

Modulation of Voltage-dependent Ca Channel Current by Arachidonic Acid and Other Long-Chain Fatty Acids in Rabbit Intestinal Smooth Muscle

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ABSTRACT The effects of arachidonic acid (AA) and other long-chain fatty acids on voltage-dependent Ca channel current (I_{Ca}) were investigated, with the whole cell patch clamp method, in longitudinal smooth muscle cells of rabbit ileum. 10–30 μ M AA caused a gradual depression of I_{Ca} . The inhibitory effect of AA was not prevented by indomethacin (10 μ M) (an inhibitor of cyclooxygenase) or nordihydroguaiaretic acid (10 μ M) (an inhibitor of lipoxygenase). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7; 25–50 μ M) or staurosporine (2 μ M) (inhibitors of protein kinase C) did not block the AA-induced inhibition of I_{Ca} , and application of phorbol ester (a protein kinase C activator) (phorbol-12,13-dibutyrate, 0.2 μ M) did not mimic the AA action. Some other *cis*-unsaturated fatty acids (palmitoleic, linoleic, and oleic acids) were also found to depress I_{Ca} , while a *trans*-unsaturated fatty acid (linolelaidic acid) and saturated fatty acids (capric, lauric, myristic, and palmitic acids) had no inhibitory effects on I_{Ca} . Myristic acid consistently increased the amplitude of I_{Ca} at negative membrane potentials. The present results suggest the possible role of AA, and perhaps other fatty acids, in the physiological and/or pathological modulation of I_{Ca} in smooth muscle.

INTRODUCTION

Arachidonic acid (AA) (20:4) is a major component of membrane lipids, and metabolites of AA, derived through cyclooxygenase and lipoxygenase pathways (AA cascade), have a variety of biological effects (Needleman, Turk, Jakschik, Morrison, and Lefkowitz, 1986; Shimizu and Wolfe, 1990), and can also act as second messengers. In some cases, the long-chain fatty acids also have effects similar to AA. For example, AA (and/or other fatty acids) is reported to activate protein kinase C (McPhail, Clayton, and Snyderman, 1984) and guanylate cyclase (Waldman and Murad, 1987), inhibit myosin light-chain kinase (Kigoshi, Uchida, Kaneko, Iwasaki, Nakano, Azukizawa, and Morimoto, 1990) and Na-K-ATPase (Swarts, Schuurmans

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Stekhoven, and De Pont, 1990), and cause mobilization of intracellular Ca (Wolf, Turk, Sherman, and McDaniel, 1986; Chow and Jondal, 1990) and superoxide release (Badwey, Curnutte, Robinson, Berde, Karnovsky, and Karnovsky, 1984; Chan, Chen, and Yu, 1988).

As regards ion channels, a 12-lipoxygenase metabolite of AA is reported to modulate S-K channels of *Aplysia* neurons (Piomelli, Volterra, Dale, Siegelbaum, Kandel, Schwartz, and Belardetti, 1987; Buttner, Siegelbaum, and Volterra, 1989), and 5-lipoxygenase metabolites of AA are also reported to be involved in the regulation of cardiac G protein-gated K channels (Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1989; Kim, Lewis, Gradziadei, Neer, Bar-Sagi, and Clapham, 1989) and of some neuronal M currents (Schweitzer, Madamba, and Siggins, 1990). Moreover, AA and some other long-chain fatty acids activate K channels of cardiac (Kim and Clapham, 1989), neuronal (Premkumar, Gage, and Chung, 1990), and amphibian gastric smooth muscle cells (Ordway, Walsh, and Singer, 1989), inhibit Cl channels of airway epithelia (Anderson and Welsh, 1990; Hwang, Guggino, and Guggino, 1990) and Na current in squid giant axon (Takenaka, Horie, Hori, and Kawakami, 1988), affect voltage-dependent Ca channel current in some neuronal cells (Linden and Routtenberg, 1989; Vacher, McKenzie, and Dufy, 1989; Keyser and Alger, 1990), and close gap-junction channels in rat lacrimal gland (Giaume, Randriamampita, and Trautmann, 1989).

AA can be liberated through several pathways (Irvine, 1982; Axelrod, 1990): (1) AA is released by phospholipase A₂ directly from membrane phospholipids that contain AA. (2) AA is also released by diacylglycerol lipase from diacylglycerol, which can be produced by phospholipase C. (3) AA is released by diglyceride lipase from phosphatidic acid generated by phospholipase D. Recent evidence shows that some receptors, such as alpha₁-adrenergic, bradykinin, and m₁-, m₃-, and m₅-muscarinic receptors, can activate phospholipase A₂ and cause release of AA (Axelrod, 1990). Low concentrations of AA and other fatty acids are present in the extracellular space under physiological conditions. Their level may increase under some pathological conditions, and consequently they could be important regulators of normal and abnormal cellular functions.

Voltage-dependent Ca channels are thought to play a major role in excitation-contraction coupling in smooth muscle, but information about the mechanisms regulating current through this channel is limited, and reports concerning its response to excitatory agonist are conflicting (Droogmans, Declerck, and Casteels, 1987; Benham and Tsien, 1988). In this study we investigated the effects of AA and other long-chain fatty acids on this current, and show that AA and some other long-chain unsaturated fatty acids inhibit, while a saturated (myristic) acid increases, L-type Ca channel current. These results suggest that AA and/or other fatty acids released by various stimuli may modulate this current.

METHODS

Preparation of Isolated Cells

The distal ileum was excised from male albino rabbits (weight 1.5–2.0 kg) under halothane anesthesia. The longitudinal muscle layer was peeled from the underlying circular muscle layer

and cut into small pieces in a low-Ca dissection solution (for composition, see below). The pieces were placed into the cell dispersion medium (for composition, see below) and incubated for 20–30 min at 37°C. After incubation, cells were dispersed by gentle pipetting and washed by centrifugation (1,500 rpm for 2 min). The isolated cells were resuspended in a high-K, 0-Ca solution (for composition, see below) and kept in a refrigerator. Cells were used within 6 h after preparation. The animals were killed by a humane procedure approved by the Animal Research Committee of the University of Virginia.

Cells were placed in a small chamber (volume 0.5 ml) on the microscope stage. After most viable cells had attached to the bottom of the chamber, the bath solution, warmed to 32–34°C, was perfused at a rate of 5 ml/min.

