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Some evidence against the involvement of arachidonic acid in muscarinic suppression of voltage-gated calcium channel current in guinea-pig ileal smooth muscle cells

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1 To see if arachidonic acid (AA) plays a role in the sustained suppression of voltage-gated calcium channel currents produced by muscarinic receptor stimulation by carbachol (CCh), the effects of AA on membrane currents were examined in whole-cell voltage-clamped smooth muscle cells of the guinea-pig ileum.

2 In cells bathed in Ba^{2+} PSS and dialysed with Cs^+-based low EGTA (0.05 mM) pipette solution, and in which Ba^{2+} current (I_{Ba}) flowing through voltage-gated calcium channels was evoked repeatedly by stepping to 0 mV from the holding potential of −60 mV, AA (1−30 μM), applied extracellularly, gradually suppressed I_{Ba} in a concentration-dependent manner. The I_{Ba} suppression was observed even with 20 mM EGTA in the pipette.

3 AA (3 μM) and CCh (10 μM) shifted the voltage-dependent inactivation curve of I_{Ba} in the negative potential direction, but the effect of AA differed from that of CCh in that an accompanying appreciable decrease in the slope was observed.

4 The sustained suppression of I_{Ba} induced by CCh (10 μM) remained almost unaltered after pretreatment with 4-bromophenacyl bromide (10 μM), an inhibitor of phospholipase A_2, or a combination of indomethacin (10 μM), an inhibitor of the cyclo-oxygenase pathway, and nordihydroguaiaretic acid (10 μM), an inhibitor of the lipoxygenase pathway.

5 In cells bathed in Ca^{2+} PSS and dialysed with K^+-based pCa 6.5 pipette solution, voltage-dependent Ca^{2+} current (I_{Ca}) and K^+ current (I_K) were recorded simultaneously. AA (3 μM) suppressed I_K as well as I_{Ca}, whereas CCh (10 μM)) suppressed I_{Ca} but not I_K.

6 We conclude from these results that AA or its metabolite is unlikely to be involved in the sustained suppression of voltage-gated calcium channel current induced by muscarinic receptor stimulation in guinea-pig ileal smooth muscle cells.

Keywords: Carbachol; arachidonic acid; calcium channel current; potassium channel current; smooth muscle, guinea-pig ileum

Introduction

Neurotransmitters or hormones activate one or more types of receptor-coupled G protein which can modulate the activity of voltage-gated calcium channels directly (a membrane-delimited pathway) as well as indirectly by regulating one or more of the intracellular second messenger systems (for reviews, see McDonald *et al.*, 1994). In longitudinal smooth muscle cells of guinea-pig ileum, muscarinic receptors have been shown to link to at least two types of G proteins (Komori *et al.*, 1992). One is sensitive to pertussis toxin (PTX) and mediates opening of non-selective cationic channels leading to the membrane depolarization (Bolton, 1972; Benham *et al.*, 1985; Inoue *et al.*, 1987). The other, which activates phospholipase C (PLC) is insensitive to this toxin. The activation of PLC then stimulates phosphatidylinositol hydrolysis and this in turn results in the formation of inositol 1,4,5,-trisphosphate (IP_3), which can release Ca^{2+} from internal stores (Bolton & Lim, 1989; Komori & Bolton, 1990; 1991; Pacaud & Bolton, 1991), and diacylglycerol (DG), which activates protein kinase C leading to phosphorylation of proteins (Nishizuka, 1992).

We have shown in the same type of smooth muscle cells that muscarinic receptor stimulation by carbachol (CCh) suppresses the voltage-gated calcium channel current in a biphasic manner; an initial transient suppression is followed by a slowly developing sustained one (Unno *et al.*, 1995). A PTX-insensitive G protein is involved in these two phases of the current suppression. The initial transient phase of the current suppression is brought about by operation of a Ca^{2+}-induced inactivation mechanism due to Ca^{2+} release by IP_3 from internal storage sites. On the other hand, how the sustained phase of the current suppression is brought about remains to be solved, although some characteristics of it have been determined. It is not mediated by the phosphatidylinositol pathway, and no involvement of cyclic AMP or cyclic GMP has been found (Unno *et al.*, 1995). The slow development, however, suggests involvement of a diffusible second messenger.

There is increasing evidence that arachidonic acid (AA) or its metabolite acts as a second messenger in modulation of ionic channels by G protein-coupled receptors (Piomelli *et al.*, 1987; Kurachi *et al.*, 1989; Graber *et al.*, 1994; Meves, 1994). Several pathways to release AA have been demonstrated (Axelrod, 1990). AA is released by phospholipase A_2 (PLA_2) directly from membrane phospholipids, indirectly by diacylglycerol lipase from DG which is formed through activation of PLC, and by diglyceride lipase from phosphatidic acid which is formed through activation of phospholipase D. In many cell types, the main route for the receptor-mediated generation of AA is the PLA_2 pathway which has been found to be Ca^{2+}-dependent (Axelrod, 1990; Kramer, 1993). More recently, Somlyo and co-workers showed that CCh increases the AA level in α-toxin-permeabilized rabbit ileal smooth muscle (Gong *et al.*, 1995), that AA gradually suppresses the voltage-gated calcium channel current in single smooth muscle cells isolated from the rabbit ileum, and that the inhibitory effect is mediated by the action of AA itself (Shimada &

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Somlyo, 1992). Under these conditions, AA is a possible candidate for a mediator of the sustained phase of the suppression of voltage-gated calcium channel current by CCh.

In the present study, the effects of CCh and AA on voltage-gated calcium channel current recorded by the whole-cell patch-clamp technique were characterized in an attempt to see whether or not AA is involved in the sustained phase of the calcium current suppression induced by muscarinic receptor stimulation in single smooth muscle cells of the guinea-pig ileum. The results are not consistent with the idea that the PLA_2-AA pathway is involved in muscarinic receptor-mediated suppression of voltage-gated calcium channel current.

Methods

Preparation of cells

Single smooth muscle cells were enzymatically isolated from the longitudinal muscle of the guinea-pig ileum as previously described (Komori *et al.*, 1992). The cells were suspended in physiological saline solution containing 0.5 mM Ca^{2+} (Ca^{2+} PSS; for composition, see below), placed on coverslips in a small aliquot and kept in a moist atmosphere at 4°C until use on the same day.

Recording of membrane currents

A coverslip with cells was placed in a small organ bath (0.8 ml) on the stage of an inverted microscope (TMD, Nikon, Chiyoda-ku, Tokyo, Japan). The organ bath was filled with Ca^{2+} PSS containing 2 mM Ca^{2+}. Whole-cell membrane-current recordings were made at room temperature (21–25°C) by use of standard patch clamp techniques (Hamill *et al.*, 1981). Patch pipettes had resistances of 4–6 MΩ when filled with pipette solution (for composition, see below). The same procedures as described previously (Unno *et al.*, 1995) were used for current recordings and data analysis.

For recordings of voltage-gated calcium channel current, cells bathed in Ca^{2+} PSS were dialysed with Cs^+-based low EGTA pipette solution (for composition, see below) to block outward potassium channel currents. Ca^{2+} current (I_{Ca}) flowing through voltage-gated calcium channels was elicited repeatedly by depolarizing pulses (30 ms duration) to 0 mV from the holding potential of −60 mV or −80 mV at a frequency of 0.25 Hz. After the amplitude of I_{Ca} was stabilized, the extracellular solution was replaced with Ba^{2+} PSS to use Ba^{2+} as a charge carrier, and effects of CCh and AA on Ba^{2+} current (I_{Ba}) flowing through voltage-gated calcium channels were investigated. The amplitude of I_{Ba} was measured as the difference from the current level ($I_{Ba}=0$) obtained by application of the depolarizing pulse to the cell in the presence of 100 μM Cd^{2+} at the end of each experiment. Although CCh induced an inward current (I_{CCh}) due to the opening of muscarinic non-selective cationic channels, measurements of I_{Ba} would be complicated by I_{CCh} only to a negligible extent because its reversal potential is close to 0 mV (Benham *et al.*, 1985; Inoue & Isenberg, 1990, Pacaud & Bolton, 1991).

