via capacitative Ca^{2+} entry in pulmonary (De La Fuente et al., 1995) and mesenteric arteries (Low, 1996), and to block spontaneous outward currents through the inhibition of capacitative Ca^{2+} entry in colonic muscularis mucosae (Hatakeyama et al., 1996).

In electrophysiological studies, non-selective cation channels are considered to be pathways responsible for capacitative Ca^{2+} entry in pancreatic acinar cells (Krause et al., 1996), endothelial cells (Inazu et al., 1995) and mouse anococcygeus muscles (Wayman et al., 1998). Furthermore, it has been reported that SK&F 96365 is effective in inhibiting capacitative Ca^{2+} entry in platelets (Vostal and Shafer, 1996), endothelial cells (Schilling et al., 1992; Inazu et al., 1995; Low et al., 1996) and mesenteric arteries (Low, 1996). Tetrandrine, a plant alkaloid isolated from Chinese herbs, attenuates thapsigargin-induced capacitative Ca^{2+} entry via non-selective cation channels in leukemic HL-60 cells (Leung et al., 1994) and endothelial cells (Low et al., 1996). Therefore, these agents may be useful to characterize Ca^{2+} entry pathways related to capacitative Ca^{2+} entry.

In rat ileal smooth muscles, we have reported that capacitative Ca^{2+} entry is activated after a transient depletion of Ca^{2+} stores with carbachol or caffeine (Ohta et al., 1995) and after continuous depletion by pretreatment with thapsigargin or ryanodine (Tabo et al., 1996). However, it has not been elucidated whether tyrosine kinases and non-selective cation channels are involved in the capacitative Ca^{2+} entry in rat ileal smooth muscles.

In our present experiments, we have examined the effects of various types of tyrosine kinase inhibitors, such as genistein, a competitive tyrosine kinase inhibitor at the ATP-binding site (Akiyama et al., 1987); tyrphostin 47, one at the substrate-binding site (Gazit et al., 1989); daidzein and tyrphostin A1, their respective inactive analogues (Casnelle, 1991); and herbimycin A, an src tyrosine kinase inhibitor (Uehara et al., 1989) on contractions mediated by capacitative Ca^{2+} entry after transient and continuous depletion of Ca^{2+} stores in rat ileal smooth muscles. In addition, the effects of SK&F 96365 and tetrandrine on contractile and $[Ca^{2+}]_i$ responses to capacitative Ca^{2+} entry were investigated in fura-2-loaded preparations.

2. Materials and methods

All experiments were carried out under the regulations of the Animal Research Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Male Wistar rats weighing 200–300 g were stunned and bled to death. The ileum, about 20 mm in length, was isolated and placed in physiological salt solution (PSS). The longitudinal smooth muscle layer was peeled from the circular muscle layer and dissected into small muscle strips (1 mm in width, 5 mm in length). Muscle tension was measured isometrically with a force transducer (BG-10, Kulite Semiconductor Products, USA) through an amplifier (DSA-601B, Minebea, Japan). Contractile and fura-2 signals were stored on a data recorder (DAC-59ESJ, Sony, Japan) and then analyzed using an analog/digital converter (MacLab, AD Instruments, Australia) in conjunction with a microcomputer (Power Macintosh G3, USA). For measurement of tension and $[Ca^{2+}]_i$ simultaneously, muscle preparations were incubated with 20 μM fura-2 acetoxymethyl ester (fura-2/AM) and 0.02% cremophore EL for 3 h at room temperature. The fura-2 method used in the present experiments was the same as that described previously (Ohta et al., 1995). Briefly, using a fluorimeter (CAF-110, Japan Spectroscopic, Japan), the muscle bundle was alternatively illuminated by 340 and 380 nm light at a frequency of 128 Hz and the fluorescent intensity at 500 nm was measured at room temperature (20–24°C). The calculated ratio of the fluorescence due to excitation at 340 nm to that at 380 nm (F340/F380) was considered to be an index of $[Ca^{2+}]_i$.

Skinned muscle preparations were obtained by exposing small muscle bundles (0.2 mm in width, 1 mm in length) to 40 μM β-escin in relaxing solution for 30 min at room temperature (Ohta et al., 1992).

The ionic composition of normal PSS was (mM): NaCl 144, KCl 5.8, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11.1, HEPES 5 (pH 7.4 with NaOH). In Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mM EGTA was added. A 40-mM KCl solution was prepared by replacing NaCl with KCl isosmotically. The relaxing solution used for skinned muscle preparations was composed of (mM): K methanesulfonate (Ms) 126, $MgMs_2$ 5.1, MgATP 3.5, EGTA 2, and PIPES 20 (pH 7.0 with KOH). In a Ca^{2+}-containing solution, 10 mM EGTA was used and a specified amount of $CaMs_2$ was added. The apparent binding constant of EGTA and Ca^{2+} was calculated by the formula reported by Harafuji and Ogawa (1980). Calmodulin (2 μM) extracted from bovine brain was added to all solutions for skinned muscle preparations.

The following chemicals were used: $ATPNa_2$ (Boehringer Mannheim, Germany), β-escin, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride (SK&F 96365, Biomol, USA), caffeine, genistein, herbimycin A, methoxyverapamil and thapsigargin (Wako, Japan), carbachol, cremophore EL, daidzein, tetrandrine, tyrphostin A1 and tyrphostin 47 (Sigma, USA), EGTA, fura-2/AM and HEPES (Dojindo, Japan).

