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Effects of Arachidonic Acid on the Calcium Channel Current (I_{Ba}) and on the Osmotic Stretch-induced Increase of I_{Ba} in Guinea-Pig Gastric Myocytes

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We employed the whole-cell patch clamp technique to investigate the effects of arachidonic acid (AA) on barium inward current through the L-type calcium channels (I_{Ba}) and on osmotic stretch-induced increase of I_{Ba} in guinea-pig antral gastric myocytes. Under isosmotic condition, AA inhibited I_{Ba} in a dose-dependent manner to 91.1±1.4, 72.0±3.2, 46.0±1.8, and 20.3±2.3% at 1, 5, 10, 30 mM, respectively. The inhibitory effect of AA was not affected by 10 μM indomethacin, a cyclooxygenase inhibitor. Other unsaturated fatty acids, linoleic acid (LA) and oleic acid (OA) were also found to suppress I_{Ba} but stearic acid (SA), a saturated fatty acid, had no inhibitory effect on I_{Ba}. The potency sequence of these inhibitory effects was AA (79.7±2.3%) > LA (43.1±2.7%) > OA (14.2±1.1%) at 30 μM. On superfusing the myocyte with hyposmotic solution (214 mOsm) the amplitude of I_{Ba} at 0 mV increased (38.0±5.5%); this increase was completely blocked by pretreatment with 30 mM AA, but not significantly inhibited by lower concentrations of AA (1, 5 and 10 μM) ($P>0.05$). Unsaturated fatty acids shifted the steady-state inactivation curves of I_{Ba} to the left; the extent of shift caused by AA was greater than that caused by LA. The activation curve was not affected by AA or LA. The results suggest that AA and other unsaturated fatty acids directly modulate L-type calcium channels and AA might modulate the hyposmotic stretch-induced increase of L-type calcium channel current in guinea-pig gastric smooth muscle.

Key Words: Calcium channel, Gastric myocytes, Osmotic stretch, Arachidonic acid

INTRODUCTION

Calcium influx through voltage-dependent Ca channels plays a central role in cellular signal transduction in various types of cells. In smooth muscle cells, the activation of Ca channels is essential for action potential generation and associated Ca influx-induced contraction (Itoh & Kuriyama, 1994). In our previous study, we reported that osmotic stretch increases voltage-dependent Ca channel current in guinea-pig gastric myocytes (Xu et al, 1996). Mechanical stress or stretch of cell membrane can modulate various signal transduction pathways, including that of phospholipase A_2 (PLA_2) (Thuren et al, 1987; Sadoshima & Izumo, 1993; Vandenburgh et al, 1993). Activation of PLA_2 will increase the amount of arachidonic acid (AA), a kind of unsaturated fatty acid in lipid membrane.

Unsaturated fatty acids are a major component of cell membrane phospholipid (Irvine, 1982) and a number of AA metabolites from cyclooxygenase and lipoxygenase pathways mediate a variety of cell signaling events under both physiological and pathological conditions (Needleman, 1986). Various modulatory effects of unsaturated fatty acids on ion channels, including voltage-dependent Ca channel, have

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recently been reported (Shimada et al, 1992; Chesnoy-Marachais et al, 1994; Nagano et al, 1995; Ordway et al, 1991, 1995; Gosling et al, 1996). Ordway et al (1995) reported that endogenous fatty acids serve as mediators for activation of a K^+ channel by membrane stretch, and both stretch and exogenously-applied fatty acids activate the channel. In human umbilical vein endothelial cells, mechanosensitive Ca^{2+} transients were inhibited by PLA_2 inhibitor and AA alone was able to induce a Ca^{2+} transient (Oike et al, 1994). In those experiments, the signal-mediating fatty acids might have been generated in response to membrane stretch by a mechanically-sensitive phospholipase A_2 (Thuren et al, 1987; Sadoshima & Izumo, 1993).

In this study, we observed the effect of unsaturated fatty acids on calcium channel current using Ba^{2+} as a charge carrier (I_{Ba}) and also tested whether AA modulates I_{Ba} increase through osmotic stretch of the myocytes membrane.