Solutions

The low-Ca solution for tissue dissection contained (mM): 136 NaCl, 6 KCl, 0.05 CaCl₂, 1.0 MgCl₂, 11 glucose, and 10 HEPES (pH 7.35 with NaOH). The cell dispersion medium contained, in addition to the above, 3 mg/ml papain, 2 mM dithiothreitol, and 3 mg/ml bovine serum albumin (pH 7.35 with NaOH).

The composition of high-K, 0-Ca solution for storing isolated cells was (mM): 85 KCl, 30 K₂HPO₄, 2 Na₂ATP, 1 MgCl₂, 5 sodium pyruvate, 5 creatine, 15 taurine, 1 EGTA, 10 glucose, and 1 mg/ml fatty acid-free bovine serum albumin (pH 7.35).

The bath perfusion solution for the measurement of Ba current through voltage-dependent Ca channels (I_{Ca}) contained (mM): 136 NaCl, 6 CsCl, 2.5 BaCl₂, 11 glucose, and 10 HEPES (pH 7.40 with NaOH). For the measurement of the voltage-dependent K current, the composition of the bath perfusion solution was (mM): 136 NaCl, 6 KCl, 2.5 MgCl₂, 11 glucose, and 10 HEPES (pH 7.40 with NaOH).

The pipette solution (intracellular solution) for the measurement of I_{Ca} contained (mM): 130 CsCl, 2 MgCl₂, 10 EGTA, 3 Na₂ATP, 0.1 NaGTP, and 10 HEPES (pH 7.20 with CsOH). The composition of the pipette solution for the measurement of the voltage-dependent K current was (mM): 130 KCl, 2 MgCl₂, 10 EGTA, 3 Na₂ATP, 0.1 NaGTP, and 10 HEPES (pH 7.20 with KOH).

Stock solutions of fatty acids were prepared in dimethylsulphoxide (DMSO) and stored under N₂ atmosphere in the dark at –20°C. Stock solutions of indomethacin, nordihydro-guaiaretic acid (NDGA), staurosporine, and phorbol-12,13-dibutyrate (PDBU) (in DMSO) were also kept dark at –20°C. The drugs were added to the bath perfusion solution from stock solutions, and the final DMSO concentration in the bath perfusion solution was 0.1%. Concentrations of fatty acids are given as the amounts added to the perfusion solution. However, due to their limited solubility, the monomeric concentration of free fatty acids in solution must necessarily be lower than the nominal concentrations indicated in the text, but on the other hand, their concentrations partitioned in the membrane may be higher due to their hydrophobicity.

Electrical Measurements and Statistical Analysis

The tight-seal whole cell patch clamp recording technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to record membrane currents. Patch pipettes were made from Corning #8161 glass with a double pull on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). The resistance of the pipettes was between 3 and 5 MΩ.

An Axopatch 1C patch clamp amplifier equipped with a CV-4 headstage (Axon Instruments, Inc., Burlingame, CA) was used. Currents were filtered at 1 kHz, and the signals were digitized and stored in an IBM-AT personal computer using the pCLAMP software and associated analog-to-digital converter and interface (Axon Instruments, Inc.). Analyses of the stored data were also performed using pCLAMP software. Linear leakage conductance was estimated by

applying 10-mV hyperpolarizing pulses from the holding potential (-60 mV), and all I_{Ca} measurements were corrected by subtracting the linear leakage current. Statistical analyses were performed by using paired or unpaired Student's t test.

Chemicals

Fatty acids (>99% pure), papain, dithiothreitol, bovine serum albumin, indomethacin, PDBU, and superoxide dismutase (SOD; from bovine erythrocytes) were obtained from Sigma Chemical Co. (St. Louis, MO). NDGA was obtained from Aldrich Chemical Co. (Milwaukee, WI). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) was from Seikagaku-kogyo (Tokyo, Japan). Staurosporine was from Kyowa Medex (Tokyo, Japan). All other chemicals were of reagent grade purity.

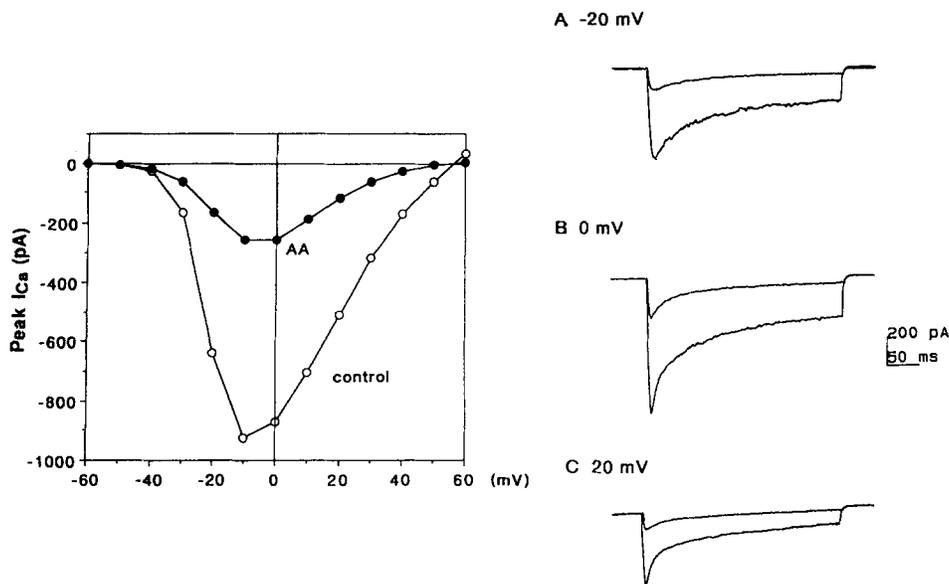


FIGURE 1. The effect of AA ($10 \mu\text{M}$) on Ba current through Ca channels (I_{Ca}) of intestinal smooth muscle cells. The bath solution contained 2.5 mM Ba^{2+} as the charge carrier. Depolarizing pulses (300 ms) ranging from -60 to $+60$ mV were applied from a holding potential of -60 mV, and peak amplitudes of I_{Ca} in the absence and presence of AA were plotted. Traces of currents that were elicited by depolarizations to -20 (A), 0 (B), and $+20$ mV (C) are also shown.