Results of the experiments were expressed as the mean ± S.E.M. Student's *t*-test was used for statistical analysis of the results and $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of tyrosine kinase inhibitors on capacitative Ca^{2+} entry and Ca^{2+} release from intracellular stores

The present experiments, unless otherwise noted, were all carried out in the presence of methoxyverapamil (10 μM) to inhibit voltage-dependent Ca^{2+} channels. First we examined the effects of genistein, a competitive tyrosine kinase inhibitor at the ATP-binding site (Akiyama et al., 1987), on responses to Ca^{2+} released from stores by carbachol and to capacitative Ca^{2+} entry after transient depletion of intracellular Ca^{2+} stores with carbachol. Since genistein showed a strong quenching effect on fura-2 fluorescence, we did not measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

In Ca^{2+}-free solution containing 2 mM EGTA, the application of a high concentration of carbachol (0.1 mM) for 1 min produced a transient contraction that was due to the release of Ca^{2+} from internal stores (Ca^{2+}-release contraction). After washout with fresh Ca^{2+}-free solution, a second application of carbachol failed to evoke any responses, because the internal stores had been depleted. Subsequently, exposure of the tissue to Ca^{2+} (2.5 mM)-containing solution (Ca^{2+}-reintroduction) for 3 min brought about a contraction, which gradually declines to the original level even in the presence of external Ca^{2+}. We have proposed that the contractile responses to Ca^{2+}-reintroduction are mainly evoked by capacitative Ca^{2+} entry after the depletion of the internal Ca^{2+} stores, because only a small response occur without preceding stimulation of carbachol releasing Ca^{2+} (Ohta et al., 1995). The administration of carbachol to the Ca^{2+}-free solution 3 min after the removal of external Ca^{2+} again caused a transient Ca^{2+}-release contraction. These contractions were repeatedly inducible by the application of carbachol in the absence of Ca^{2+} and by the reintroduction of Ca^{2+}. This protocol was used to examine the effects of genistein on the contractions induced by capacitative Ca^{2+} entry and by Ca^{2+}-release. As shown in Fig. 1A, the contractile response to Ca^{2+}-reintroduction was strongly suppressed by genistein (100 μM). Even when the contraction evoked by Ca^{2+}-reintroduction was completely abolished by genistein, the following Ca^{2+}-release contraction induced by carbachol was reduced only to 93.5 ± 2.0% ($n = 5$) of the control (Fig. 1Aa, indicated by an arrow). If the magnitude of Ca^{2+}-release contraction reflects Ca^{2+} content in the store, the result may imply that genistein has less effect on Ca^{2+}-refilling into the stores.

Next, to examine the effect of genistein on Ca^{2+}-release contractions induced by carbachol, genistein was applied to the Ca^{2+}-free solution 3 min before and during carbachol stimulation (Fig. 1Ab). Genistein reduced, but not abolished, the Ca^{2+}-release contraction to about 60% of the control. Fig. 1B shows the genistein concentration–inhibition curves for contractions induced by Ca^{2+}-reintroduction and by Ca^{2+}-release with carbachol. These data indicate that genistein is much more effective in inhibiting capacitative Ca^{2+} entry than carbachol-induced Ca^{2+}-release from the stores.