Steady-state inactivation curves of I_{Ba} were obtained before and after drug application by means of a two-pulse protocol consisting of a conditioning pulse to various voltages between −80 mV and +20 mV (2 s duration) followed with a 5 ms interpulse interval by a constant test pulse from −80 mV to 0 mV (30 ms duration).

To record I_{Ca} and K^+ current (I_K) flowing through potassium channels simultaneously, Ca^{2+} PSS for the extracellular solution and K^+-based pCa 6.5 pipette solution (for composition, see below) for the intracellular solution were used, and depolarizing pulses to 0 mV (2 s duration) from the holding potential of −80 mV were applied with a frequency of 0.05 Hz. Recording of I_K alone was achieved by replacement of the extracellular solution with Mn^{2+} PSS to suppress I_{Ca} and muscarinic non-selective cationic current (Inoue, 1991). The amplitude of I_K was measured by subtracting a leakage component from the evoked net outward current. The leakage component was estimated in each experiment as follows. A 10 mV hyperpolarizing pulse was applied at the holding potential (−80 mV) and the amplitude of the current was regarded as a leakage current for each 10 mV step. The leakage current commensurate with the required step was then subtracted from the outward current evoked by the depolarizing pulse, assuming that the leakage did not rectify over the potential range studied.

The values in the text are presented as means ± s.e.mean. Statistical significance was tested by Student's unpaired or paired *t* test and differences were considered significant when $P<0.05$.

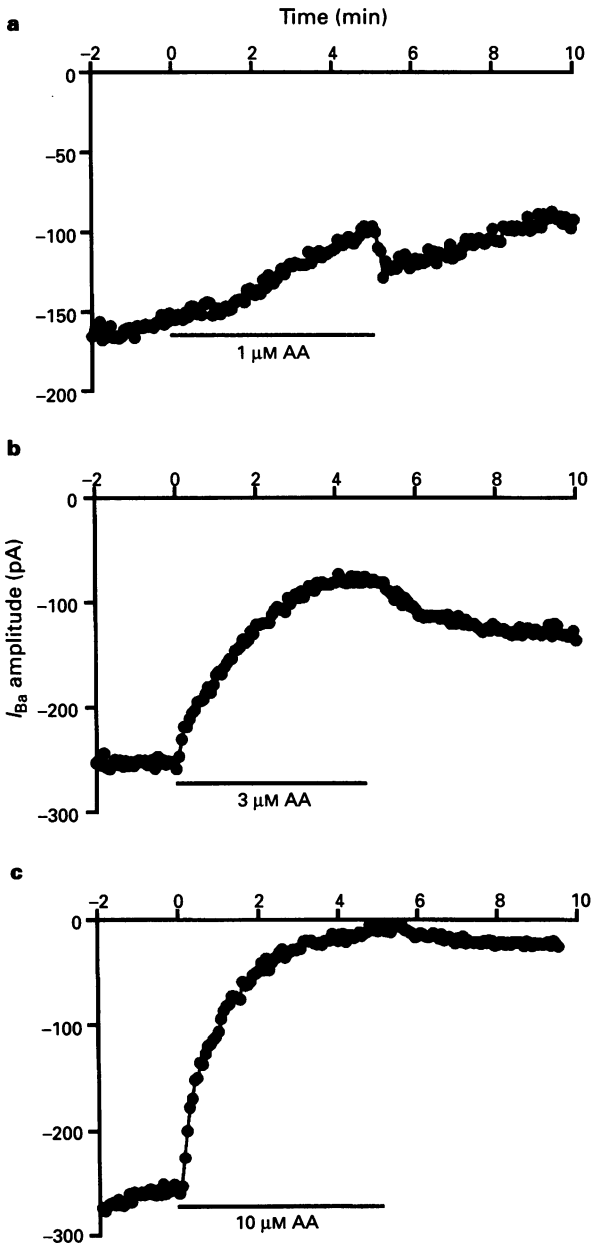

Figure 1 Effect of arachidonic acid (AA) on the Ba^{2+} current (I_{Ba}) in single smooth muscle cells isolated from guinea-pig ileum. The cells were dialysed with Cs^+-based low EGTA pipette solution and bathed in Ba^{2+} PSS. I_{Ba} was elicited by depolarizing pulses (30 ms duration) to 0 mV from a holding potential of −60 mV at 0.25 Hz. (a), (b) and (c), the change in I_{Ba} amplitude (●) with time (the beginning of AA application was taken as zero), when AA was applied extracellularly at (a) 1 μM, (b) 3 μM or (c) 10 μM. AA gradually suppressed I_{Ba} in a concentration-dependent manner. Data in (a), (b) and (c) are from different cells.

Solutions and drugs

Ca^{2+} PSS used in the experiments had the following composition (mM): NaCl 126, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1.2, glucose 14, HEPES 10.5 (titrated to pH 7.2 with NaOH). When Ba^{2+} and Mn^{2+} were used for extracellular solution, the $CaCl_2$ was replaced with an equimolar solution of $BaCl_2$ (Ba^{2+} PSS) and $MnCl_2$ (Mn^{2+} PSS), respectively. Composition of patch-pipette solutions (mM) was as follows. Cs^+-based low EGTA solution: CsCl 134, $MgCl_2$ 1.2, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5 and EGTA 0.05 (titrated to pH 7.2 with CsOH). Cs^+-based high EGTA solution: CsCl 100, $MgCl_2$ 1.2, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5 and EGTA 20 (titrated to pH 7.2 with CsOH). K^+-based pCa 6.5 solution: KCl 80, $MgCl_2$, 2.5, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5, BAPTA 20 and $CaCl_2$ 13.3 (titrated to pH 7.2 with KOH). In this solution, calcium-BAPTA buffer was used to maintain the ionized calcium concentration at a level of pCa 6.5. For calculation we used stability constants for BAPTA described by Tsien (1980). K^+-based high EGTA solution: KCl 80, $MgCl_2$ 1.5, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5 and EGTA 20 (titrated to pH 7.2 with KOH).

Drugs and chemicals used were collagenase (type XI), papain (from *papaya* latex), magnesium adenosine 5'-triphosphate (MgATP), sodium guanosine 5'-triphosphate (NaGTP), 1,2-*bis* (2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid (BAPTA), arachidonic acid (AA), 4-bromophenacyl bromide (4-BPB), indomethacin, nordihydroguaiaretic acid (NDGA), eicosatetraynoic acid (ETYA) (all from Sigma, St Louis, MO, U.S.A.), carbachol chloride (CCh), tetraethylammonium chloride (TEA) (Wako, Osaka City, Osaka, Japan), O,O'-bis (2-aminoethyl) ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA; Dojin Kagaku, Kamimasushiro-gun, Kumamoto, Japan). AA, 4-BPB, indomethacin, NDGA and ETYA were dissolved in 100% dimethyl sulphoxide (DMSO) to make a concentrated solution (the final concentration of DMSO was reduced to less than 0.1% which had no effect on membrane currents). All other drugs were dissolved in distilled water as a stock solution.

Application of drugs

Extracellular application of drugs was carried out by replacing the bath solution in the recording chamber with drug-containing solution more than five times. For pretreatment of cells with 4-BPB, indomethacin and NDGA, these drugs were applied extracellularly more than 10 min before a rupture of the patch membrane was made and continued to be present throughout the period during which effect of CCh was tested (CCh was applied 15–20 min after the pretreatment). In the case of 4-BPB, it was also applied intracellularly via the pipette filled with the drug-containing solution.