METHODS

Preparation of cells

Guinea-pigs of either sex weighing 300～350 g were exsanguinated after stunning. The antral part of the stomach was cut and the mucosal layer was removed from muscle layers in Ca-free physiological salt solution (Ca-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and was cut into small segments (2×3 mm). These were incubated for 30 min at 4°C in a medium modified from Kraft-Brühe (K-B) medium (Isenberg, & Klöckner, 1982). They were then incubated for 15～25 min at 35°C in digestion medium (Ca-free PSS) containing 0.1% collagenase (Wako, Japan), 0.05% dithioerythreitol, 0.1% trypsin inhibitor and 0.2% bovine serum albumin. After digestion, the supernatant was discarded and the softened muscle segments were transferred into modified K-B medium; single cells were dispersed by gentle agitation with a wide-bored glass pipette. Isolated gastric myocytes were kept in modified K-B medium at 4°C until use. All experiments were carried out at room temperature within 12 hours of harvesting cells.

Whole-cell voltage clamp

Isolated cells were transferred to a small chamber on the stage of an inverted microscope (IMT-2, Olympus, Japan) and perfused with PSS (2～3 ml/min). Glass pipettes with a resistance of 2～5 MΩ were used to make a giga seal of 5～10 GΩ. An Axopatch-1C patch-clamp amplifier (Axon instruments, USA) was used to record membrane currents and command pulses were applied using an IBM-compatible 486-grade computer and pClamp software v.5.5.1(Axon instruments, USA). Data were displayed on a digital oscilloscope (PM 3350, Philips, Netherlands), and a computer monitor.

Solutions

Ca-free PSS containing (in mM) NaCl 131, KCl 4.5, glucose 5, and *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) 10 was adjusted to pH 7.4 with tris[hydroxymethyl]aminomethane (TRIZMA). Modified K-B solution containing (in mM) L-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, HEPES 10, and ethyleneglycol bis-(β-aminoethyl ether-*N,N,N',N*-tetraacetic acid (EGTA) 0.5 was adjusted to pH 7.4 with KOH. External solutions containing (in mM) NaCl 80, KCl 4.5, HEPES 10, $BaCl_2$ 10, $MgCl_2$ 1, and glucose 10 were adjusted to hypotonic solution and isosmotic solution with sucrose, and pH was adjusted to 7.4 with tris. The osmolality of control, hypo- and hyperosmotic solution was measured using an osmometer (Advanced Digimatic Osmometer, Model 3D2, USA). Pipette solution containing (in mM) CsCl 110, TEA 20, EGTA 10, HEPES 10, Na_2ATP 3, and $MgCl_2$ 3.5 was adjusted to pH 7.3 with tris.

Drugs

All drugs used in this study were purchased from Sigma (USA). Arachidonic acid, linoleic acid and oleic acid were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stearic acid was dissolved in alcohol at 10 mM. The vehicle alone had no effect on whole-cell calcium currents at the concentrations used (0.3%, $P>0.05$, n = 8) and was always added in control solutions. The stock solutions of unsaturated fatty acids were tightly sealed in ampules and stored at −20°C until use.

Statistics

The data are expressed as mean±SEM. Statistical significance was estimated by Student's paired *t* test; P values less than 0.05 were considered to be statistically significant.

RESULT

The effect of AA on I_{Ba} under an isosmotic condition

10 μM AA, an unsaturated fatty acid with four double bonds, slowly decreased both peak and steady-state amplitude of I_{Ba} and such effect was partially reversible after washout(69.5±1.3%, n = 8) (Fig. 1A). As shown in Fig. 1 B, AA inhibited I_{Ba} in a dose-dependent manner to 91.1±1.4, 72.0±3.2, 46.0±1.8, and 20.3±2.3% at 1, 5, 10, 30 μM, respectively (mean±SEM, n = 10). This inhibitory effect of AA was not affected by indomethacin, a cyclo-oxygenase inhibitor. 15 minutes after pretreatment with 10 μM of this inhibitor, 10 μM AA still inhibited I_{Ba} to similar extent (47.3±2.5%, n = 6, Fig. 1C).