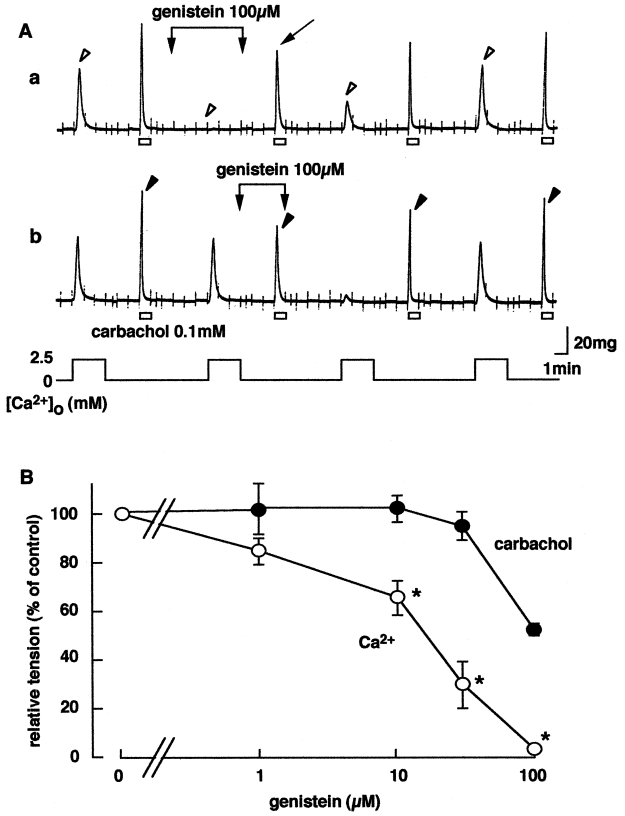

Fig. 1. Effects of genistein on contractions elicited by Ca^{2+}-reintroduction and carbachol in the Ca^{2+}-free solution. (A) After depletion of Ca^{2+} stores with carbachol (0.1 mM) in Ca^{2+}-free solution containing 2 mM EGTA, reintroduction of Ca^{2+} (2.5 mM) for 3 min elicited a transient contraction due to capacitative Ca^{2+} entry. The tissues were then washed out for 3 min with the Ca^{2+}-free solution and were stimulated by carbachol for 1 min (open bars). Ca^{2+} concentration in the solution is illustrated in the lowest trace. Genistein (100 μM) was applied 3 min before and during responses as shown in each trace. In (a), open arrowheads indicate contractions evoked by Ca^{2+} reintroduction. In (b), filled arrowheads show carbachol-induced contractions utilizing Ca^{2+} released from internal stores. Vertical spiky lines in this and following figures indicate artifacts due to solution exchange. Note that even after contraction evoked by Ca^{2+} reintroduction was completely abolished by genistein, the following carbachol-induced response was only slightly decreased (in (a), indicated by an arrow). (B) Concentration–inhibition relations for genistein on contractions evoked by Ca^{2+}-reintroduction (Ca^{2+}), and by carbachol-induced Ca^{2+} release (carbachol). $^*P < 0.05$; significantly different when compared with responses to Ca^{2+}-reintroduction and to carbachol at each given concentration of genistein. Results are expressed as mean ± S.E.M. ($n = 5$).

Rat ileal smooth muscles have caffeine-sensitive Ca^{2+} stores (Ohta et al., 1993) and capacitative Ca^{2+} entry is also activated by the depletion of these stores (Ohta et al., 1995). We then examined the effects of genistein on the responses to caffeine-induced Ca^{2+}-release and to subsequent capacitative Ca^{2+} entry. The same experimental protocol as shown in Fig. 1 was carried out except that

caffeine (30 mM) was used instead of carbachol. Fig. 2A and B show original recordings and the concentration–inhibition curves for genistein, respectively. Similar to the case of carbachol, genistein suppressed the contraction induced by Ca^{2+}-reintroduction to greater extent than that by Ca^{2+}-release with caffeine. Ca^{2+}-refilling into the stores also seemed to be less influenced by genistein, because the Ca^{2+}-release contraction evoked by caffeine slightly declined to $90.6 \pm 3.2\%$ ($n = 5$) of the control (Fig. 2Aa, indicated by an arrow).

We investigated the effects of tyrphostin 47 and herbimycin A, other tyrosine kinase inhibitors, and daidzein and tyrphostin A1, respective inactive analogues of genistein and tyrphostins. Fig. 3 shows the summarized effects of these drugs at the same concentration (100 μM) on Ca^{2+}-release contractions evoked by carbachol and caffeine, and the following respective contractions induced by

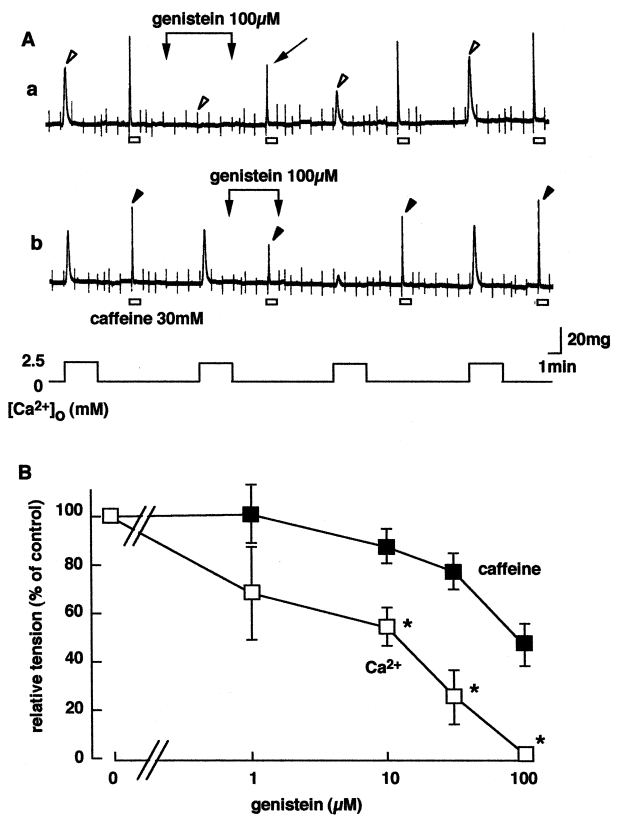

Fig. 2. Effects of genistein on contractions elicited by Ca^{2+}-reintroduction and caffeine in the Ca^{2+}-free solution. (A) Original chart records. The same experimental protocol as shown in Fig. 1 was used with caffeine (30 mM) instead of carbachol. In (a), open arrowheads show contractions evoked by Ca^{2+}-reintroduction. As shown by an arrow, genistein abolished the preceding contraction evoked by Ca^{2+} reintroduction, but not the caffeine-induced contraction. In (b), filled arrowheads show caffeine-induced contractions utilizing Ca^{2+} released from the stores. (B) Concentration–inhibition relations for genistein on contractions evoked by Ca^{2+}-reintroduction (Ca^{2+}) and by caffeine-induced Ca^{2+} release (caffeine). $^*P < 0.05$; significantly different when compared with responses to Ca^{2+}-reintroduction and to caffeine at each given concentration of genistein. Results are expressed as mean ± S.E.M. ($n = 5$).