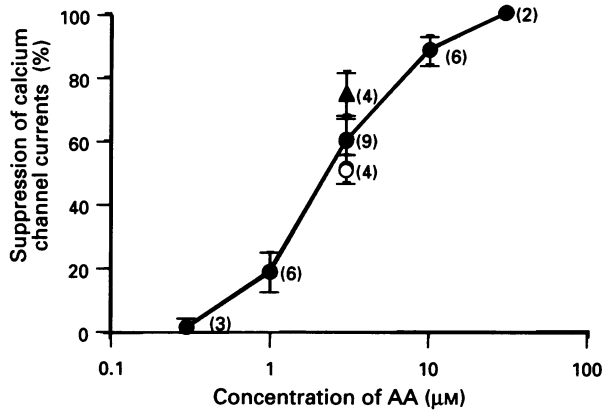

Figure 2 Concentration-response curve for arachidonic acid (AA)-induced suppression of the Ba^{2+} current. The mean percentage suppression of I_{Ba} measured 5 min after the application of AA was plotted against AA concentration. (●) Mean percentage suppression of I_{Ba} in cells bathed as in Figure 1; (○) mean percentage suppression of Ca^{2+} current (I_{Ca}) in cells bathed in Ca^{2+} PSS (no replacement with Ba^{2+} PSS); (▲) mean percentage suppression of I_{Ba} in cells dialysed with Cs^+-based high EGTA pipette solution. A single concentration of AA was applied to each cell. The number of cells used are shown in parentheses by each value. Vertical lines indicate s.e.mean. See text for details.

Results

Effects of carbachol and arachidonic acid on calcium channel currents

Carbachol (CCh; 10 μM), applied extracellularly to a cell 2 min after replacement of Ca^{2+} with Ba^{2+}, induced a biphasic suppression of Ba^{2+} current (I_{Ba}) flowing through voltage-gated calcium channels, as previously described (Unno *et al.*, 1995); an initial transient component was followed by a more sustained one. I_{Ba} amplitude was decreased by 60.8±6.2% (n=8) in the initial transient phase and by 49.5±2.2% in the sustained phase evaluated 2 min after the application of CCh.

When CCh (10 μM) was applied 5 min after replacement of Ca^{2+} with Ba^{2+}, I_{Ba} suppression was of the sustained type (without its transient component) because of a great loss of stored Ca^{2+} during this period (5 min) in a Ca^{2+}-free environment. I_{Ba} amplitude was decreased with time after the application of CCh to reach a plateau within 90 s. The mean percentage suppression of I_{Ba} amplitude measured 2 min after CCh application in five cells was 53.2±4.0%, which was not

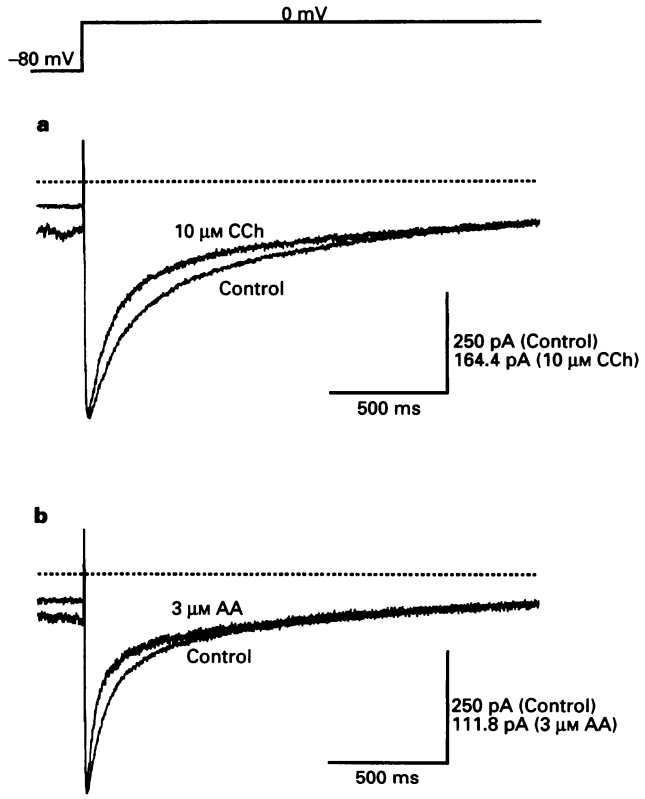

Figure 3 Effects of carbachol (CCh) and arachidonic acid (AA) on the inactivation of the Ba^{2+} current. I_{Ba} was evoked by a depolarizing pulse (2 s duration) to 0 mV from a holding potential of −80 mV. (a) Effect of CCh (10 μM); (b) effect of AA (3 μM). The peak of I_{Ba} in the presence of either drug was adjusted to that in the absence of the drug. CCh and AA accelerated the inactivation of I_{Ba} during the stepping pulse duration. Data in (a) and (b) are from different cells. Note the difference of size calibrations in the absence and presence of the drug.

significantly different from that of the sustained component of I_{Ba} suppression induced by CCh applied 2 min after replacement of Ca^{2+} with Ba^{2+} (49.5±2.2%, $n=8$).

In the following experiments, CCh was applied 5 min after replacement of Ca^{2+} with Ba^{2+}, unless otherwise stated, to obtain the sustained type of I_{Ba} suppression.

Figure 1 shows the inhibitory effects of arachidonic acid (AA) on I_{Ba}, when it was applied at three different concentrations of 1, 3 and 10 μM. The inhibitory effect at 1 μM AA started with a delay of more than 1 min after the application of AA and never reached an appreciable plateau until the end of AA application (Figure 1a). As the concentration of AA was increased, the delay markedly decreased. At 3 μM and higher concentrations of AA, I_{Ba} suppression developed with a roughly exponential time course to reach a plateau (Figure 1b and c). The time constant (τ) for the development of I_{Ba} suppression was decreased with increasing AA concentration (τ = 108.9±9.7 s for 3 μM ($n=9$), 77.0±8.1 s for 10 μM ($n=6$) and 9.4±4.2 s for 30 μM ($n=2$)). Recovery of I_{Ba} from the suppression was usually observed after the removal of AA. It took place slowly, especially at higher concentrations of AA, and partial recovery was achieved after 6 min (the longest time observed in the present study). In some cells, the suppression remained almost unaltered after the removal of AA. No noticeable change in the holding current occurred after application of AA at any concentration used.

The concentration-response curve for the action of AA (0.3–30 μM) is shown in Figure 2. The inhibitory effect was measured 5 min after the application of AA and the response to one concentration of AA was evaluated in one cell because of the incomplete recovery from the effect after washout of AA. An allowance of run-down of I_{Ba} at a rate of 15.6±4.8% ($n=5$) for 5 min was made for evaluation of the percentage suppression of I_{Ba} by AA. The suppression of I_{Ba} induced by