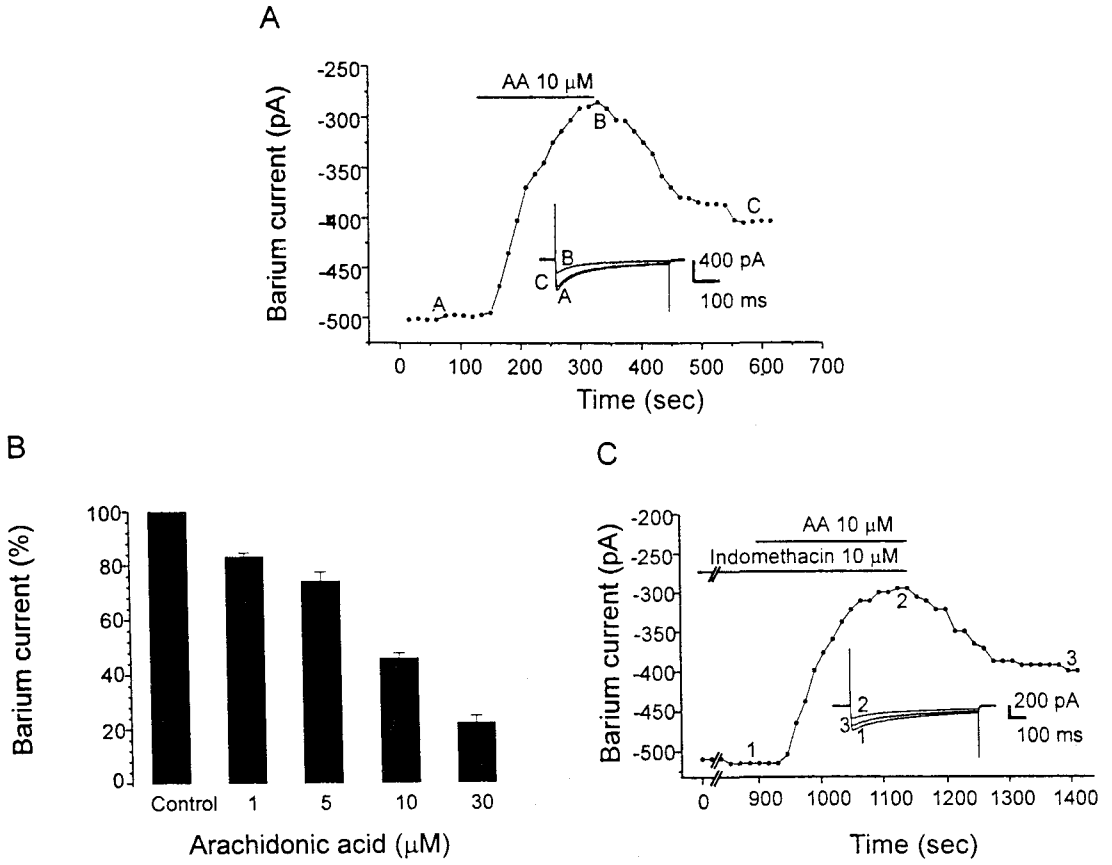

Fig. 1. Effects of arachidonic acid (AA) on I_{Ba} through Ca channels. I_{Ba} was elicited by 500 ms depolarization from −80 to 0 mV every 20 seconds. (A) shows the effect of AA on I_{Ba} in the myocytes isolated from guinea-pig stomach. AA (10 μM) was applied during the period indicated by a horizontal bar. I_{Ba} decreased significantly and then partially recovered following washout of AA. Time 0 indicates the start of I_{Ba} recording, with potassium current blocked by internal diffusion of Cs from the recording pipette for about 3 min after rupture of patch membrane. (B) shows the dose-dependent inhibitory effect of AA on I_{Ba}. AA decreased I_{Ba} in a dose-dependent manner; the half inhibitory concentration of AA was about 10 μM. (C) shows that 10 μM indomethacin was added to the external solution, and after about 10 minutes, AA was also administered. Indomethacin did not block this inhibitory effect of AA on I_{Ba}.

Effect of other fatty acids on I_{Ba}

Linoleic acid (LA), another unsaturated fatty acid with two double bonds, also reversibly decreased I_{Ba} (Fig. 2A) and dose-dependently inhibited I_{Ba} to 90.7 ±1.4, 82.7±1.6, 71.4±2.9 and 53.0±2.7% at 1, 5, 10 and 30 μM, respectively (n = 6, Fig. 2B). We also tested the effects of 30 μM oleic acid (OA), an unsaturated fatty acid with one double bond, and of 30 μM stearic acid (SA), a saturated fatty acid, on I_{Ba}. SA had no effect on I_{Ba}. The inhibitory effects of three unsaturated fatty acids were compared (Fig. 3A). The potency sequence of the inhibitory effects