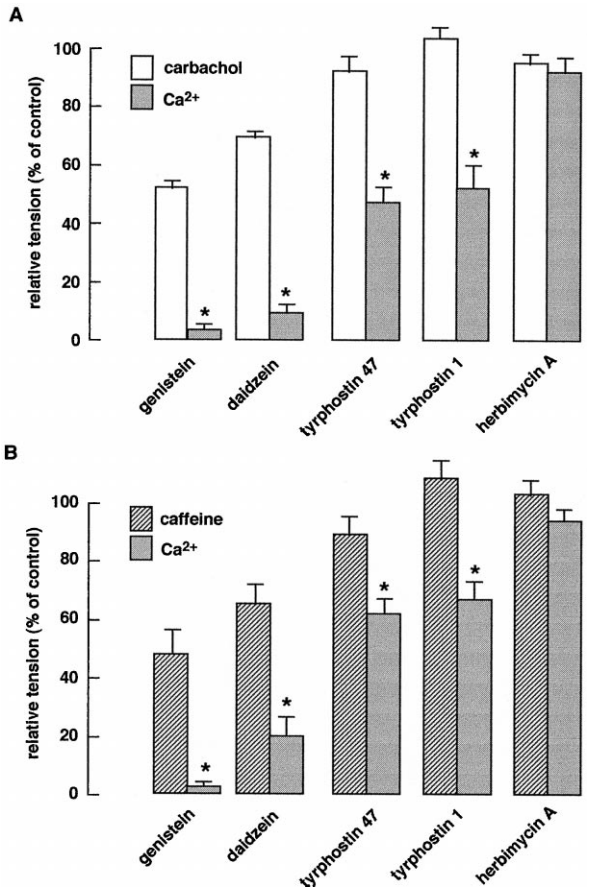

Fig. 3. Comparison of the effects of various tyrosine kinases and their inactive analogues on the contractions elicited by carbachol (A) or caffeine (B) in the absence of Ca^{2+}, and respective following contractions produced by Ca^{2+}-reintroduction. The relative amplitudes of tension responses in the presence of genistein, daidzein, tyrphostin 47, tyrphostin A1 and herbimycine A (100 μM) are plotted as a percentage of each contraction obtained in their absence. The experimental procedure was the same as that show in Figs. 1 and 2. (A) Open and dotted columns show carbachol-induced contractions in the absence of Ca^{2+} (carbachol) and the subsequent contractions evoked by Ca^{2+}-reintroduction (Ca^{2+}), respectively. (B) Hatched and dotted columns show caffeine-induced contractions in the absence of Ca^{2+} (caffeine) and the following contractions evoked by Ca^{2+}-reintroduction (Ca^{2+}), respectively. $^*p < 0.05$; significantly different when compared with responses evoked by Ca^{2+} release from the stores and by Ca^{2+}-reintroduction for each drug. Results are expressed as mean ± S.E.M. ($n = 4–5$).

Ca^{2+}-reintroduction. The inhibitory effects of genistein and tyrphostin 47 on the contractile response to Ca^{2+}-reintroduction were always larger than on Ca^{2+}-release responses, though tyrphostin 47 was less potent than genistein. Regardless of their lack of inhibitory action on tyrosine kinase, daidzein and tyrphostin A1 produced inhibitory effects on contractions induced by Ca^{2+}-reintroduction to the same extent as genistein and tyrphostin 47, respectively. On the other hand, herbimycin A exerted almost no effect on both responses to Ca^{2+}-reintroduction and to Ca^{2+}-release with carbachol or caffeine.

3.2. Effects of tyrosine kinase inhibitors on long-lasting capacitative Ca^{2+} entry after treatment with thapsigargin

Unlike carbachol and caffeine, thapsigargin, a Ca^{2+}-pump ATPase inhibitor of the internal stores (Thastrup et al., 1990) leads to continuous depletion of the stores under Ca^{2+}-free condition. As shown in Fig. 4, the muscle preparations were first challenged with 40 mM KCl to check their contractility, and then external solution was switched from normal solution to the Ca^{2+}-free solution containing 2 mM EGTA. Methoxyverapamil (10 μM) was added to all the solutions thereafter. Under these conditions, tissues were treated with thapsigargin (10 μM) for 30 min and then washed with fresh Ca^{2+}-free solution without thapsigargin for 5 min. In the tissues pretreated with thapsigargin, the internal stores were completely depleted, assessed by the abolition of Ca^{2+}-release contractions with carbachol or caffeine (Tabo et al., 1996). Subsequently, readministration of Ca^{2+} (2.5 mM) produced a marked and sustained contraction. Fig. 4B is a representative original trace showing inhibitory effects of increasing concentrations of genistein on the sustained contractile responses to Ca^{2+}-reintroduction in thapsigargin-pretreated tissues. Genistein inhibited the contraction in a dose-dependent manner and complete inhibition was observed at 100 μM. After the washout of genistein, the amplitude of contraction returned to the control level prior to the application of genistein. Concentration–inhibition curves for genistein, daidzein, tyrphostin 47, tyrphostin A1 and herbimycin A are depicted in Fig. 4C. These drugs, except for herbimycin A, decreased the contractions evoked by Ca^{2+}-reintroduction in a concentration-dependent manner, though the inhibitory potency of tyrphostin A1 was somewhat weaker than the others.

3.3. Effects of genistein on the contractile machinery in skinned muscle preparations

To determine whether genistein exerts a direct inhibitory action on contractile machinery of rat ileal smooth muscle, the effect of genistein on Ca^{2+}-induced contraction was examined in skinned muscle preparations treated with β-escin (40 μM). Application of 0.6 μM Ca^{2+}, which concentration caused 50–75% of the maximal Ca^{2+}-induced contraction in skinned muscles, induced a contraction that reached its peak in about 3 min. As shown in Fig. 5A, genistein (100 μM) produced a slight inhibition of Ca^{2+}-induced contractions. The summarized results are depicted in Fig. 5B.