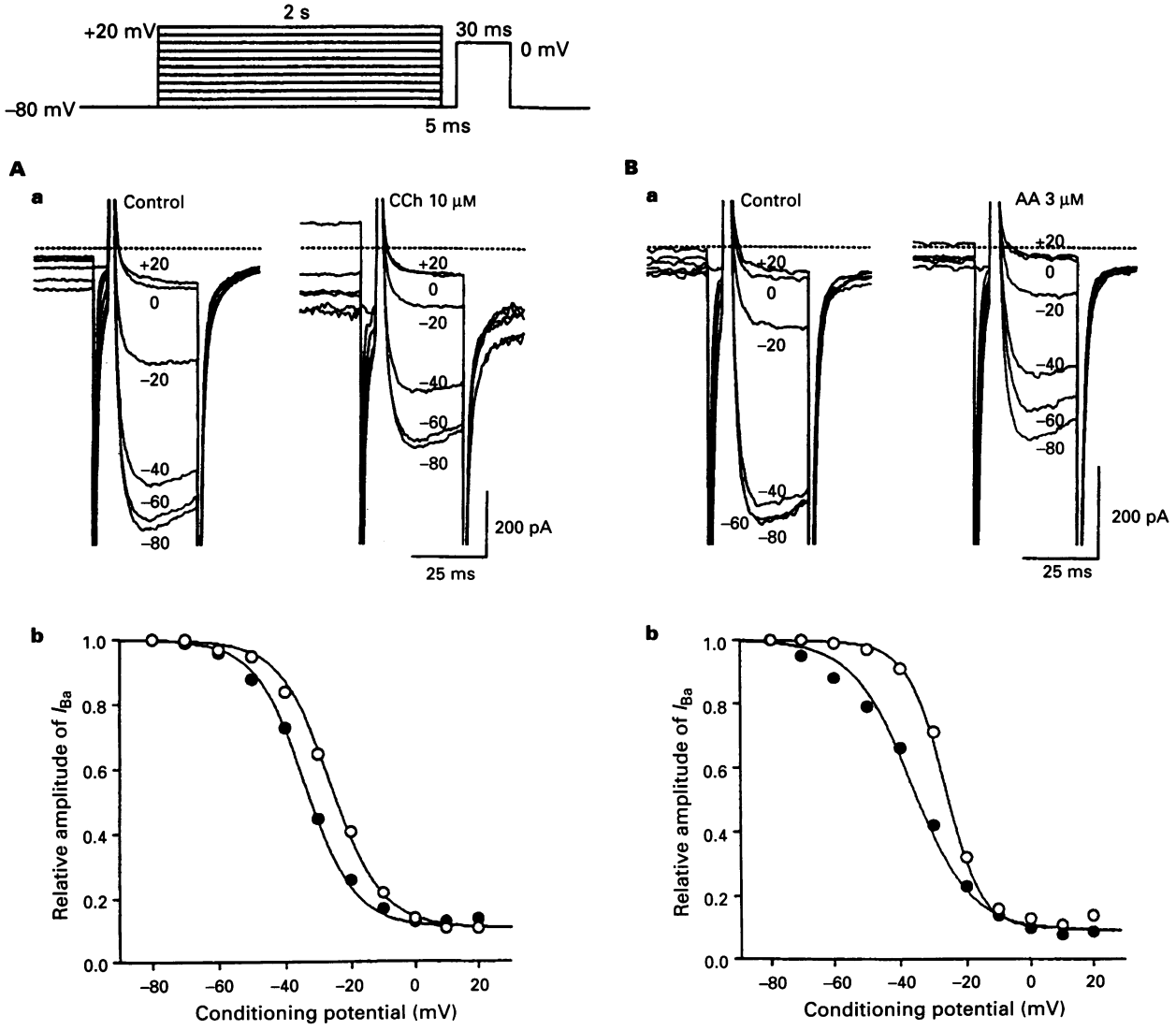

Figure 4 Effects of carbachol (CCh) and arachidonic acid (AA) on the voltage-dependent inactivation of Ba^{2+} current. (A) Effect of CCh; (a) superimposed current traces recorded from a cell in which the membrane potential was held at different levels (−80 to +20 mV) during a 2 s conditioning pulse and then stepped to 0 mV for 30 ms after a 5 ms interpulse interval; Left, the control; right, in the presence of CCh (10 μM). (b) The relationships between the relative amplitude of I_{Ba} and the membrane potential attained by the conditioning pulse in the absence (○) and presence (●) of CCh. (B) Effect of AA; (a) superimposed current traces in the same way as in (Aa). Left, the control; right, in the presence of AA (3 μM). (b) The relationships between the relative amplitude of I_{Ba} and the membrane potential attained by the conditioning pulse in the absence (○) and presence (●) of AA. Attached figures in current traces represent the voltages of the conditioning pulses and interrupted lines indicate zero I_{Ba} levels. In (Ab) and (Bb), the amplitudes of I_{Ba} evoked by stepping to 0 mV from −80 mV without conditioning pulse in the absence and presence of the drug were taken as 1.0, respectively. Points were fitted by the Boltzmann equation. See text for details.

3 μM AA (60.4±6.3%, $n=9$) was nearly equivalent to 10 μM CCh-induced suppression (53.2±4.0%, $n=5$). AA 3 μM was used in the following experiments.

AA (3 μM), applied without replacement of Ca^{2+} with Ba^{2+}, also suppressed the amplitude of the Ca^{2+} current (I_{Ca}) flowing through voltage-gated calcium channels by 51.2±4.5% ($n=4$, Figure 2), which was not significantly different from that of I_{Ba} (60.4±6.3%, $n=9$). In cells dialysed with a pipette solution containing 20 mM EGTA, the mean percentage suppression of I_{Ba} was 74.8±6.8% ($n=4$, Figure 2), which was not significantly different from that observed in cells dialysed with low EGTA pipette solution. Thus the inhibitory effect of AA on calcium channel currents irrespective of I_{Ca} and I_{Ba} did not depend on $[Ca^{2+}]_i$. In this respect, AA differed from CCh as the inhibitory effect of CCh on I_{Ca} and I_{Ba} is abolished with 20 mM EGTA in the pipette (Unno *et al.*, 1995).

Effects of CCh and AA on the inactivation of I_{Ba}

A depolarizing pulse from a holding potential of −80 mV to 0 mV with a long duration of 2 s was used to elicit I_{Ba}, and the effects of CCh and AA on the inactivation kinetics of I_{Ba} were investigated. Figure 3 shows representative traces of I_{Ba} before and after application of CCh or AA. To compare the decay time course of I_{Ba}, the peak of I_{Ba} in the presence of either drug was adjusted to that in the absence of the drug. The decay time course of I_{Ba} could be approximated well by the sum of two exponentials (fast and slow components) as observed previously (Droogmans & Callewaert, 1986). In five cells, the mean time constant was 153.7±18.2 ms for the fast component and 2250.7±290.0 ms for the slow component in the absence of CCh, and 94.7±14.2 ms and 2273.2±313.7 ms in the presence of 10 μM CCh, respectively. The difference between the two means for the fast component was statistically significant. In another four cells, the mean time constants for the fast and slow components were 137.0±15.5 ms and 2135.7±100.6 ms in the absence of AA, and 63.6±5.5 ms and 2056.5±58.6 ms in the presence of 3 μM AA, respectively. The difference between the two means for the fast component was statistically significant. As can be also seen from Figure 3, neither CCh nor AA changed noticeably the activation time course of I_{Ba}.

Effects of CCh and AA on the voltage-dependence of inactivation of I_{Ba} were examined by use of a conventional two-pulse protocol. A 2 s conditioning pulse, varying from −80 mV to +20 mV in 10 mV increments was applied and then a constant test pulse (30 ms duration) stepping to 0 mV from the holding potential (−80 mV) was applied (Figure 4). The I_{Ba} elicited by the test pulse was decreased in amplitude when the potential of the conditioning pulses was increased. The I_{Ba} was still evoked by the test pulse even when the conditioning potential was positive. The amplitude of the I_{Ba} (I) evoked by the test pulse was normalized by taking the amplitude of I_{Ba} (I_{max}) evoked by the test pulse with a conditioning pulse of −80 mV as 1.0, and plotted against the potential of the conditioning pulse (V). The data could be fitted by a least squares method with a Boltzmann function: $I/I_{max}=(1-C)/\{1+\exp[(V-V_{0.5})/S]\}+C$, where C, $V_{0.5}$, and S are the fraction of I_{Ba} which was not inactivated by the conditioning pulse, the potential at which the inactivating current was half-inactivated, and the slope factor, respectively. The inactivation curve of I_{Ba} was shifted by about 7 mV in the negative direction without any appreciable change in the slope after application of 10 μM CCh as illustrated in Figure 4Ab. Similar results were obtained in four other cells. The mean values of $V_{0.5}$, S and C in the absence of CCh were −23.7±1.5 mV, 7.0±0.3 mV and 0.11±0.01, respectively, and corresponding mean values in the presence of 10 μM CCh were −30.0±2.6 mV, 7.4±0.4 mV and 0.12±0.01 ($n=5$), respectively. The difference between the mean $V_{0.5}$ values was statistically significant. When AA (3 μM) instead of 10 μM CCh was used, the inactivation curve was shifted by 10 mV in the negative direction with an appreciable change of the slope (Figure 4Bb). Four experiments gave mean values for $V_{0.5}$, S and C as −23.8±1.3 mV, 7.1±0.4 mV and 0.11±0.01 in the absence of AA and as −33.5±1.2 mV, 10.6±0.4 mV and 0.07±0.01 in the presence of AA, respectively. The differences between the mean values for $V_{0.5}$ and between the mean values for S were statistically significant.