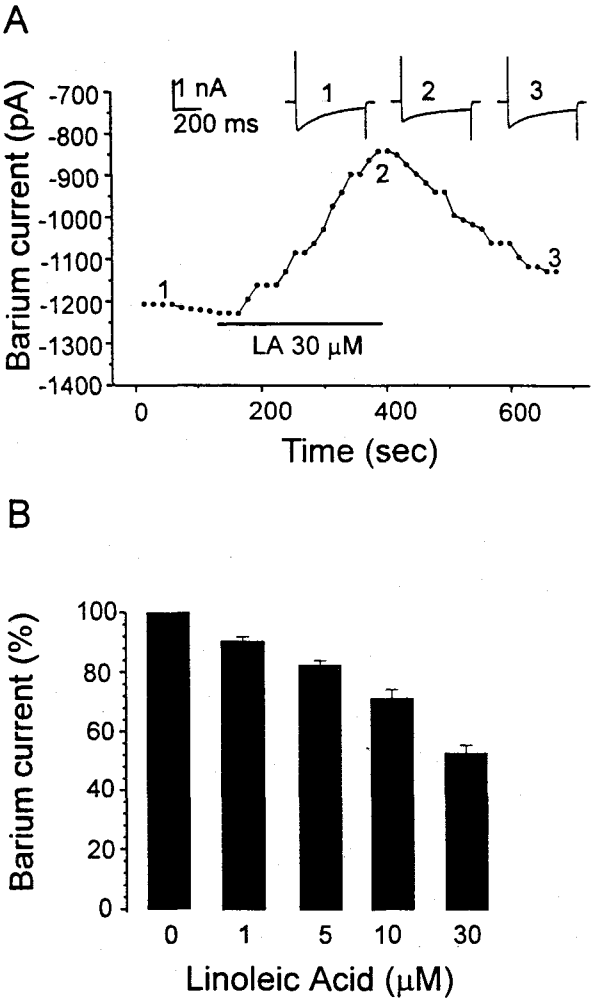

Fig. 2. Effect of another unsaturated fatty acid, linoleic acid (LA), on I_{Ba}. (A) shows the inhibitory effect of LA on I_{Ba}. LA (30 (M) was applied during the period indicated by a horizontal bar. I_{Ba} was significantly inhibited, and then recovered partially after washout of LA. (B) shows the concentration-dependence of the inhibitory effect of LA on I_{Ba}. LA also decreased I_{Ba} in a dose-dependent manner; the half inhibitory concentration of LA was about 30 μM.

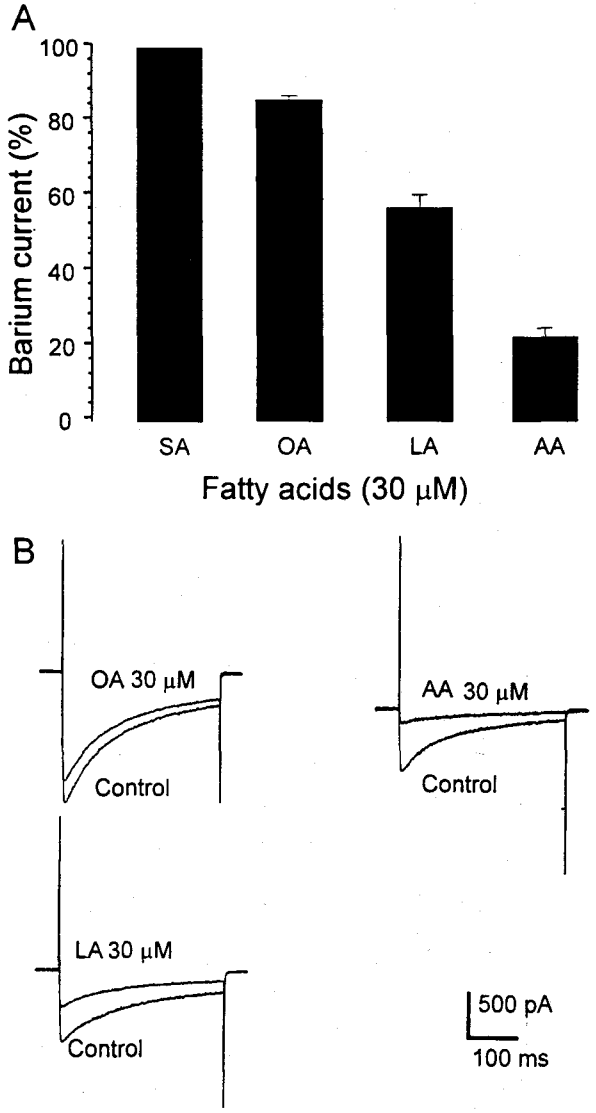

Fig. 3. Comparison of inhibitory effects of unsaturated fatty acids with that of saturated fatty acid on I_{Ba}. (A) shows the inhibitory effects of various fatty acids (30 μM) on I_{Ba}. Unsaturated fatty acid decreased I_{Ba} but a saturated fatty acid, stearic acid (SA), had no effect. This inhibitory effect seemed to be related to the number of double bonds in a fatty acid chain; the potency of inhibition was AA (4 double bonds) > LA (2 double bonds) > OA (1 double bond). (B) shows representative current traces which represented one cell in each group, respectively.

was AA (79.7±2.3%) > LA (43.1±2.7%) > OA (14.2±1.1%) at 30 μM (n = 6) and this sequence was in accordance with the number of double bonds in the fatty acid chain. Fig. 3B shows representative raw traces of I_{Ba}.