3.4. Effects of SK&F 96365 and tetrandrine on contraction and $[Ca^{2+}]_i$ increase produced by capacitative Ca^{2+} entry

To study the characteristics of the Ca^{2+} entry pathways involved in capacitative Ca^{2+} entry in rat ileal smooth muscles, the effects of SK&F 96365 and tetrandrine on the contractile and $[Ca^{2+}]_i$ responses to Ca^{2+}-reintroduction were examined in fura-2-loaded tissues. The experi-

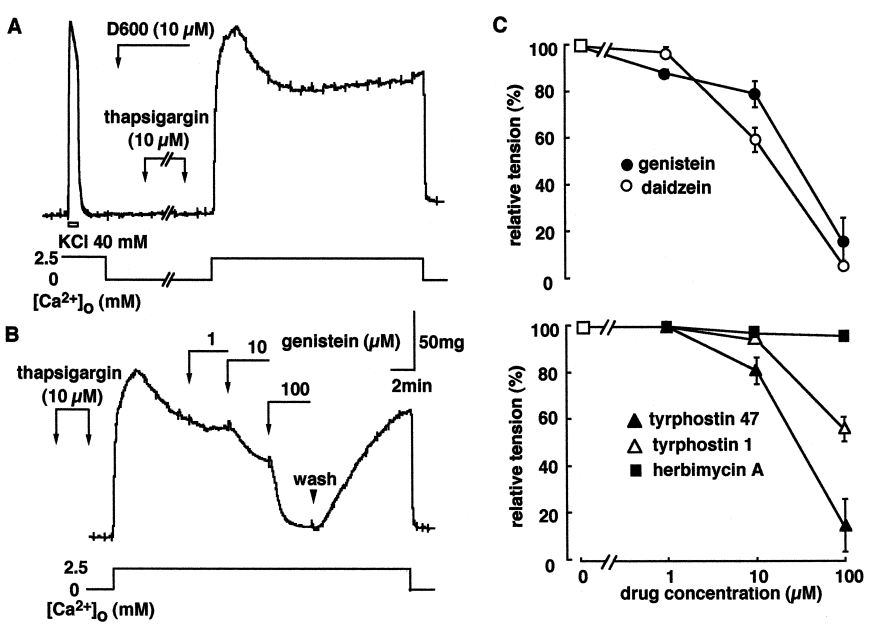

Fig. 4. Effects of various tyrosine kinases and their inactive analogues on sustained contractions in tissues whose internal stores were depleted by pretreatment with thapsigargin. (A) Tissues were first stimulated with KCl (40 mM, 1 min) and then incubated with the Ca^{2+}-free solution containing EGTA (2 mM) together with methoxyverapamil (D600, 10 μM). The following experiment was carried out in the presence of D600. After 30-min treatment with thapsigargin (10 μM), tissues were washed with the fresh Ca^{2+}-free solution without thapsigargin. The application of 2.5-mM Ca^{2+} produced a sustained contraction due to the capacitative Ca^{2+} entry. Ca^{2+} concentration in the solution is shown in a lower trace. (B) Genistein dose-dependently suppressed the sustained contraction evoked by Ca^{2+}-reintroduction in tissues pretreated with thapsigargin. (C) Concentration–inhibition relations of genistein and daidzein (upper) and tyrphostin 47, tyrphostin A1 and herbimycin A (lower). Results are expressed as mean ± S.E.M. ($n = 4$).

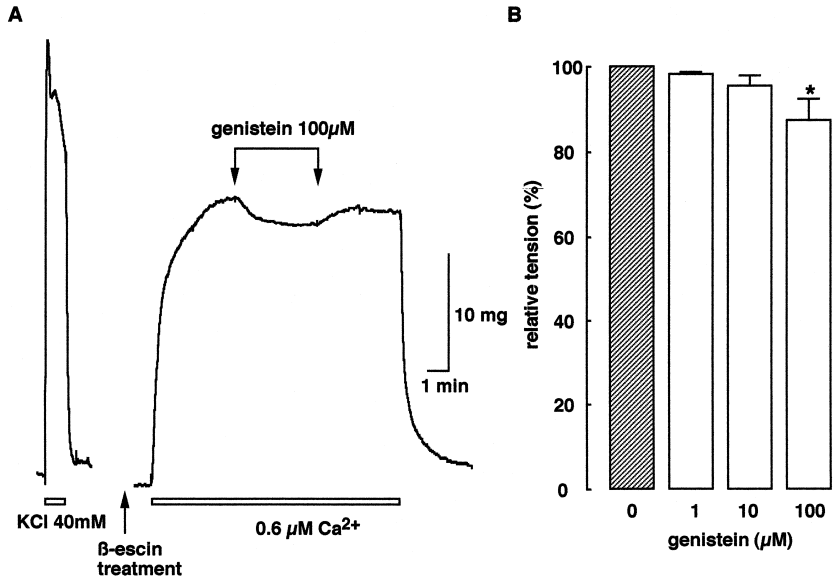

Fig. 5. Effect of genistein on the Ca^{2+}-induced contraction in skinned muscles. (A) After stimulation with KCl (40 mM), tissues were immersed in relaxing solution containing β-escin (40 μM) for 30 min, then washed with fresh relaxing solution without β-escin. After 0.6 μM Ca^{2+}-induced contraction reached a steady level, genistein (100 μM) was added. Note that this concentration of genistein produced complete inhibition of contraction due to capacitative Ca^{2+} entry (Figs. 1–4). (B) The relative amplitudes of Ca^{2+}-induced contractions in the presence of various concentrations of genistein. * $p < 0.05$; significantly different when compared with responses in the absence of genistein. Results are expressed as mean ± S.E.M. ($n = 5$).