These results indicate that both CCh and AA affect the inactivation kinetics of I_{Ba}. However, the mode of action of AA may be somewhat different from that of CCh, as AA caused an appreciable change in the slope of the inactivation curve.

Effects of inhibitors of phospholipase A_2, cyclo-oxygenase and lipoxygenase on CCh-induced suppression of I_{Ba}

4-BPB (10 μM), an inhibitor of the phospholipase A_2 pathway, itself had no effect on I_{Ba} and CCh-induced non-selective cationic channel current (I_{CCh}). The mean current density of I_{Ba} was 7.6±1.3 pA/pF in cells treated with 4-BPB (mean cell capacitance: 46.2±5.5 pF, $n=6$), which was not significantly different from that of 5.7±0.6 pA/pF in untreated cells (mean cell capacitance: 49.4±2.4 pF, $n=8$). The mean transient and sustained suppressions of I_{Ba} by CCh (10 μM) in 4-BPB treated cells were 51.1±10.6% ($n=6$) and 48.0±7.4% ($n=6$), respectively, neither significantly different from the values in untreated cells (60.8±6.2 and 49.5±2.2%, $n=8$). The mean transient and sustained I_{CCh} of 332.3±67.9 pA and 87.3±15.1 pA ($n=6$), respectively, in 4-BPB treated cells were comparable to those in untreated cells (297.5±28.6 pA and 76.3±11.1 pA, $n=8$).

In cells pretreated with a combination of indomethacin (10 μM), an inhibitor of the cyclo-oxygenase pathway, and NDGA (10 μM), an inhibitor of the lipoxygenase pathway, the current density of I_{Ba} of 2.4±0.6 pA/pF (mean cell capacitance: 48.5±2.4 pF, $n=6$) was significantly smaller than that in untreated cells (5.7±0.6 pA/pF), and the amplitude of the transient and sustained I_{CCh} in response to CCh (10 μM) were 349.9±46.7 pA and 27.4±9.5 pA ($n=6$), respectively. The sustained I_{CCh} was significantly decreased in amplitude (76.3±11.1 pA, $n=8$ in the absence of drugs). However, the transient and sustained suppressions of I_{Ba} by CCh were 73.3±8.5% ($n=6$) and 55.6±3.8% ($n=6$), respectively, neither significantly different from the control.

As shown by Shimada & Somlyo (1992), the inhibitory effect of AA on I_{Ba} was not related to metabolism of AA through the cyclo-oxygenase or lipoxygenase pathways, since ETYA (3 or 10 μM), an AA analogue which is not a substrate for these enzymes, also produced a similar effect on I_{Ba}. The mean percentage suppression of I_{Ba} by 3 or 10 μM ETYA was 50.9±5.4% ($n=5$) and 70.2±10.5% ($n=3$), respectively, neither significantly different from the values at corresponding concentrations of AA (60.4±6.3% ($n=9$) at 3 μM and 88.3±4.5% ($n=6$) at 10 μM).

These results suggest that the phospholipase A_2-AA pathway and AA metabolites through cyclo-oxygenase and lipoxygenase pathways are unlikely to be involved in the muscarinic suppression of calcium channel current.

Effects of CCh and AA on potassium channel currents

AA suppresses voltage-gated potassium channel current (I_K) in rabbit ileal smooth muscle cells (Shimada & Somlyo, 1992). If CCh causes accumulation of AA, the drug could exert the same action as AA. The effects of CCh and AA on I_K were examined in cells in which $[Ca^{2+}]_i$ was clamped at a certain level by use of a calcium-BAPTA buffered pipette solution to avoid complications arising from $[Ca^{2+}]_i$ change.

In cells dialysed with K^+-based pCa 6.5 pipette solution and bathed in Ca^{2+} PSS, a 2 s depolarizing pulse to 0 mV from the holding potential of −80 mV elicited a transient inward current occurring immediately after the capacitive current, followed by a sustained outward current decaying slowly. The