Effect of arachidonic acid on osmotic stretch-induced I_{Ba} increase

Membrane potential was held at −80 mV and step pulses to 0 mV were applied repetitively every 20 seconds. Cell membrane stretch was elicited by superfusing the myocytes with hyposmotic solution (214 mOsm). I_{Ba} increased under these conditions and the maximal increase (38.0±5.5%, n = 10) was obtained within 1 min of hyposmotic superfusion (Fig. 4A). In Fig. 4B, 30 μM AA was added to an isosmotic superfusing solution (290 mOsm) and I_{Ba} decreased slowly from 1186 pA to 286 pA; under these conditions, hyposmotic stretch caused no increase of I_{Ba}. After washout of AA, however, hyposmotic superfusion increased I_{Ba} from 335 pA to 536 pA. We performed similar experiments at various concentrations of AA and the extents of I_{Ba} increase were compared (Fig. 4C). The effect of AA on hyposmotic stretch-induced I_{Ba} increase was insigni-

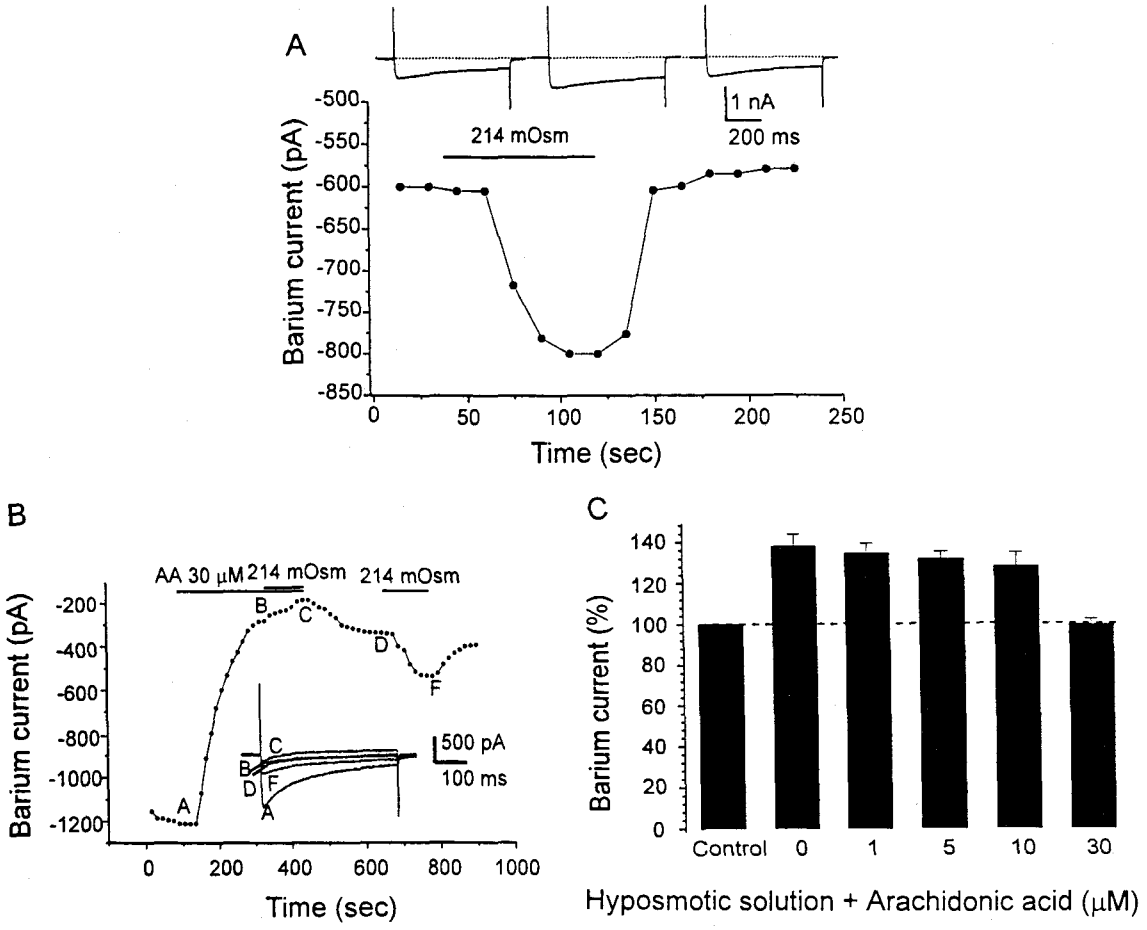

Fig. 4. Effect of AA on osmotic stretch-induced calcium current increase. I_{Ba} was elicited by 500 ms depolarization from −80 to 0 mV every 20 seconds. (A) shows that osmotic stress significantly increased I_{Ba}, which was completely recover under the isosmotic condition. (B) shows that AA (30 μM) completely blocked the increase of I_{Ba} induced by osmotic stress, and that I_{Ba} was still enhanced by osmotic stress after washout of AA. (C) shows the concentration-dependence of AA-induced I_{Ba} inhibition. Osmotic stress-induced increase of I_{Ba} was not affected by AA at 1, 5 and 10 μM. However, osmotic stress-induced enhancement of I_{Ba} was completely blocked at 30 μM of AA (mean±SEM, n = 6).

