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SUPPRESSION OF STEADY MEMBRANE CURRENTS BY ACETYLCHOLINE IN SINGLE SMOOTH MUSCLE CELLS OF THE GUINEA-PIG GASTRIC FUNDUS

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SUMMARY

1. Single smooth muscle cells from the fundus region of the guinea-pig stomach, which showed contractile responses to acetylcholine (ACh) at concentrations $\geqslant 10^{-7}$ mol/l, were obtained by enzymatic digestion using highly purified collagenase and papain. They were studied by recording membrane currents under voltage clamp with the patch pipette technique in the whole-cell configuration at 25–28 °C.

2. By applying voltage jumps from negative holding levels (−70 to −60 mV) to more positive levels, we identified two major activating currents: an initial inward Ca^{2+} current (I_{Ca}) was followed, and partly overlapped, by an outward K^+ current (I_K).

3. Cholinergic effects on membrane currents were investigated in the range of negative membrane potentials by determining current–voltage relations in the absence of ACh and during its continuous presence in the bathing fluid.

4. ACh induced a decrease in the steady-state conductance which was reversibly blocked by atropine. At physiological external K^+ concentration ($[K^+]_o = 6$ mmol/l), the reversal potential (E_{rev}) of the current suppressed by ACh (3×10^{-6} mol/l) was about 20 mV more positive than the calculated K^+ equilibrium potential (E_K).

5. When $[K^+]_o$ was increased, E_{rev} was shifted positively; but at each $[K^+]_o$, E_{rev} was more positive than E_K.

6. Like ACh (10^{-6} mol/l), tetraethylammonium (TEA, 1 mmol/l) also suppressed a current with a reversal potential that was, at physiological $[K^+]_o$, 20 mV more positive than E_K. ACh (10^{-5} mol/l) applied in the presence of 1 mmol/l TEA suppressed a pure K^+ current ($E_{rev} = E_K$), which was also suppressed by 10 mmol/l TEA.

7. When K^+ in the pipette and in the bathing solution was completely replaced by Na^+, both ACh (10^{-5} mol/l) and TEA (1 mmol/l) caused a reduction of the membrane conductance that appeared to be identical. TEA added to the bathing solution in the presence of ACh did not produce a significant additional conductance decrease. These results did not depend on whether Cl^- was present as a charge carrier or not.

8. It is concluded that in fundus muscle of the guinea-pig stomach a major mechanism underlying muscarinic activation is a decrease of a K^+ conductance. In

addition the results indicate a suppression of a small Na^+ conductance which is made up by a population of channels that are also blocked by TEA.

INTRODUCTION

Contraction of visceral smooth muscle evoked by muscarinic receptor activation is generally accompanied by membrane depolarization. Apart from this common feature there is a large degree of diversity in the electrical responses to cholinergic agonists of isolated preparations of different origin, which is also seen in their pattern of spontaneous activity (see Bolton, 1981). From early experiments on multicellular mammalian smooth muscle preparations it was concluded that the mechanism underlying the membrane depolarization is a conductance increase to a mixed current carried by K^+, Na^+ and/or Ca^{2+} (Bolton, 1972, 1981). This view has recently been strengthened by a study in single freshly dispersed cells from the rabbit jejunum applying the patch clamp technique in its whole-cell configuration (Benham, Bolton & Lang, 1985). Inoue, Kitamura & Kuriyama (1987) found that in single cells from the guinea-pig ileum, ACh activates Na^+ channels which also permit the passage of K^+. However, it has also been reported that, in isolated gastric smooth muscle cells from the toad, the depolarization induced by ACh is caused by suppression of a K^+ conductance (Sims, Singer & Walsh, 1985), a mechanism already identified in the excitatory muscarinic action in certain neurons (Kobayashi & Libet, 1970; Brown & Adams, 1980). Determinations of current–voltage relations in isolated smooth muscle cells eliminate uncertainties inherent in the interpretation of electrical data obtained from whole-tissue preparations, due to the tissue's syncytial nature (Bolton, Tomita & Vassort, 1981; Lammel 1981*a*, *b*). The apparent existence of two fundamentally different membrane effects of ACh underlying depolarization therefore raises the question whether qualitative differences like those between mammalian and amphibian intestinal smooth muscle cells may also be present in mammalian preparations of different origin, possibly reflecting specialized physiological functions. The present study contributes to this question by investigating ACh actions on the membrane conductance of single smooth muscle cells enzymatically dispersed from the muscular wall of the guinea-pig stomach. Our experiments have been restricted to cells isolated from the fundus region, because it is known from observations in multicellular preparations that spontaneous activity as well as responses to agonists differ in different regions of this organ (Golenhofen, von Loh & Milenov, 1970; Wagner, 1976; Golenhofen, Wagner & Weston, 1977). With regard to the divergent results mentioned (Benham *et al.* 1985; Sims *et al.* 1985; Inoue *et al.* 1987) we concentrated on cholinergic effects on the steady-state conductance near the resting potential, i.e. in the range of negative potentials. Our results indicate the existence of a conductance reduction mechanism similar to that found by Sims *et al.* (1985) in toad stomach, although some noticeable dissimilarities were also observed. A brief account of this work has been published previously (Deitmer, Noack, Boev, Golenhofen & Lammel, 1988).