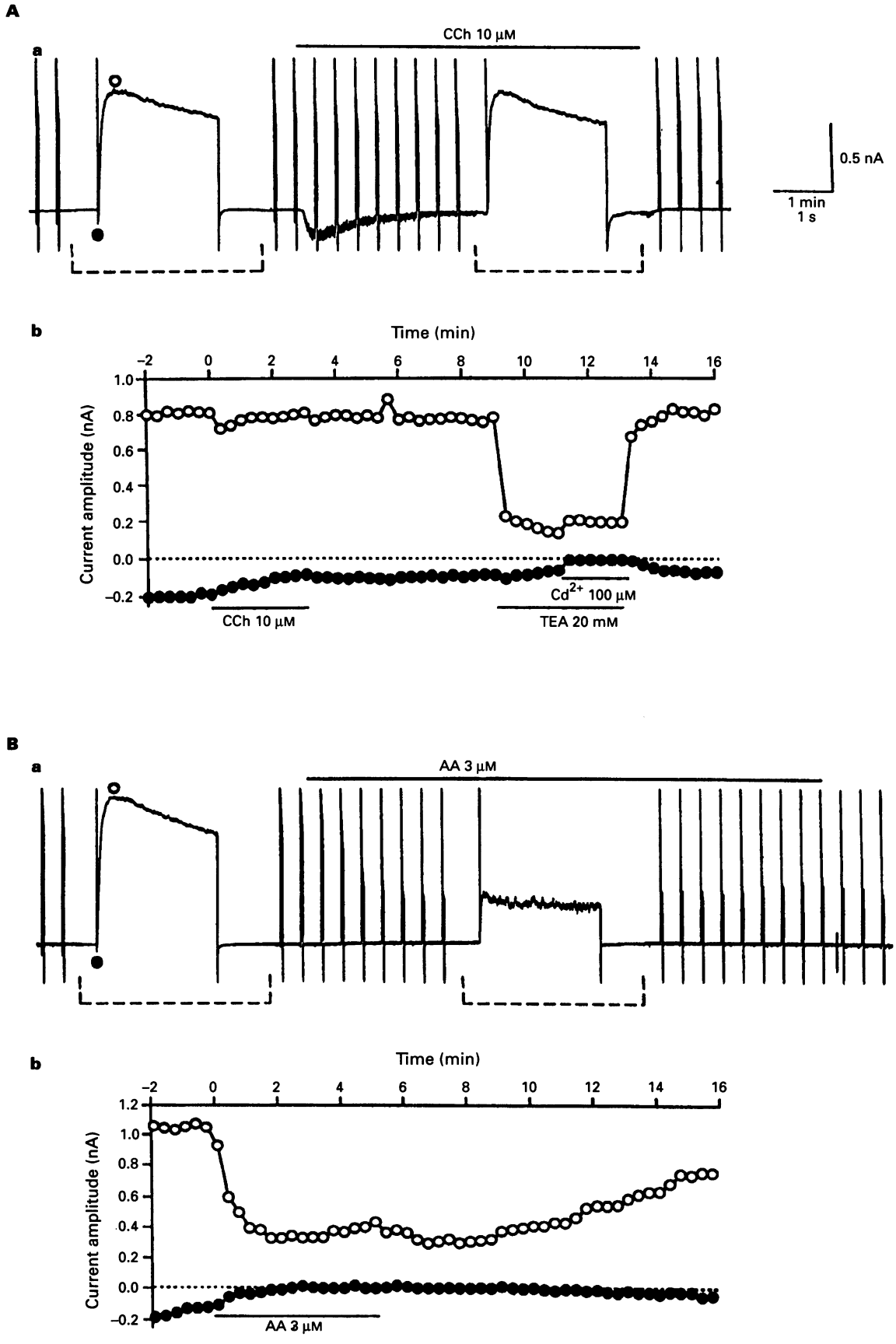

Figure 5 Effects of carbachol (CCh) and arachidonic acid (AA) on Ca^{2+} current (I_{Ca}) and K^+ current (I_K) recorded simultaneously from one cell. Cells were dialysed with K^+-based pCa 6.5 pipette solution and bathed in Ca^{2+} PSS. I_{Ca} and I_K were evoked by depolarizing pulses (2 s duration) to 0 mV from the holding potential of −80 mV at 0.05 Hz. (A) Effect of CCh; (a) current traces before and after application of 10 μM CCh; (b) plots of changes in amplitude of inward I_{Ca} (●) and outward I_K (○) against time (the beginning of CCh application was taken as zero). The I_K was markedly inhibited by application of tetraethylammonium (TEA; 20 mM) and the I_{Ca} was abolished by application of Cd^{2+} (100 μM). (B) Effect of AA; (a) current traces before and after application of 3 μM AA; (b) plots of changes in amplitude of inward I_{Ca} (●) and outward I_K (○) against time (the beginning of AA application was taken as zero). Every sharp current deflection in (Aa) and (Ba) shows overlapped I_{Ca}, I_K and

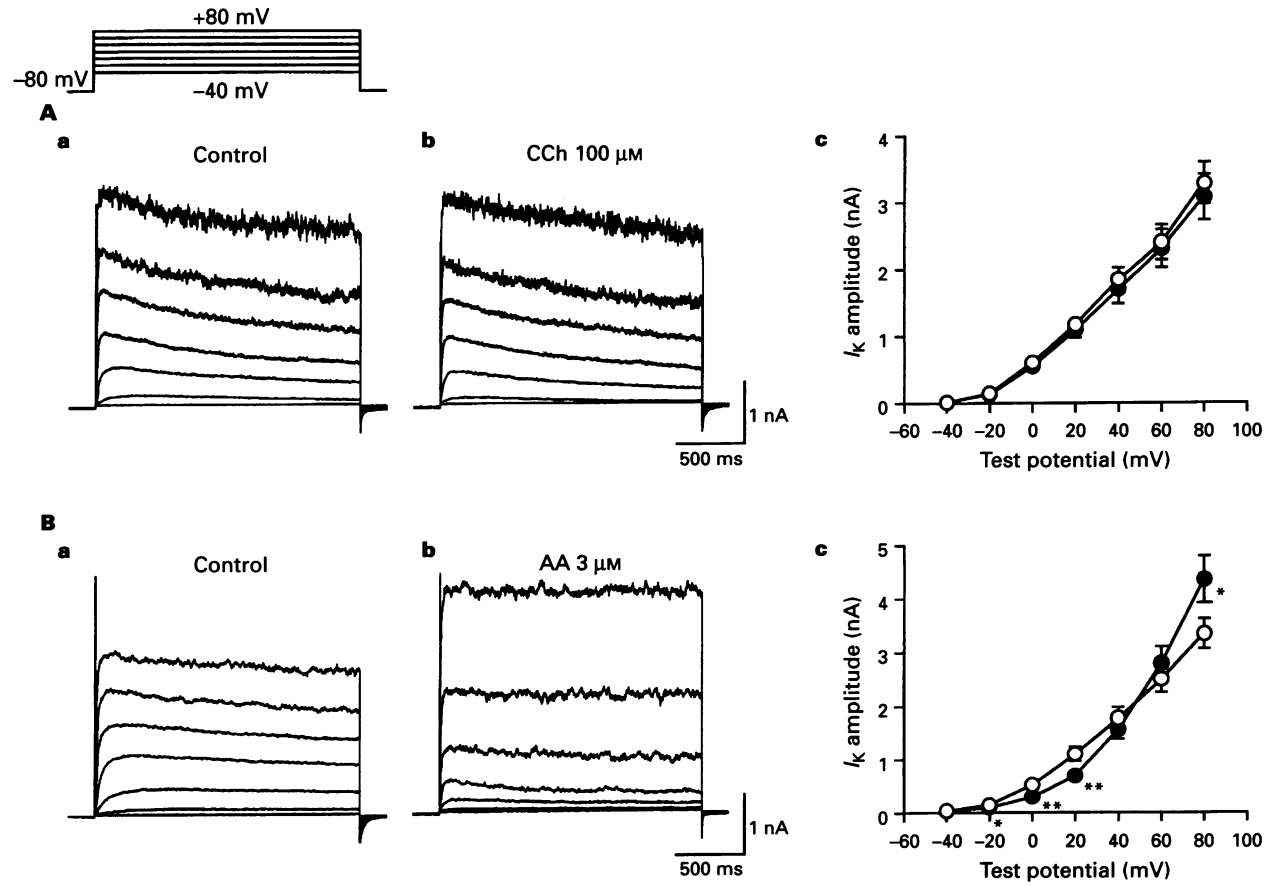

Figure 6 Effects of carbachol (CCh) and arachidonic acid (AA) on current-voltage relationships of K^+ current. Cells were dialysed with K^+-based pCa 6.5 pipette solution and bathed in Mn^{2+} PSS. I_K was evoked by depolarizing pulses (2 s duration) to test potentials of −40 mV to +80 mV in 20 mV increments from the holding potential of −80 mV in the absence and presence of either drug. (A) Effect of CCh; (a) and (b), current traces before and after application of CCh (100 μM), respectively; (c) the current-voltage relationships of I_K in the absence (○) and presence (●) of CCh. (B) Effect of AA; (a) and (b), current traces before and after application of AA (3 μM), respectively; (c) the current-voltage relationships of I_K in the absence (○) and presence (●) of AA. In (Ac) and (Bc), the amplitude of I_K evoked by stepping to different potentials is plotted against the potential attained by stepping. Each point indicates the mean ± s.e.mean (shown by vertical lines) of five (for CCh) or six (for AA) measurements in different cells. Asterisks in (Bc) represent statistically significant differences from the control (*$P<0.05$; **$P<0.01$, paired t test). CCh had little or no effect on I_K, but AA was capable of inhibiting and potentiating I_K in a voltage-dependent manner.

inward current was abolished by application of 100 μM Cd^{2+} and the outward current was markedly inhibited by application of 20 mM TEA (Figure 5A). Thus, the inward current represents the opening of voltage-gated calcium channels and the outward current the opening of voltage-gated potassium channels (Droogmans & Callewaert, 1986; Ohya *et al.*, 1986).