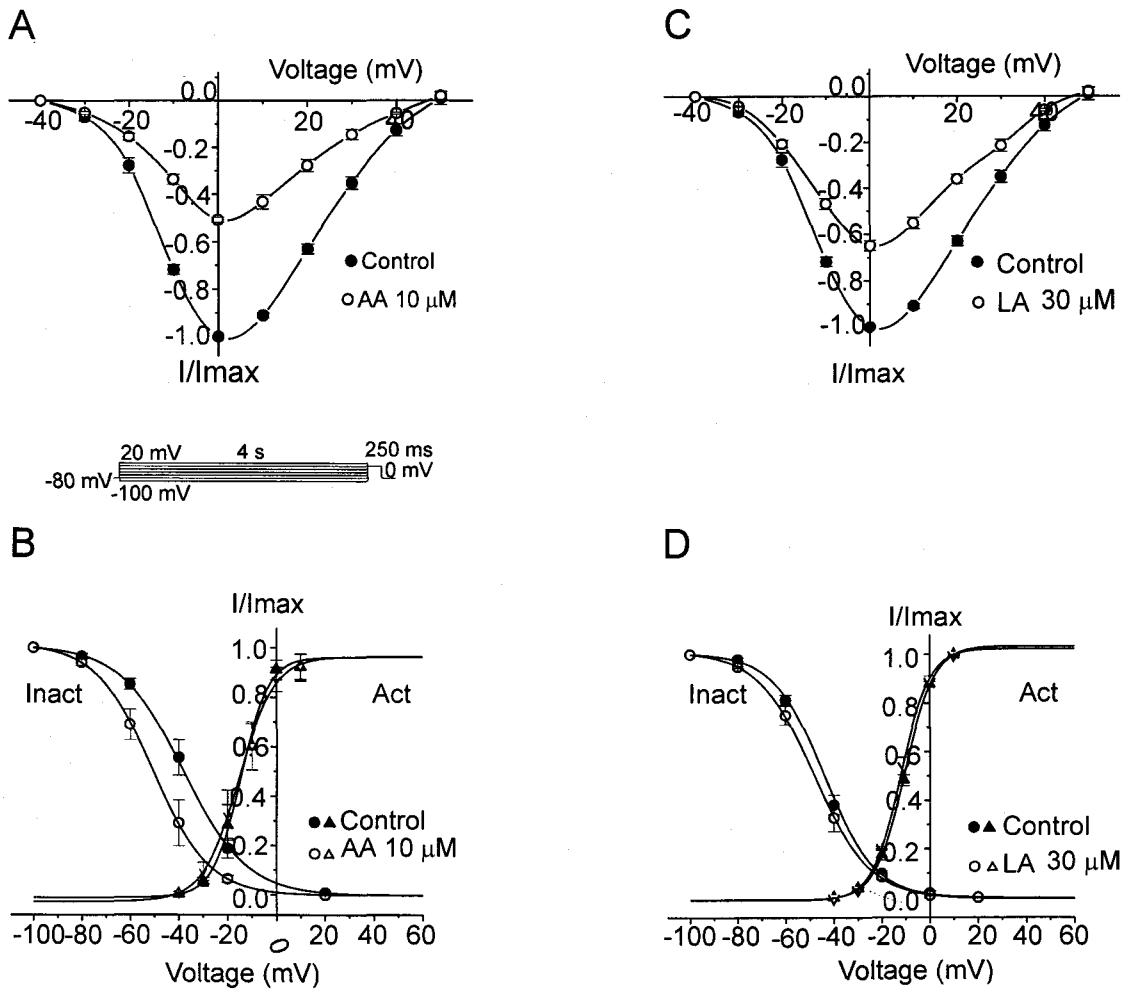

Fig. 5. Current-voltage (*I/V*) relationships for cells exposed to control (filled symbol) and AA (open symbol) (mean ± SEM, n = 8). Peak values were normalized to the values obtained at 0 mV under control conditions and in 10 μM AA (A). Steady-state activation and inactivation curves for the cells exposed to control solution (*filled symbols*) and to 10 μM AA (*open symbols*) are shown. For the steady-state activation relationship, peak conductance was determined from peak inward current, corrected for the change in driving force at each test potential and normalized to 1. Driving force was obtained from the difference between test potential and observed reversal potential (50 mV). For the steady-state inactivation relationship, peak currents were obtained using a two-pulse protocol (4 seconds of prepulse potentials from −100 to +20 mV followed by a 7 ms interpulse interval at +80 mV) and membrane potential was raised to a test potential of 0 mV for 250 ms (see inset). The difference between peak current and late current present before the end of the test pulse was normalized to 1 and plotted against the prepulse potential. The data from each group were fitted by the Boltzmann equation: f_∞ (V) or d_∞ (V) = $\{1+\exp[(V-V0.5)/k]\}^{-1}$, where f_∞ (V) and d_∞ (V) are activation and inactivation parameters, respectively, $V0.5$ is the voltage of half activation, and k the slope factor (see *Result*). In addition, the combined data are fitted by the same equation and are drawn as solid lines. AA decreased I_{Ba} at each membrane potential level but did not shift the *I/V* relationship (A). 10 μM AA significantly shifted the steady-state inactivation curve to the left but did not affect steady-state activation curve (B). 30 μM LA also inhibited I_{Ba} at each voltage level but did not shift the *I/V* curve (C). Like AA, LA also shifted the steady-state inactivation curve to the left but did not affect the steady-state activation curve (D).

ficant at lower concentrations (1~10 μM, P > 0.05, Fig. 4C).