RESULTS

Fig. 1 shows the effect of $10 \mu\text{M}$ AA on Ba current through Ca channels (I_{Ca}). The inward current was generated by depolarizing pulses ranging between -60 and $+60$ mV (duration 300 ms) in the absence and presence of AA ($10 \mu\text{M}$) from a holding potential of -60 mV. Since there is no evidence of a low-threshold Ca current (T-type current) in this tissue (Terada, Kitamura, and Kuriyama, 1987; Inoue, Xiong, Kitamura, and Kuriyama, 1989), the high-threshold, dihydropyridine-sensitive Ca

current (L-type current) is the dominant inward current observed in this study. Application of AA in the bath reduced the amplitude of the current. Current responses to -20 , 0 , and $+20$ mV are shown on the right side of this figure. AA inhibited the peak amplitude of I_{Ca} by 75% at -20 mV, 70% at 0 mV, and 77% at $+20$ mV. The inhibition of the current amplitude at the end of the depolarizing pulse (300 ms) was 81% at -20 mV, 84% at 0 mV, and 80% at $+20$ mV. The results in a total of four similar experiments (Table I) indicated that inhibition was significantly greater at the end of the pulse than during the peak current. The I - V relationship of the peak I_{Ca} shows that AA depresses I_{Ca} at all command voltages tested, and that the reversal potential is not significantly altered by AA. The level of the holding current was not altered by $10 \mu\text{M}$ AA (Fig. 1), indicating that the inhibition of I_{Ca} is not due to cell membrane breakdown. In some experiments, we also tried to apply AA (10 – $30 \mu\text{M}$) intracellularly through the patch pipette, but under these conditions giga-seal formation and maintenance of the patch were very difficult. Therefore, we did not perform a systematic analysis of the effect of intracellularly applied AA, although, in a few successful experiments, intracellular AA also depressed I_{Ca} (data not shown).

The time course of the effect of AA on the peak I_{Ca} is shown in Fig. 2. In this series

TABLE I
Inhibition (Percent) of Voltage-gated Current through Ca^{2+} Channels by AA ($10 \mu\text{M}$)

mV	Peak	300 ms	
-20	67.0 ± 8.2	71.7 ± 4.7	
-10	62.2 ± 3.9	82.1 ± 2.8	$P < 0.01$
0	61.9 ± 3.7	83.7 ± 3.3	$P < 0.03$
$+10$	64.4 ± 3.4	82.2 ± 3.2	$P < 0.02$

Values are means \pm SE in four cells. Holding potential -60 mV.

of experiments, cells were held at -60 mV, and 300-ms command pulses to 0 mV were applied every 8 s. After the amplitude of I_{Ca} was stabilized (a gradual increase of I_{Ca} was usually observed during the first 2–3 min after the formation of whole cell clamp configuration), we began to store the current responses (time 0 in Fig. 2), and $10 \mu\text{M}$ AA was added to the bath. The peak amplitude of I_{Ca} was gradually depressed by AA by nearly 80% in this cell. Partial recovery of I_{Ca} was also observed after the elimination of AA from the perfusion solution. Inclusion of fatty acid-free albumin ($100 \mu\text{M}$) accelerated the recovery phase (data not shown). Three traces of I_{Ca} (before application of AA [A], after application of AA [B], and after washout of AA [C]) are also shown (right side of Fig. 2).

The relative amplitudes of the peak I_{Ca} after 4 min application of 0.3 – $30 \mu\text{M}$ AA are summarized in Table II. For this series of experiments the cells were held at -60 mV and I_{Ca} was elicited by 300-ms depolarizing pulses to 0 mV every 8 s. After the amplitude of I_{Ca} was stabilized, each concentration of AA was added to the bath for up to 4 min and I_{Ca} was monitored. Due to the slow time course of the AA effect (cf. Fig. 2), the effect of a single concentration of AA was examined in each cell. When we applied 0.1% DMSO (vehicle of AA) alone in the bath, the relative peak amplitude of

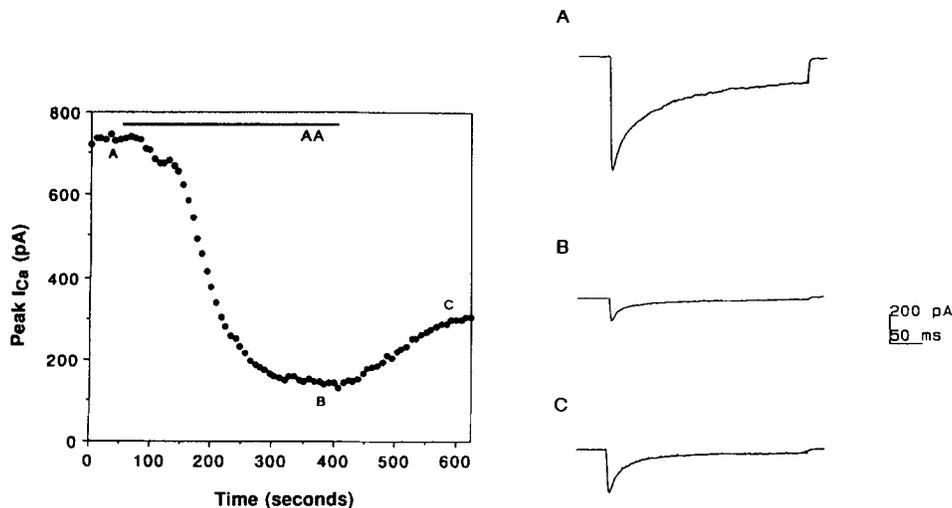


FIGURE 2. Time course of the AA-induced inhibition of I_{Ca} . The effect of AA (10 μ M) on I_{Ca} was monitored by repeated depolarizing pulses (300 ms, 0.125 Hz) to 0 mV from a holding potential of -60 mV, and absolute values of the amplitude of I_{Ca} were plotted. Three traces (A, B, and C) are also shown.

I_{Ca} after 4 min application was 0.91 ± 0.03 ($n = 15$ cells) compared with I_{Ca} before the application of DMSO. Therefore, we corrected the experimental I_{Ca} amplitudes by this average value. As seen in Table II, 10 μ M AA inhibited I_{Ca} by $\sim 65\%$ in 4 min, and 30 μ M AA inhibited it almost completely. Lower concentrations (0.3–3 μ M) of AA had no significant inhibitory effect on I_{Ca} , and in some cells we even observed a slight increase in I_{Ca} .