As shown in Figure 5A, 10 μM CCh induced a sustained I_{CCh} (upper panel, a) and a gradual decrease in the amplitude of I_{Ca} (lower panel, b). The inhibitory effect of CCh on I_{Ca} reached a plateau within 2 min after its application, whereas the amplitude of I_K was not noticeably changed throughout the presence of CCh. However, in some cells, as suppression of I_{Ca} developed, the rate of generation and amplitude of I_K increased to some extent. The mean percentage suppression of I_{Ca} in eight cells was 59.7 ± 6.9%, and that of I_K was only 1.2 ± 2.2% (these values were evaluated 3 min after the application of CCh). With 100 μM CCh, the mean percentage suppression of I_{Ca} was 88.3 ± 8.5% ($n=5$) and that of I_K was 5.2 ± 7.3% ($n=5$). In contrast, 3 μM AA strongly suppressed both I_{Ca} and I_K (Figure 5B). The suppression of I_{Ca} developed progressively and within 3 min it reached a maximal and plateau level of 86.6 ± 10.0% suppression ($n=6$). The effect of AA persisted beyond the period of the presence of AA. The suppression of I_K attained a peak of 56.4 ± 5.3% suppression ($n=6$) within 2 min after AA application and then 3 min later decreased to 36.3 ± 13.7% suppression ($n=6$) even in the presence of AA. After the removal of AA, I_K was decreased in amplitude again before its transition to recovery from the suppression (Figure 5Bb).

In cells bathed in Ca^{2+}-free, Mn^{2+} PSS and dialysed with a K^+-based pCa 6.5 pipette solution, I_K was evoked by stepping with 2 s-depolarizing pulses to −40 mV or more positive (up to +80 mV) in 20 mV increments from the holding potential of −80 mV. I_K increased in amplitude in a voltage-dependent manner, it decayed gradually during the stepping pulse duration and it was accompanied by an appreciable noise when evoked at very positive potentials (Figure 6A). CCh (100 μM) had little or no effect on the voltage-dependent I_K and produced a very small or no I_{CCh} because of its inhibition by Mn^{2+} possibly through direct interaction of the ion with the channel

capacitive current and the areas of interrupted line represent expanded time scale as 1 s for time calibration. Time and size calibrations in (Ba) are the same as in (Aa). Data in (A) and (B) are from different cells. Note CCh-induced I_{Ca} suppression without any appreciable effect on I_K.

protein (Inoue, 1991). AA (3 μM) suppressed the amplitude and hastened the decaying rate of I_K evoked at potentials ranging from −20 mV to +40 mV, but potentiated the amplitude and slowed the decaying rate of I_K evoked at more positive potentials (Figure 6B). Figure 6Bc shows plots of the mean amplitude of I_K against the potential in the absence and presence of AA. Thus the effect of AA on I_K changed voltage-dependently in a biphasic manner.

When K^+-based high EGTA pipette solution was used to eliminate the Ca^{2+}-dependent component of I_K which flows through Ca^{2+}-dependent potassium channels, voltage-gated, Ca^{2+}-independent potassium currents (I_{KV}) were evoked with little current noise even at very positive potentials (Figure 7A). CCh (100 μM) had little or no effect on I_{KV} (Figure 7A), but AA (3 μM) suppressed I_{KV} as shown in Figure 7B and changed the time-dependent inactivation of I_{KV}, suggesting that AA affects channel kinetics of I_{KV}. The inhibitory effect was apparently voltage-independent.

These results suggest that the potentiating effect of AA on the total voltage-dependent potassium channel currents is attributable to Ca^{2+}-dependent potassium channels.

Discussion

The aim of the present study was to examine whether or not muscarinic receptor-mediated sustained suppression of the voltage-gated calcium channel current is brought about via the phospholipase A_2 (PLA_2)-arachidonic acid (AA) pathway in guinea-pig ileal smooth muscle cells. Exogenously-applied AA, like carbachol (CCh), was very effective in suppressing Ba^{2+} current (I_{Ba}) flowing through voltage-gated calcium channels. However, the AA-induced suppression of I_{Ba} was different in some aspects from CCh-induced sustained suppression of I_{Ba}. The I_{Ba} suppression produced by 3 μM AA was a well matched-response with 10 μM CCh-induced sustained suppression of I_{Ba}, but the former developed with a slower time course ($\tau = 109$ s) than the latter ($\tau = 26$ s) (Unno *et al.*, 1995). AA reduced the slope of the voltage-dependent inactivation curve of I_{Ba}, whereas CCh did not affect it, although both drugs shifted the curve in the negative potential direction. AA exerted an inhibitory effect on I_{Ba} even in the virtual absence of cytosolic free Ca^{2+} (with 20 mM EGTA in the pipette), but CCh has been shown to be without effect on I_{Ba} under such intracellular ionic conditions (Unno *et al.*, 1995). The different properties of the I_{Ba} suppression by AA from that by CCh suggest that AA is not involved in the signal transduction for the inhibitory effect of CCh on I_{Ba}. This is supported by the findings that AA, unlike CCh, was capable of modifying the K^+ current (I_K) as well as I_{Ba}, and that the inhibitor of PLA_2, 4-BPB, did not reduce the CCh-induced suppression of I_{Ba}.

In many studies, receptor-mediated release of AA has been found to be dependent on Ca^{2+} at concentrations within the range of $[Ca^{2+}]_i$ levels attained when receptors are stimulated (Axelrod, 1990; Kramer, 1993). In human cultured airway epithelial cells stimulation of purinoceptors causes a rise of