mental protocol shown in Fig. 1 was used to evoke responses to capacitative Ca^{2+} entry and to Ca^{2+}-release with carbachol. As shown in Fig. 6A, reintroduction of Ca^{2+} (2.5 mM) evoked a transient contraction and increase of $[Ca^{2+}]_i$ that exceeded the original level of $[Ca^{2+}]_i$. SK&F 96365 (100 μM) greatly inhibited the tension and $[Ca^{2+}]_i$ increases produced by Ca^{2+}-reintroduction. No $[Ca^{2+}]_i$ increase over the original level occurred in the presence of SK&F 96365. Furthermore, as shown by arrowheads in Fig. 6A, the following Ca^{2+}-release re-

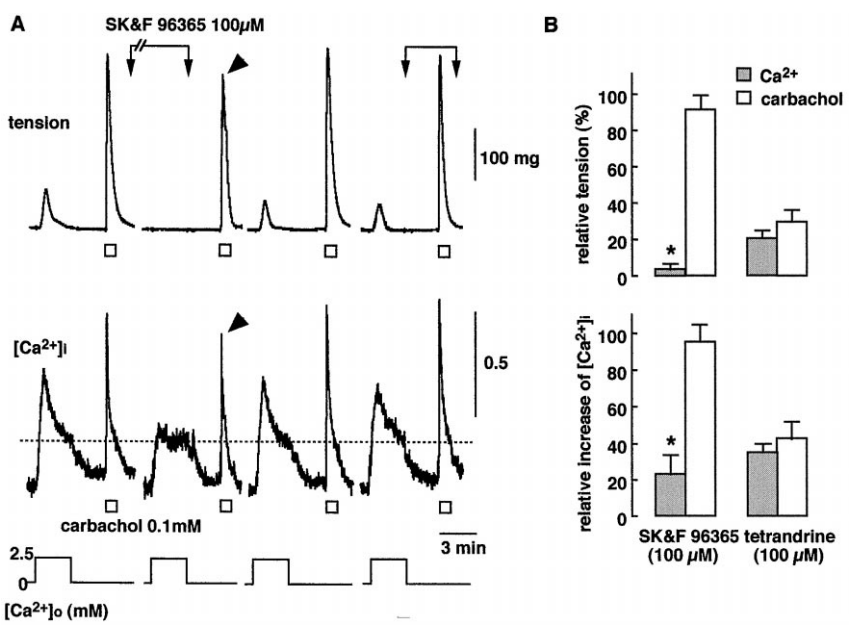

Fig. 6. Effects of SK&F 96365 on contractile and $[Ca^{2+}]_i$ responses to Ca^{2+}-reintroduction and subsequently applied carbachol in the Ca^{2+}-free solution in fura-2-loaded rat ileal smooth muscle. (A) From top to bottom: tension, $[Ca^{2+}]_i$ and the concentration of external Ca^{2+}. The same experimental protocol as shown in Fig. 1 was employed. The dotted line indicates the original $[Ca^{2+}]_i$ level in normal PSS. Note that even after contraction evoked by Ca^{2+}-reintroduction was completely abolished by SK&F 96365, the following carbachol-induced responses were only slightly decreased (indicated by arrowheads). (B) Summarized effects of SK&F 96365 (100 μM, left) and tetrandrine (100 μM, right) on peak amplitude of tension (upper) and that of $[Ca^{2+}]_i$ (lower) responses over the original level to Ca^{2+}-reintroduction (dotted columns) and carbachol (open columns) in the Ca^{2+}-free solution. * $p < 0.05$; significantly different when compared with responses evoked by Ca^{2+} release from the stores and by Ca^{2+}-reintroduction for each drug. Results are expressed as mean ± S.E.M. ($n = 5$).