Since the onset of the inhibition of I_{Ca} by AA is gradual, as seen in Fig. 2, we tested whether repeated depolarizing pulses are needed for the AA effect (Fig. 3A) by holding the cell for 3.5 min at -60 mV in the absence of depolarizing pulses and in the presence of AA (30 μ M). Command pulses to 0 mV (300 ms, every 8 s) were restarted after the 3.5-min exposure to AA. As seen in this figure, the peak I_{Ca} was significantly depressed at this time in the absence of repeated pulses; this result fails to show any use dependence of the AA-induced inhibition of I_{Ca} , although we cannot rule out the possibility that channels that open, albeit very infrequently, at the negative holding potential (-60 mV) are the sites of AA-induced block.

TABLE II

Relative Peak Amplitude of I_{Ca} after a 4-min Application of AA

0.3 (μ M)	1.05 ± 0.07 (6)
1	1.08 ± 0.04 (6)
3	1.02 ± 0.08 (11)
10	0.34 ± 0.06 (7)
30	0.02 ± 0.01 (5)

Holding potential -60 mV, test potential 0 mV. Values are shown as means \pm SE (number of cells examined).

Fig. 3 B shows inactivation curves of I_{Ca} in the absence and presence of AA (10 μ M). For this series of experiments cells were held at -80 mV and depolarizing pulses to 0 mV (300 ms) were preceded by 2-s conditioning pulses to various voltages (-80 to $+40$ mV). These data were fitted to the Boltzmann function:

$$I_{Ca.test}/I_{Ca.control} = 1/[1 + \exp [(V - V_{1/2})/K]]$$

In the absence of AA, $V_{1/2}$ was -18.50 mV and the slope factor (K) was 7.28 . In the

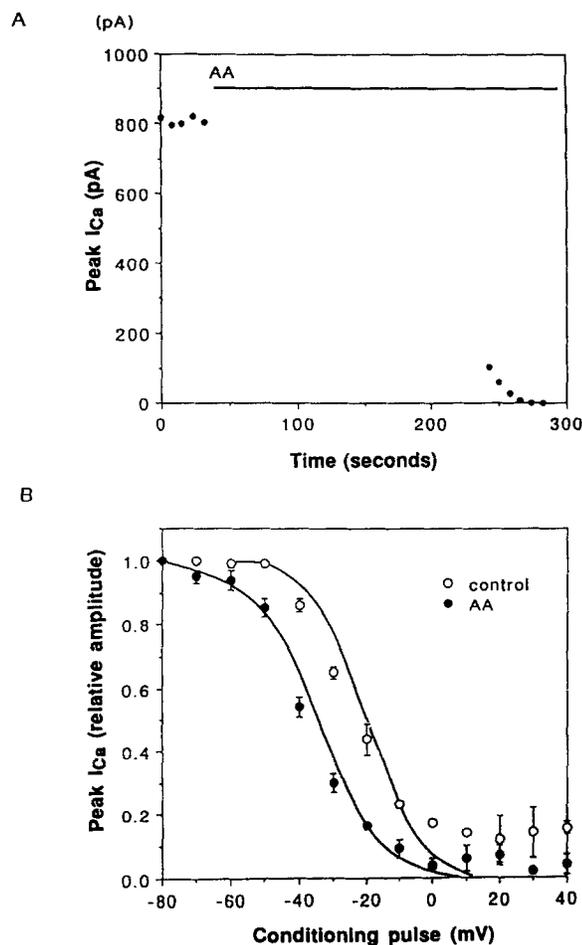


FIGURE 3. (A) The effect of AA (30 μ M) in the absence of repeated test pulses. The cell was held at -60 mV and AA was applied in the absence of depolarizing pulses. After 3.5 min of AA application, depolarizing pulses to 0 mV (300 ms, 0.125 Hz) from a holding potential of -60 mV were reapplied and absolute values of the I_{Ca} amplitude were plotted. (B) Inactivation curves of I_{Ca} in the absence of AA (control) and presence of AA (10 μ M). Test pulses to 0 mV (300 ms) were preceded by 2-s conditioning pulses (-80 to $+40$ mV), and mean relative amplitudes of I_{Ca} compared with the amplitude of I_{Ca} without conditioning pulse were plotted. Vertical bars indicate values of SE ($n = 4$ cells).

presence of AA, $V_{1/2}$ was -34.75 mV and the slope factor was 9.92 . Thus, application of AA caused a left shift of the inactivation curve, indicating that the AA effect is voltage dependent.

We next examined possible mechanisms of the AA-induced inhibition of I_{Ca} . Since AA is a substrate for cyclooxygenase and lipoxygenases, and metabolites of these pathways (AA cascade), such as prostaglandins and leukotrienes, have known biological activities (Needleman et al., 1986; Shimizu and Wolfe, 1990), we tested the effects

of indomethacin, a cyclooxygenase inhibitor, and NDGA, a lipoxygenase inhibitor, on the AA-induced inhibition of I_{Ca} .

Pretreatment with indomethacin (10 μ M) for 12 min before the addition of AA (10 μ M) had no significant effect on the inhibition of I_{Ca} by AA (Fig. 4A). The relative peak amplitude of I_{Ca} after a 4-min application of AA to the cells treated with