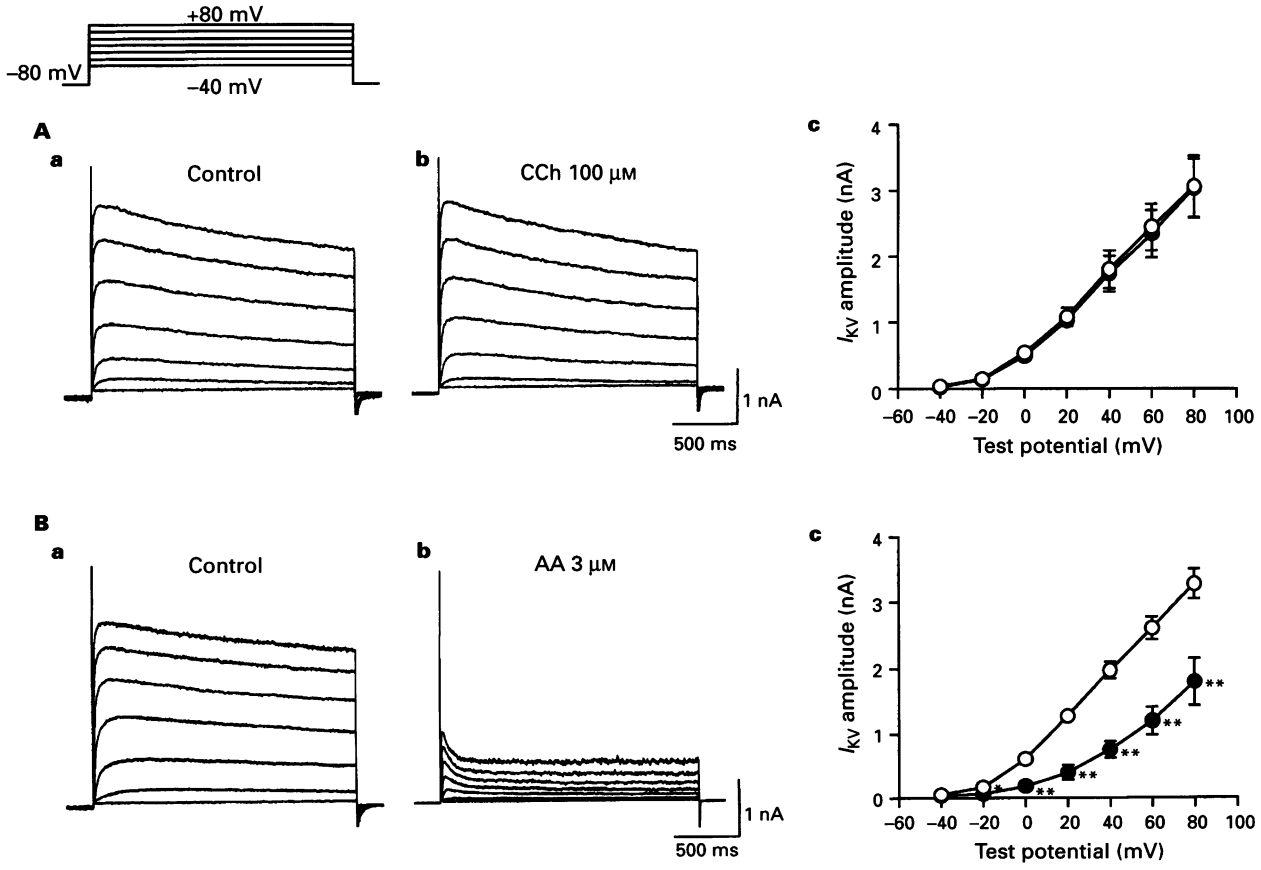

Figure 7 Effects of carbachol (CCh) and arachidonic acid (AA) on current-voltage relationships of voltage-gated, Ca^{2+}-independent potassium channel current (I_{KV}). Cells were dialysed with K^+-based high EGTA pipette solution and bathed in Mn^{2+} PSS. I_{KV} was evoked in the same way as in Figure 6. (A) Effect of CCh; (a) and (b), current traces before and after application of CCh (100 μM), respectively; (c) the current-voltage relationships of I_{KV} in the absence (○) and presence (●) of CCh. (B) Effect of AA; (a) and (b), current traces before and after application of AA (3 μM), respectively; (c) the current-voltage relationships of I_{KV} in the absence (○) and presence (●) of AA. In (Ac) and (Bc), the amplitude of I_{KV} evoked by stepping to different potentials is plotted against the potential attained by stepping. Each point indicates the mean ± s.e.mean (shown by vertical lines) of four (for CCh) or five (for AA) measurements in different cells. Asterisks in (Bc) represent statistically significant differences from the control (*$P<0.05$; **$P<0.01$, paired t test). AA suppressed I_{KV} elicited at every potential.

$[Ca^{2+}]_i$ resulting from Ca^{2+} release by IP_3 from internal stores, which plays an essential role in the activation of PLA_2 and the subsequent generation of AA (Lazarowski *et al.*, 1994). If this is applicable to muscarinic receptors in guinea-pig ileal muscle cells which can mediate Ca^{2+} release in the same way as purinoceptors, the sustained suppression of I_{Ba} produced by CCh should be greater in the presence of stored Ca^{2+}. However, this is not the case, as judged by comparison between the I_{Ba} suppression before ($49.5 \pm 2.2\%$) and that after ($53.2 \pm 4.0\%$) depletion of stored Ca^{2+}.

Treatment with a combination of indomethacin and NDGA which inhibit the cyclo-oxygenase and lipoxygenase pathway, respectively resulted in a marked decrease in amplitude of the sustained component of CCh-induced non-selective cationic channel current (I_{CCh}). The inhibitors of AA metabolism may exert an inhibitory effect through the accumulation of AA. In fact, exogenously applied AA (3 μM) was capable of inhibiting I_{CCh} in the present experiments (data are not shown). Taken together, there is a possibility that the generation of AA may occur following stimualtion of muscarinic receptors by CCh. Nevertheless, the CCh-induced I_{Ba} suppression remained unaltered after treatment with a combination of indomethacin and NDGA. This makes it unlikely that AA or its metabolites through the cyclo-oxygenase and lipoxygenase pathways serve as a mediator of the muscarinic suppression of I_{Ba}.

Although the G protein subtype involved in PLA_2 regulation is not identified yet and the mechanism by which G protein activates PLA_2 is still unknown, Gi-like proteins have been suggested to regulate receptor-coupled PLA_2 on the basis that the AA release induced by receptor stimulation is inhibited by pertussis toxin (PTX) (Axelrod, 1990; Ito *et al.*, 1993; Kramer, 1993; Yousufzai & Abdel-Latif, 1993). Indeed, in the longitudinal smooth muscle of guinea-pig ileum the increase in PLA_2 activity judged by release of AA has been shown to depend on a PTX-sensitive G protein (Wang *et al.*, 1993; Murthy *et al.*, 1995). On the other hand, Unno *et al.* (1995) demonstrated in single cells from the same type of smooth muscle that the sustained suppression of I_{Ba} by CCh is mediated via a PTX-insensitive G protein. These previous findings are not incompatible with the present idea that the PLA_2-AA pathway is not involved in the signal transduction of the muscarinic suppression of I_{Ba}.

Muscarinic receptor stimulation increases tissue levels of AA in intestinal smooth muscle (Wang *et al.*, 1993; Gong *et al.*, 1995). However, endogenous AA, unlike exogenously-applied AA, might be incapable of suppressing the calcium channel current. This issue is reminiscent of that for the action of adenosine 3':5'-cyclic monophosphate (cyclic AMP) on the contractility of cardiac muscle. It is well established that activaiton of adenylate cyclase by β_1-adrenoceptor stimulation results in an increase in the cyclic AMP level and cyclic AMP causes an increase of the activity of voltage-gated calcium channels leading to a positive inotropic effect. However, β_2-adrenoceptor stimulation can only elicit a weak positive inotropic effect even though the cyclic AMP level is elevated to the same extent as in β_1-adrenoceptor stimulation (Yanagisawa *et al.*, 1989). This discrepancy has been explained by assuming a compartmentalization of cyclic AMP: cyclic AMP produced through stimulation of β_1-adrenoceptors may well be coupled to the system responsible for the positive inotropic effect, but that through β_2-adrenoceptors may not (Hayes & Brunton, 1982; Yanagisawa *et al.*, 1989). If AA is released by muscarinic receptor stimulation in guinea-pig ileal muscle cells, it could not gain access to voltage-gated calcium channels. Spatial separation between the AA-generating system and the channels, and the existence of proteins which bind cytosolic fatty acids would buffer their activity effectively (Matarese *et al.*, 1989).

The effect of AA on the inactivation curve of I_{Ba} has a close resemblance to the effect of lidocaine on the inactivation curve of voltage-gated sodium channel current in frog sciatic nerve fibres (Hille, 1977) and rabbit cardiac myocytes (Starmer *et al.*, 1990). Starmer *et al.* (1990) showed that the reduction of the slope of the inactivation curve results from the interaction of the charged form of lidocaine with the sodium channels in a voltage-dependent manner. AA may act on the calcium channels as free acid form (Meves, 1994) to reduce the slope of the inactivation curve of I_{Ba}, as does lidocaine on the sodium channels.

AA-potentiated I_K, consisting of Ca^{2+}-dependent and Ca^{2+}-independent components, evoked by depolarizing pulses to 60 mV or more positive potentials from the holding potential (−80 mV). The disappearance of the effect of AA in the virtual absence of cytosolic free Ca^{2+} suggests that AA may increase the activity of the Ca^{2+}-dependent potassium channels, as demonstrated in vascular smooth muscle cells (Kirber *et al.*, 1992).

CCh suppressed Ca^{2+} current (I_{Ca}) flowing through voltage-gated calcium channels even in cells in which $[Ca^{2+}]_i$ was clamped at a certain level using calcium-BAPTA buffered pipette solution, suggesting that the suppression occurs without a change in $[Ca^{2+}]_i$. In addition, some of the characteristics of this CCh-induced suppression are in common with those of the oxotremorine-induced suppression of I_{Ca} in rat superior cervical ganglion cells (Bernheim *et al.*, 1991; Beech *et al.*, 1992): it develops slowly, is mediated via a PTX-insensitive G protein and involves neither the phosphatidylinositol pathway, the cyclic AMP pathway, nor the cyclic GMP pathway in its signal transduction.

In summary, although the second messenger underlying the suppression of voltage-gated calcium channel current induced by stimulation of muscarinic receptors remains to be identified, the present observations may exclude AA from the list of substances which can act as this second messenger and impose some restrictions on possible mediators involved.

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