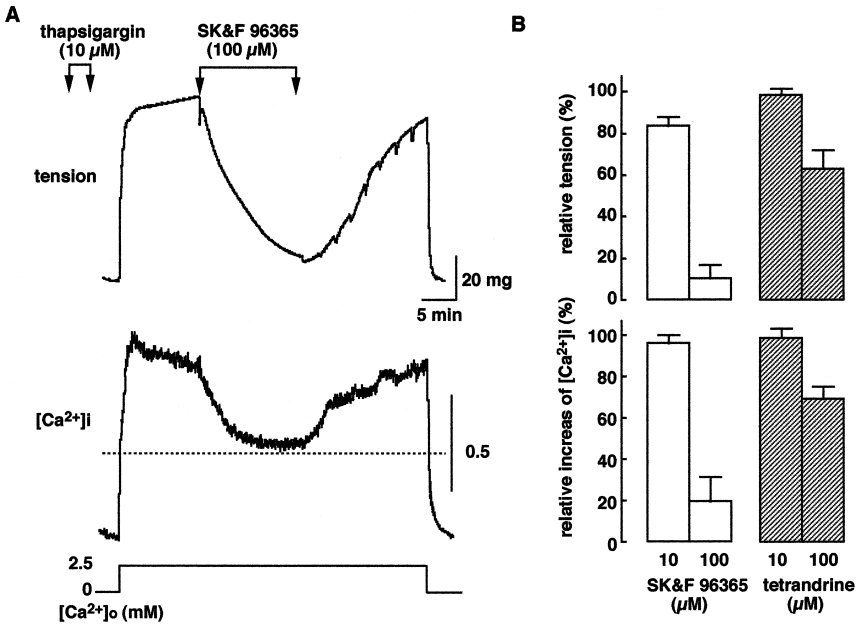

Fig. 7. Effects of SK&F 96365 and tetrandrine on the contraction and $[Ca^{2+}]_i$ increase induced by Ca^{2+}-reintroduction to tissues pretreated with thapsigargin. (A) From top to bottom: tension, $[Ca^{2+}]_i$ and the concentration of external Ca^{2+}. The same experimental protocol as shown in Fig. 4 was employed. Ca^{2+}-reintroduction produced a sustained contraction concomitant with an increase of $[Ca^{2+}]_i$ unless SK&F 96365 was added. The dotted line indicates the original $[Ca^{2+}]_i$ level in normal physiological salt solution. (B) Summarized effects of SK&F 96365 (left, 10 and 100 μM) and tetrandrine (right, 10 and 100 μM) on contraction and increase of $[Ca^{2+}]_i$ over the original level induced by Ca^{2+}-reintroduction. Results are expressed as mean ± S.E.M. ($n = 5$).

sponses to carbachol were slightly decreased to 92.2 ± 2.3% ($n = 5$) for contraction and to 93.8 ± 1.3% for $[Ca^{2+}]_i$ increase of the control, indicating that SK&F 96365 does not inhibit Ca^{2+} refilling into the stores. Carbachol-induced Ca^{2+} release from the stores was almost unaffected in the presence of SK&F 96365 (Fig. 6A,B). On the other hand, tetrandrine suppressed not only responses to capacitative Ca^{2+} entry but also those to Ca^{2+}-release induced by carbachol to the same degree shown in Fig. 6B.

The effects of SK&F 96365 and tetrandrine on capacitative Ca^{2+} entry in tissues with the continuous depletion of the Ca^{2+} stores were examined (Fig. 7). In tissues pretreated with thapsigargin for 30 min under the Ca^{2+}-free conditions, Ca^{2+}-reintroduction produced a sustained contraction concomitant with an increase of $[Ca^{2+}]_i$. SK&F 96365 diminished the sustained contraction evoked by capacitative Ca^{2+} entry and the rise in $[Ca^{2+}]_i$ to near the original level. Summarized data of the effects of SK&F 96365 on contractile and $[Ca^{2+}]_i$ responses to capacitative Ca^{2+} entry are shown in Fig. 7B. Similar inhibitory effects were observed with tetrandrine, though its inhibitory potency for capacitative Ca^{2+} entry was much weaker than that of SK&F 96365.

4. Discussion

In the presence of voltage-dependent Ca^{2+} channel blockers, we previously reported that the contractile responses to Ca^{2+}-reintroduction in rat ileal smooth muscles, of which internal stores were depleted transiently by carbachol or caffeine and continuously by thapsigargin, were mainly mediated by capacitative Ca^{2+} entry (Ohta et al., 1995; Tabo et al., 1996), which was recently reviewed in various types of smooth muscles (Gibson et al., 1998). In the present experiments, genistein, one of the most commonly used tyrosine kinase inhibitor, reversibly inhibited contractions mediated by capacitative Ca^{2+} entry in tissues depleted of Ca^{2+} stores either transiently or continuously. Tyrphostin 47, a tyrosine kinase inhibitor structurally unrelated to genistein (Gazit et al., 1989), also suppressed these contractions mediated by capacitative Ca^{2+} entry, though the potency of inhibitory action of tyrphostin 47 in transiently store-depleted tissues was different from that in continuously store-depleted ones. This difference might be due to a different state of Ca^{2+}-ATPase, which is intact even after the stimulation with carbachol or caffeine, but is continuously impaired by thapsigargin. It has also been reported that the level of tyrosine phosphorylation is different between capacitative Ca^{2+} entry evoked by agonist stimulation and by thapsigargin in rat thyroid cell line (Meucci et al., 1995). These results obtained with genistein and tyrphostins 47 are apparently favorable to the hypothesis that capacitative Ca^{2+} entry is modulated by tyrosine kinase, as reported in a variety of cells (Vostal et al., 1991; Schilling et al., 1992; Lee et al., 1993; Sargeant et al., 1993; Montero et al., 1994; Sharma and Davis, 1996)

including smooth muscles (De La Fuente et al., 1995; Hatakeyama et al., 1996; Low, 1996).

In this study, however, daidzein and tyrphostin A1, compounds lacking tyrosine kinase inhibitory action (Casnelle, 1991), unexpectedly suppressed capacitative Ca^{2+} entry. Moreover, no inhibitory effects were observed with herbimycin A, a src tyrosine kinase inhibitor (Uehara et al., 1989), on the capacitative Ca^{2+} entry. Similar to our present results, in rat mesenteric artery, these inactive analogues have potent inhibitory action on high K^+- and phenylephrine-induced contractions (Toma et al., 1995). Yokoshiki et al. (1996) suggested the possibility that there are different tyrosine kinase isoforms that are inhibited by both genistein and daidzein. In addition, tyrphostins have been reported to have considerable differences in potency in their action against different tyrosine kinases (Yaish et al., 1988; Gazit et al., 1989).