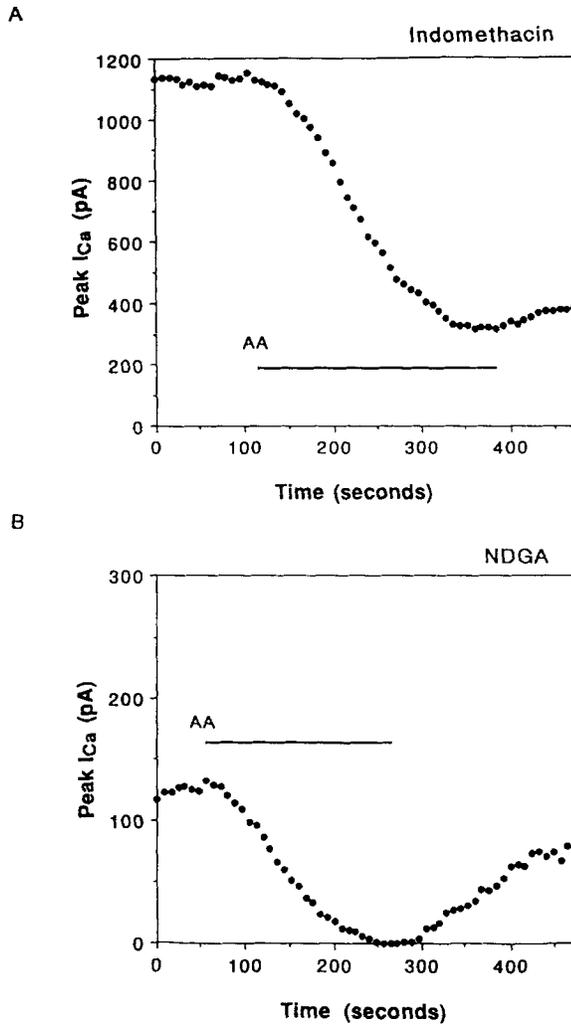


FIGURE 4. (A) The effect of indomethacin (10 μ M) on the AA-induced inhibition of I_{Ca} . The cell was pretreated with 10 μ M indomethacin for 12 min before the application of AA (10 μ M). Depolarizing pulses (300 ms, 0.125 Hz) to 0 mV were applied from a holding potential of -60 mV to monitor I_{Ca} , and absolute values of peak amplitudes of I_{Ca} were plotted. (B) The effect of NDGA (10 μ M) on the AA-induced inhibition of I_{Ca} . The cell was pretreated with 10 μ M NDGA for 10 min before the application of AA (10 μ M). Depolarizing pulses (300 ms, 0.125 Hz) to 0 mV were applied from a holding potential of -60 mV to monitor I_{Ca} , and absolute values of peak amplitudes of I_{Ca} were plotted.

indomethacin was 0.36 ± 0.05 ($n = 6$ cells), which was not significantly different from experiments without indomethacin (cf. Table II). Because NDGA itself also has an inhibitory effect on Ca channel current (Korn and Horn, 1990), it was difficult to quantitate precisely the effect of NDGA on the AA-induced inhibition of I_{Ca} . However, AA (10 μ M) further inhibited the residual component of I_{Ca} remaining

after pretreatment with NDGA (10 μM) for 10 min before the addition of AA as shown in Fig. 4 B (representative of four experiments).

AA is also reported to activate protein kinase C (McPhail et al., 1984) and inhibit Ca current of some neuronal cells through this mechanism (Linden and Routtenberg, 1989; Keyser and Alger, 1990). In these neuronal cell types, phorbol ester (a protein kinase C activator) (Castagna, Takai, Kaibuchi, Sano, Kikkawa, and Nishizuka, 1982) is reported to suppress Ca current (Linden and Routtenberg, 1989; Doerner, Abdel-Latif, Rogers, and Alger, 1990). Therefore, we tested the effect of PDBU (0.2 μM) on I_{Ca} , but found that it had no significant effect on I_{Ca} . The relative peak amplitude of I_{Ca} after a 4-min application of PDBU was 0.97 ± 0.07 ($n = 8$ cells). We also examined the effects of H7 and staurosporine, inhibitors of protein kinase C

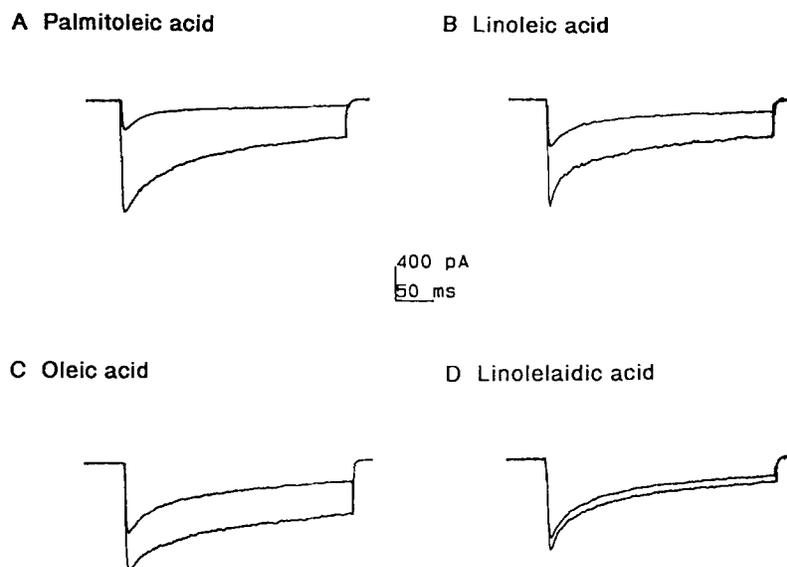


FIGURE 5. Effects of unsaturated fatty acids on I_{Ca} . Current traces before and after a 4-min application of each fatty acid are shown. Holding potential was -60 mV and test potential was 0 mV. (A) Palmitoleic acid (16:1, *cis*); (B) linoleic acid (18:2, *cis*); (C) oleic acid (18:1, *cis*); and (D) linolelaidic acid (18:2, *trans*).

(Kawamoto and Hidaka, 1984; Tamaoki, Nomoto, Takahashi, Kato, Morimoto, and Tomita, 1986) on the AA-induced inhibition of I_{Ca} . AA (10 μM) inhibited I_{Ca} even when the pipette solution (intracellular solution) contained 25 μM H7 (AA was applied 4–5 min after the formation of whole cell clamp configuration in this series of experiments). The relative peak amplitude of I_{Ca} after a 4-min application of AA (10 μM) was 0.43 ± 0.04 ($n = 4$ cells). When 50 μM H7 was contained in both the bath and pipette solutions, the relative peak amplitude of I_{Ca} after a 4-min application of AA (10 μM) was 0.12 ± 0.06 ($n = 3$ cells); this suppression of I_{Ca} by AA was significantly greater than in the absence of H7 (c.f. Table II). When the pipette solution contained 2 μM staurosporine, I_{Ca} was always small (50–100 pA). However, AA (10 μM) further suppressed, almost completely, the residual I_{Ca} ($n = 5$ cells).

Oxygen free radicals are also reported to be mediators of the AA-induced inhibition of Ca current in hippocampal CA1 pyramidal cells (Keyser and Alger, 1990). Therefore, we examined the effect of SOD on the AA effect on I_{Ca} . In this series of experiments, 90 U/ml of SOD (from bovine erythrocytes) was added to both the bath perfusion solution and pipette solution. However, SOD did not prevent the AA-induced inhibition of I_{Ca} . The relative peak amplitude of I_{Ca} after a 4-min application of AA (10 μ M) in the presence of SOD was 0.21 ± 0.01 ($n = 3$ cells).