The effects of AA and LA on steady-state voltage-dependent inactivation and activation of I_{Ba}

In order to assess whether the changes in peak I_{Ba} caused by AA were due to shifts in the current/voltage (*I/V*) relationship of I_{Ba}, currents were also elicited over a wide range of test potentials. Fig. 5A shows *I/V* relationships for normalized I_{Ba} in control solution and on treatment with 10 μM AA. Activation of inward current elicited from a holding potential of −80 mV began at approximately −40 mV, maximum I_{Ba} occurred at 0 mV, and observed reversal potential was about +50 mV. AA decreased I_{Ba} throughout the test potential range and mean normalized peak current at 0 mV decreased from 1 to 0.50±0.01 (n = 8). However, there was no parallel shift in *I/V* relations in control solution and on treatment with AA.

A double-pulse protocol was used to measure the steady-state inactivation of I_{Ba} as a function of membrane potential. Prepulse potentials ranging from −100 mV to +40 mV were applied for a duration of 4 seconds, and following a 7 ms interpulse interval at a potential of −80 mV, membrane potential was again raised to a test potential of 0 mV for 250 ms. Currents were then normalized to the current obtained at −100 mV prepulse potential (*I/I*max) and plotted against each prepulse potential. Plotted data were well fitted by a Boltzmann equation, with a half inactivation voltage ($V_{0.5}$) of −38.5±0.7 mV in control solution and −50.8±0.3 mV in 30 μM AA ($P<0.05$, n = 6), and slope factor (k) of 12.5±0.5 in control solution and 11.5±0.1 ($P>0.05$) in 30 μM AA (Fig. 5B).

Steady-state activation curves were estimated from the peak conductance at each potential by using the following equation: $I_{Ba} = g_{Ba} \times (V\text{-}E_{rev})$ where g_{Ba}, V, and E_{rev} are peak conductance, test potential and observed reversal potential, respectively. Half activation values were −14.5±1.4 and −15.3±1.1 mV ($P>0.05$, n = 6) with slope factors (k) of 5.7±0.7 and 7.3±0.9 ($P>0.05$) in control solution and in 30 μM AA, respectively (Fig. 5B).