Recently, pharmacological approaches using genistein have raised doubts about the regulatory role of tyrosine kinase in capacitative Ca^{2+} entry. In platelets, Vostal and Shafer (1996), who first hypothesized the involvement of tyrosine kinase in capacitative Ca^{2+} entry, have reported that genistein inhibits capacitative Ca^{2+} entry without inhibition of protein tyrosine phosphorylation. In mouse pancreatic acinar cells, genistein, but not other tyrosine kinase inhibitors, suppressed capacitative Ca^{2+} entry (Pfeiffer et al., 1995; Krause et al., 1996). Taken together, these results suggest that a more direct interaction between channels and genistein rather than the inhibition of tyrosine kinase is probably responsible for the inhibition of capacitative Ca^{2+} entry in rat ileal smooth muscles. It should be noted that the interpretation of data obtained with 'specific inhibitors' of tyrosine kinase should be carefully assessed.

Ca^{2+}-induced contraction in β-escin-treated skinned muscles was suppressed only a little by genistein even at a concentration producing almost complete suppression of capacitative Ca^{2+} entry. On the other hand, genistein partly inhibited carbachol- or caffeine-induced contraction due to Ca^{2+} release from the internal stores. It has been reported that genistein inhibits IP_3 synthesis in platelets (Ozaki et al., 1993) and Ca^{2+} release evoked through IP_3 in arterial cells (Nelson et al., 1997).

Tetrandrine, an alkaloid isolated from Chinese herbs and being used in the treatment of hypertension and angina pectoris, is shown to block capacitative Ca^{2+} entry via non-selective cation channels in leukemic HL-60 cells (Leung et al., 1994) and endothelial cells (Low et al., 1996). In the present study, tetrandrine suppressed capacitative Ca^{2+} entry-induced contractions and increases in $[Ca^{2+}]_i$, and its inhibitory action was greater in tissues with transiently depleted Ca^{2+} stores than those with continuously depleted ones. There might be some different properties in Ca^{2+} entry mechanisms between transiently store-depleted tissues and continuously depleted ones with thapsigargin. It has been reported that tetrandrine inhibits IP_3 synthesis in human lymphocytes (Ioannoni et al., 1989). In fact, tetrandrine decreased carbachol-induced Ca^{2+}-release and capacitative Ca^{2+} entry to the same extent. Therefore, despite the fact that tetrandrine showed an inhibitory action on capacitative Ca^{2+} entry, this drug does not seem suitable to characterize capacitative Ca^{2+} entry.

SK&F 96365, originally introduced as a blocker for receptor-operated Ca^{2+} channels (Merritt et al., 1990), has been shown to inhibit capacitative Ca^{2+} entry via non-selective cation channels (Schilling et al., 1992; Inazu et al., 1995; Low, 1996; Low et al., 1996). In the present experiments, SK&F 96365 inhibited transient contractions and increases in $[Ca^{2+}]_i$ induced by Ca^{2+}-reintroduction with a slight inhibition of responses to carbachol-induced Ca^{2+}-release. Furthermore, sustained contractile and $[Ca^{2+}]_i$ responses to Ca^{2+}-reintroduction in tissues pretreated with thapsigargin were also reduced by SK&F 96356. These data suggest that non-selective cation channels are one of the Ca^{2+} entry pathways related to capacitative Ca^{2+} entry in rat ileal smooth muscles. In the presence of SK&F 96365, $[Ca^{2+}]_i$ did not exceed the original level during reintroduction of Ca^{2+}. Similar results were obtained when low concentration of $LaCl_3$ was added during Ca^{2+}-reintroduction (data not shown). Furthermore, even after SK&F 96365 inhibited contraction and $[Ca^{2+}]_i$ increase evoked by Ca^{2+}-reintroduction, it produced only a slight reduction of the following Ca^{2+}-release responses. These results indicate that SK&F 96365 selectively inhibits Ca^{2+} entry pathways responsible for contraction induced by Ca^{2+}-reintroduction but not those for refilling Ca^{2+} into the stores in rat ileal smooth muscles. Similar phenomena were observed with genistein (Figs. 1 and 2). It seems likely, therefore, that Ca^{2+} entry pathways involved in contractile and $[Ca^{2+}]_i$ responses to Ca^{2+}-reintroduction are different from those related to Ca^{2+} refilling into the stores. This may support our previous report that an increase of the extracellular K^+ concentration inhibits capacitative Ca^{2+} entry-mediated contraction and $[Ca^{2+}]_i$ increase, but has little affect on Ca^{2+}-refilling into the stores (Tabo et al., 1996).

Recently, it has been suggested that an extracellular membrane Ca^{2+} pool sensitive to EGTA (1 mM) is present in canine bronchial and lower esophageal sphincter muscles (Bazan-Perkins et al., 1998; Salapatek et al., 1998). Interestingly, this pool can release Ca^{2+} in response to receptor agonists and uptake without Ca^{2+} pump activity. However, in this experiment, as we used 2 mM EGTA for Ca^{2+}-free solution, the possibility of the involvement of this kind of pool in the Ca^{2+} stores sensitive to carbachol and caffeine, if any, may be minimal or negligible.

The present results indicating that genistein and tyrphostin 47, as well as their inactive analogues, inhibited capacitative Ca^{2+} entry with little influence on Ca^{2+} release from the stores may imply a direct interaction of these compounds with Ca^{2+} entry pathways related to capacitative Ca^{2+} entry. Non-selective cation channels are

possible Ca^{2+} entry pathways for capacitative Ca^{2+} entry, particularly for producing contraction evoked by Ca^{2+}-re-introduction in rat ileal smooth muscles.

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