To explore the specificity of the effect of AA, we also determined the effects of other fatty acids on I_{Ca} . The results obtained with some long-chain unsaturated fatty acids are shown in Fig. 5. Cells were held at -60 mV, and depolarizing pulses to 0 mV were applied in the absence and presence of 10 μ M palmitoleic acid (16:1, *cis*), linoleic acid (18:2, *cis*), oleic acid (16:1, *cis*), or linolelaidic acid (18:2, *trans*). Palmitoleic acid and linoleic acid depressed I_{Ca} , and oleic acid also caused a mild

TABLE III
Relative Peak Amplitude of I_{Ca} after a 4-min Application of Unsaturated Fatty Acids

Myristoleic acid (14:1, <i>cis</i>)	3 (μ M)	1.10 ± 0.02 (4)
	10	1.03 ± 0.07 (5)
Palmitoleic acid (16:1, <i>cis</i>)	3 (μ M)	1.10 ± 0.04 (5)
	10	0.32 ± 0.05 (4)
	30	0.02 ± 0.01 (5)
Oleic acid (18:1, <i>cis</i>)	3 (μ M)	1.09 ± 0.05 (4)
	10	0.84 ± 0.04 (5)
	30	0.74 ± 0.03 (4)
Linoleic acid (18:2, <i>cis</i>)	3 (μ M)	1.03 ± 0.08 (5)
	10	0.54 ± 0.17 (4)
	30	0.11 ± 0.03 (5)
Linolelaidic acid (18:2, <i>trans</i>)	3 (μ M)	1.01 ± 0.08 (5)
	10	1.01 ± 0.09 (5)
	30	1.08 ± 0.13 (5)

Holding potential -60 mV, test potential 0 mV. Values are shown as means \pm SE (number of cells examined).

inhibition of I_{Ca} , while linolelaidic acid had only little or no effect. The time course of these effects was also slow, as in the case of AA (data not shown).

The relative peak amplitudes of I_{Ca} after a 4-min application of each unsaturated fatty acid are summarized in Table III. Depolarizing pulses to 0 mV (duration 300 ms) from a holding potential of -60 mV were applied every 8 s in these experiments, and the effect of a single concentration of each fatty acid was examined in each cell. The experimental I_{Ca} amplitude was corrected using the data obtained in the experiments with DMSO alone as in the experiments summarized in Table II. Table III shows that 10–30 μ M palmitoleic acid and linoleic acid had significant inhibitory effects on I_{Ca} , while oleic acid had a weaker effect. On the other hand, myristoleic acid (14:1, *cis*) and linolelaidic acid had no significant effect even at a concentration of 30 μ M.

To determine whether saturated fatty acids also have effects similar to AA and other unsaturated fatty acids, we tested the effects of some long-chain saturated fatty acids. At first we examined the effect of myristic acid (MA) (14:0) on I_{Ca} . In Fig. 6, the cell was held at -60 mV and depolarizing pulses (300 ms) ranging from -60 to $+60$ mV were applied in the absence or presence of MA ($10 \mu\text{M}$). $10 \mu\text{M}$ MA caused a slight increase in the peak amplitude of I_{Ca} , especially at negative voltages. In this particular cell, a nearly twofold increase in the peak current amplitude was observed at -20 mV, while it was almost the same at $+20$ mV. Maximum current was observed at 0 mV in the control condition, while it was -10 mV in the presence of MA. MA also caused a slight left shift of the reversal potential by ~ 5 mV in this cell.

Fig. 7 shows the time course of the MA ($10 \mu\text{M}$) effect on I_{Ca} . The cell was held at

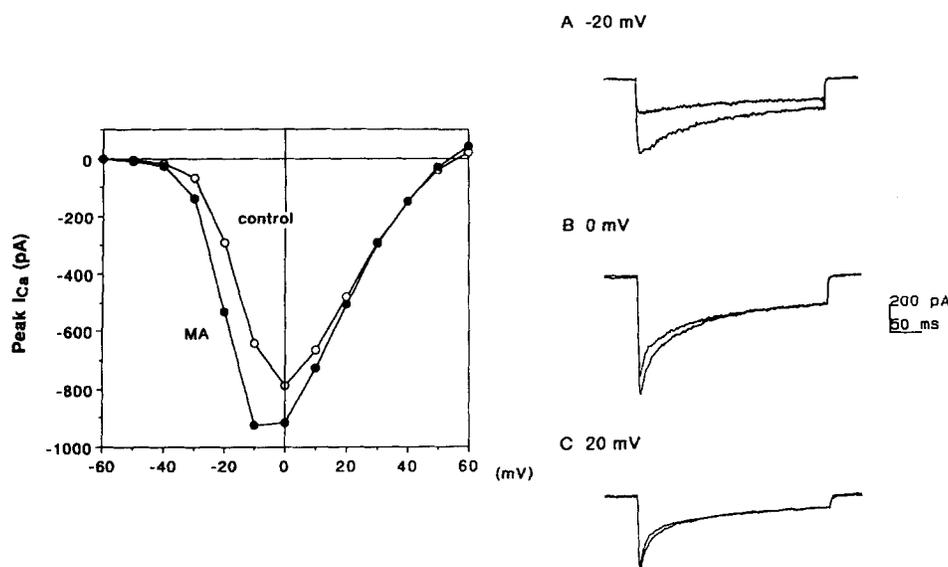


FIGURE 6. Effect of MA ($10 \mu\text{M}$) on I_{Ca} . Depolarizing pulses ranging from -60 to $+40$ mV were applied from a holding potential of -60 mV, and peak amplitudes of I_{Ca} in the absence and presence of MA were plotted. Traces of currents that were elicited by depolarizations to -20 (A), 0 (B), and $+20$ mV (C) are also shown.

-60 mV, and 300-ms command pulses to 0 mV were applied every 8 s. Bath application of $10 \mu\text{M}$ MA caused a slight increase in the peak amplitude of I_{Ca} within 1–2 min, as seen in this figure; after removal of MA, the amplitude of I_{Ca} gradually declined. Two traces (before application of MA [A] and after application of MA [B]) are also shown.