30 μM LA also inhibited I_{Ba} throughout the test potential range (Fig. 5C) and also shifted the inactivation curve of I_{Ba} to the left (Fig. 5D). In the control solution, half inactivation voltage ($V_{0.5}$) was changed from −43.4±1.8 mV to −49.9±1.5 mV ($P<0.05$, n = 6) by 30 μM LA. The extent of suppression of the *I-V* relationship and the shift in the inactivation curve were smaller than those induced by 30 μM AA. There was no parallel shift of the *I/V* relationship and the steady-state activation curves were not changed by LA (half activation voltages were −10.4±0.3 and −11.9±0.4 mV ($P>0.05$) in control solution and on treatment with LA, respectively (Fig. 5D).

DISCUSSION

In this study, exogenously applied AA (30 μM) completely blocked osmotic stretch-induced increase of voltage-operated calcium channel current. Direct inhibitory effects of unsaturated fatty acids on calcium channel current were also observed, and these were similar to those described in previous reports concerning other kinds of cells (Shimada & Somlyo, 1992; Nagano et al, 1995; Petit-Jacques & Hartzell, 1996).

Although the mechanism of this inhibitory effect of AA on calcium channel current is still unclear, we suppose that the effect of AA on I_{Ba} was a direct one as we found that indomethacin, a cyclooxygenase inhibitor, did not block the effect of AA on I_{Ba} and other unsaturated fatty acids, LA and OA, also inhibited calcium channel current and shifted the steady-state inactivation curve. Previous investigators also observed an inhibitory effect of AA on calcium channel current (Shimada & Somlyo, 1992 in guinea- pig intestinal smooth muscle; Nagano et al, 1995 in guinea-pig vas deferens smooth muscle; Petit-Jacques & Hartzell, 1996, in frog cardiac myocytes) and suggested that the fatty acid might elicit its effects by interacting with the channel itself, or by altering the lipid bilayer in guinea-pig vas deferens smooth muscle (Ordway et al, 1991; Nagano et al, 1995). The direct action of AA on membrane phospholipid may imply an effect on the bulk properties of membrane lipid that is commonly referred to as 'membrane fluidity' (Anthony & Donald, 1986; Robert et al, 1986) and this possibility is strengthened by observed results that the potencies of these inhibitory effects and the extent to which the steady-state inactivation curve shifted were related to the number of double

bonds in the fatty acid chain (Fig. 5B and Fig. 5D). Many other mechanisms which may be responsible for the effect of AA on ionic currents have been reported; these include the activation of particulate (Wallach & Pastan, 1976) or soluble guanylate cyclase (Gerzer et al, 1986), activation of protein kinase C (McPhail et al, 1984), superoxide radicals derived from AA oxidation (Nagano et al, 1995) and the stimulation of protein phosphatase activity (Petit-Jacques & Hartzell, 1996). All these possible mechanisms, which may explain the effect of AA and unsaturated fatty acids, cannot at present be ruled out; as the pipette solution contained a high concentration of EGTA (10 mM) and 10 mM Ba was used as a charge carrier, only the Ca-induced inactivation of Ca channels (Ohya et al, 1988), resulting from the disturbance of intracellular Ca homeostasis by AA or other fatty acids, could be excluded.

In gastrointestinal smooth muscle, muscarinic stimulation was reported to activate PLA_2, with resultant increase of endogenous AA (Wang et al, 1993). Such an increase will probably have a physiological effect on membrane excitability of this smooth muscle. In this study, we found that relatively high concentrations of AA completely blocked the increase in I_{Ba} caused by osmotic stretch. At first, we thought it possible that inhibition of PLA_2 caused by osmotic stretch reduced AA concentration in membrane so that I_{Ba} was increased in guinea-pig gastric myocytes. This possibility was unlikely, however, because there was always a shift in the I_{Ba} inactivation curve, while in our previous study, no such shift of voltage-dependence during hyposmotic stimulation was observed (Xu et al, 1996). The present results thus suggest that AA levels in cell membrane may merely affect the sensitivity of smooth muscle membrane under the physiological or experimental stimuli. Further AA-related experiments should consider the effect of AA on other ion channels including potassium channels (Kirber et al, 1992; Ordway et al, 1992; Kim et al, 1995; Petrou et al, 1995).

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