The effects of different concentrations of MA, and the effects of some other saturated fatty acids are summarized in Table IV. The relative peak amplitudes of I_{Ca} after a 2-min application of these fatty acids (I_{Ca} reached a plateau within 1–2 min) were calculated as in the experiments summarized in Table II. Since the relative amplitude of I_{Ca} after a 2-min application of 0.1% DMSO alone (vehicle of fatty acids) was 0.95 ± 0.02 ($n = 15$ cells), we corrected the obtained data of I_{Ca} amplitude by

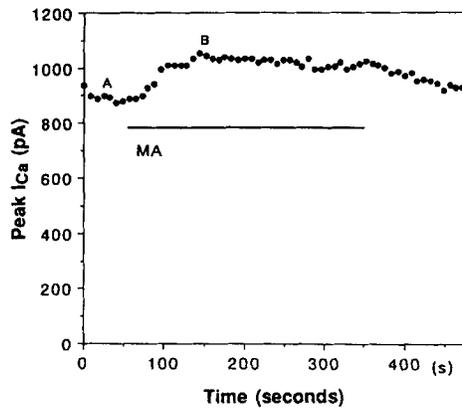


FIGURE 7. Time course of the MA effect on I_{Ca} . The effect of MA ($10 \mu\text{M}$) on I_{Ca} was monitored by repeated depolarizing pulses (300 ms, 0.125 Hz) to 0 mV from a holding potential of -60 mV, and absolute values of peak amplitudes were plotted. Two traces (A and B) are also shown.



this average value. The increases in the amplitude caused by $10\text{--}30 \mu\text{M}$ MA were statistically significant ($P < 0.01$), and the stimulatory effect was larger at the test potential of -20 mV. Among other saturated fatty acids, $30 \mu\text{M}$ lauric acid (12:0) also caused a significant increase in the amplitude of I_{Ca} ($P < 0.01$), while capric acid (10:0) and palmitic acid (16:0) had no significant effects. However, we could not test

TABLE IV
Relative Peak Amplitude of I_{Ca} after a 2-min Application of Myristic and Other Saturated Fatty Acids

Myristic acid	0.3 (μM)	0.96 ± 0.06 (4)
(14:0)	1	1.00 ± 0.02 (6)
	3	1.01 ± 0.02 (6)
	10	1.13 ± 0.03 (8)*
	30	1.30 ± 0.05 (7)*
	10 (test potential -20 mV)	1.48 ± 0.12 (7)*
	30 (test potential -20 mV)	1.66 ± 0.08 (7)*
Capric acid	10 (μM)	1.00 ± 0.03 (4)
(10:0)	30	1.00 ± 0.04 (8)
Lauric acid	10 (μM)	1.09 ± 0.05 (4)
(12:0)	30	1.18 ± 0.04 (9)*
Palmitic acid	10 (μM)	0.98 ± 0.03 (4)
(16:0)		

Holding potential -60 mV, test potential 0 mV. Data to the test potential of -20 mV were also shown in the case of myristic acid (10 and $30 \mu\text{M}$). Values are shown as means \pm SE (number of cells examined).

*Statistically significant current increase ($P < 0.01$).

the effect of 30 μM palmitic acid due to its insolubility, and the lack of effect at 10 μM may also have been due to its insolubility.

Finally, to evaluate the specificity of AA effect on membrane currents, we examined the effects of AA and MA on the voltage-dependent K current. For this series of experiments no Ca was added to the bath perfusion solution and the KCl-based pipette solution contained a high concentration of EGTA (10 mM). The cell was held at -60 mV, and depolarizing pulses to $+20$ mV were applied every 8 s to monitor the outward current at $+20$ mV. As shown in Fig. 8, 10 μM AA caused a reduction in the current amplitude (39% inhibition at the peak and 41% inhibition at the end of pulse in this cell). AA also changed the shape of the evoked current, suggesting that AA also affects the kinetics of the K current, whereas 10 μM MA was without significant effect. The peak amplitude of the current was 0.53 ± 0.05 ($n = 4$ cells) of the control

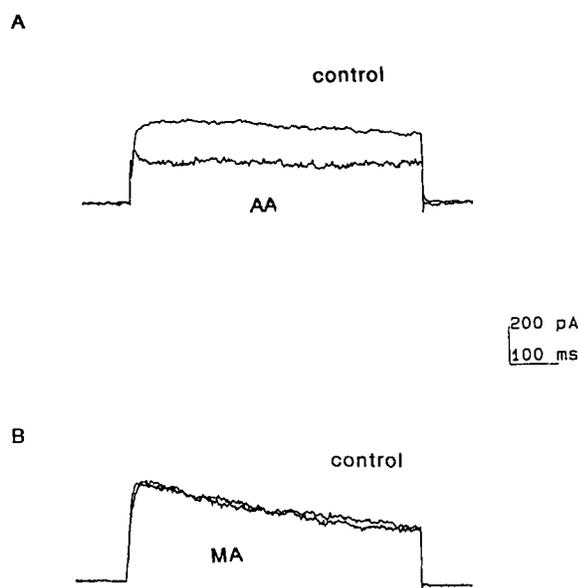


FIGURE 8. Effects of AA (10 μM) (A) and MA (10 μM) (B) on voltage-dependent K current of the intestinal smooth muscle cells. Two traces are superimposed before and after a 3-min application of AA or MA. Holding potential was -60 mV and test potential was $+20$ mV.

after a 2-min application of 10 μM AA and 0.94 ± 0.03 ($n = 4$ cells) after a 2-min application of 10 μM MA. This result indicates that AA can affect other membrane currents, as well as I_{Ca} , in the present preparation.

DISCUSSION

This study has shown the following effects of AA and other long-chain fatty acids on Ba current through Ca channels (I_{Ca}) in intestinal smooth muscle cells of rabbit: (1) Bath application of AA (10–30 μM) reduced the amplitude of I_{Ca} . (2) We could not find positive evidence for the involvement of metabolites of the AA cascade or protein kinase C in the inhibitory effect of AA on I_{Ca} . (3) Some other long-chain *cis*-unsaturated fatty acids (palmitoleic, linoleic, and oleic acids) also inhibited I_{Ca} , while a *trans*-unsaturated fatty acid (linoleic acid) did not. (4) Saturated fatty acids had

no inhibitory effects on I_{Ca} , and myristic acid and lauric acid caused a slight increase in the amplitude of I_{Ca} . (5) AA also inhibited the voltage-dependent K current.

The effects of fatty acids on voltage-dependent Ca channels in other cell types are variable. AA (1 μ M) increases Ca current in GH₃ pituitary cells (Vacher et al., 1989), while *cis*-unsaturated fatty acids inhibit Ca current in mouse neuroblastoma cells (Linden and Routtenberg, 1989), and AA also inhibits Ca current in hippocampal CA1 pyramidal cells (Keyser and Alger, 1990). It has been suggested that these inhibitory effects of *cis*-unsaturated fatty acids on Ca current are mediated by protein kinase C (Linden and Routtenberg, 1989), or a combination of protein kinase C activation and superoxide anion generation in the AA cascade (Keyser and Alger, 1990).

In this study indomethacin and NDGA failed to block the inhibitory effect of AA on I_{Ca} , and similar inhibitory effects on I_{Ca} were observed with other *cis*-unsaturated fatty acids that are not substrates for either cyclooxygenase or lipoxygenase. NDGA and staurosporine, inhibitors of lipoxygenase and kinase C, respectively, inhibited the voltage-gated current through Ca channels (this study), presumably as the result of nonspecific effects of these compounds. Furthermore, NDGA is reported to inhibit both lipoxygenation and cyclooxygenation (Payan, Goldman, and Goetzl, 1984). Yet in spite of their relatively nonselective blocking and nonspecific inhibitory effects, these compounds did not interfere with the inhibitory effect of AA, supporting the conclusion that it is not mediated by an AA metabolite or by a kinase C (see below) that is inhibited by staurosporine. Obviously we cannot exclude the possible involvement of some metabolites of AA generated by pathways of AA metabolism that are not inhibited by the inhibitors at the concentrations used in this study.

Inhibitors of protein kinase C did not prevent the inhibitory effect of AA on I_{Ca} in the present preparation, unlike the results obtained in some neuronal cell types (Linden and Routtenberg, 1989; Keyser and Alger, 1990). Since AA selectively activates the gamma isoform of protein kinase C that is rich in neuronal cells (Shearman, Naor, Sekiguchi, Kishimoto, and Nishizuka, 1989), the apparent lack of an effect mediated by protein kinase C in our experiments may reflect differences between neuronal and smooth muscle protein kinase C isozymes. However, phorbol ester at a concentration that inhibits the Ca current in neuronal cells (Linden and Routtenberg, 1989; Doerner et al., 1990) also had no effects on I_{Ca} in our preparation, and we note that even in neuronal cells high concentrations of diacylglycerol analogues can inhibit I_{Ca} through a mechanism independent of protein kinase C activation (Hockberger, Toselli, Swandulla, and Lux, 1989; Doerner et al., 1990). Our results do not exclude the possibility that kinase C, while not mediating the suppression of the Ca current by AA, can affect the L-type channels in ileal smooth muscle; indeed, the inhibitory effect of staurosporine itself and the potentiation of the inhibitory effect of AA by H7 could be so interpreted. However, this interpretation would require the existence of a (hitherto unreported) kinase C isozyme that is constitutively active at the low Ca concentrations present in our patched cells and is not further stimulated by phorbol ester.

We also consider it unlikely that the AA-induced inhibition of I_{Ca} in rabbit ileal smooth muscle cells is mediated by oxygen radical species, because SOD also had no effect on the AA-induced inhibition of I_{Ca} . Similarly, the possibility that the release of

Ca from intracellular stores (sarcoplasmic reticulum; Somlyo and Somlyo, 1971) by AA causes "Ca-induced inactivation of Ca current" (Ohya, Kitamura, and Kuriyama, 1988) is not likely, because the pipette solution contained a high concentration of EGTA (10 mM) and 2.5 mM Ba was used as the charge carrier.

A significant measure of specificity of AA action is suggested by the fact that it enhances a K channel current in amphibian stomach (Ordway et al., 1989), but not the voltage-dependent K current in mammalian neuronal (Keyser and Alger, 1990) and intestinal smooth muscle (this study). Furthermore, in atrial cardiac muscle, AA selectively affects certain, but not all, K channels (Kim and Clapham, 1989) and, unlike in amphibian smooth muscle (Ordway et al., 1989), it is active only when applied to the cytoplasmic side. Our results do not allow us to determine whether the effects of fatty acids on I_{Ca} are direct or mediated by some second messenger systems. However, the slow onset of the AA effects suggests that, if direct binding to the channel does occur, it is to a buried, hydrophobic domain of the channel, and takes place after incorporation of AA into the bilayer. In view of the tissue to tissue variability and specificity of some of these effects, it seems unlikely that they are due solely to a general physicochemical change, such as a general increase in membrane lipid fluidity induced by *cis*-saturated fatty acids (Klausner, Kleinfeld, Hoover, and Karnovsky, 1980; Karnovsky, Kleinfeld, Hoover, and Klausner, 1982).

Activation of certain receptors can release AA from the cell membrane (Axelrod, 1990), and may increase the local concentration of AA just beneath the cell membrane sufficiently to affect membrane current. In some smooth muscle cells, excitatory agonists have been reported to have inhibitory effects on L-type Ca channel current. Noradrenaline reduces the amplitude of L-type Ca channel current in cultured smooth muscle cells of rat portal vein (Pacaud, Loirand, Mironneau, and Mironneau, 1987) and in smooth muscle cells of guinea pig vas deferens (Imaizumi, Takeda, Muraki, and Watanabe, 1991). These phenomena were explained by "Ca-induced inactivation of Ca current" mechanism, because excitatory agonists increase intracellular Ca concentration by releasing Ca from the intracellular stores. In the intestinal smooth muscle cells of rabbit (the present preparation), reduction of Ca current by an excitatory (muscarinic) agonist is observed only when EGTA in the pipette solution is low and the charge carrier is Ca (unpublished observation). Although this could be interpreted as evidence of "Ca-induced inactivation of Ca current," it does not exclude an alternative mechanism mediated by AA (or other fatty acids), because phospholipase A₂ requires some Ca even when activated by receptor stimulation (Axelrod, 1990). The potential effects of fatty acids and other second messengers, including calcium, on ion channel currents are likely to contribute to the complexity of the electrical responses that are associated with pharmacomechanically coupled (Somlyo, Kitazawa, Kobayashi, Gong, and Somlyo, 1991), agonist-induced contractions of smooth muscle.

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