METHODS

Cell preparation. Guinea-pigs (180–250 g) were stunned and bled. Several strips (total weight 40–50 mg) of the muscle layer of the gastric fundus were separated from the mucosa by means of

fine scissors. Care was taken not to pierce the mucosal layer during this dissection. The procedure of enzymatic cell dispersion was similar to that described by Maruyama *et al.* for taenia coli (Maruyama, Yoshida, Kobayashi, Oyamada & Momose, 1987). After equilibrating the tissues for 75 min in 50 ml of Tyrode HEPES solution (composition in mmol/l: NaCl, 137; KCl, 2·7; $CaCl_2$, 1·8; $MgCl_2$, 1·0; glucose, 5·6; HEPES, 4·2; pH 7·4), they were exposed for 30 min to 50 ml of the same medium with Ca^{2+} omitted. Following this, the muscle strips were transferred for 45 min to Ca^{2+}-free KCl-Tyrode HEPES solution (NaCl replaced by KCl). They were then minced into small pieces (about 2×2 mm) and successively incubated in two different digestion solutions. Incubation for 30 min in a Ca^{2+}-free KCl-Tyrode HEPES solution containing bovine serum albumin (Boehringer Mannheim, fraction V, 10 mg/ml) and purified collagenase (Sigma, type VII, 100 units/ml) was followed by exposure for another 30 min to Ca^{2+}-free KCl-Tyrode HEPES solution containing papain (Sigma, 15 units/ml) and collagenase inhibitor (Sigma, 200 μg/ml). All solutions were kept at 37 °C and bubbled by O_2. Enzymatic treatment was terminated by centrifuging twice at 1100 r.p.m. for 1 min, discarding the supernatant and resuspending the pellet in 1 ml Ca^{2+}-free KCl-Tyrode HEPES solution. Mechanical agitation was then applied by repeatedly sucking the tissue into a fire-polished Pasteur pipette until the solution became cloudy from the cells released into it. Undigested tissue pieces were removed by filtration through a 200 μm nylon mesh. During this procedure the solution was diluted to a volume of 4 ml by adding 'KB-medium' of the following composition (mmol/l): KCl, 85; K_2HPO_4, 30; $MgSO_4$, 5; Na_2ATP, 2; sodium pyruvate, 5; creatine, 5; taurine, 20; β-OH-butyrate, 5; and 1 g/l fatty acid-free albumin; pH 7·2 (Klöckner & Isenberg, 1985). The cells were stored in this solution at 4 °C before their use for experiments within 12 h. The total yield from 50 mg of tissue was $0{\cdot}5–1 \times 10^5$ cells.

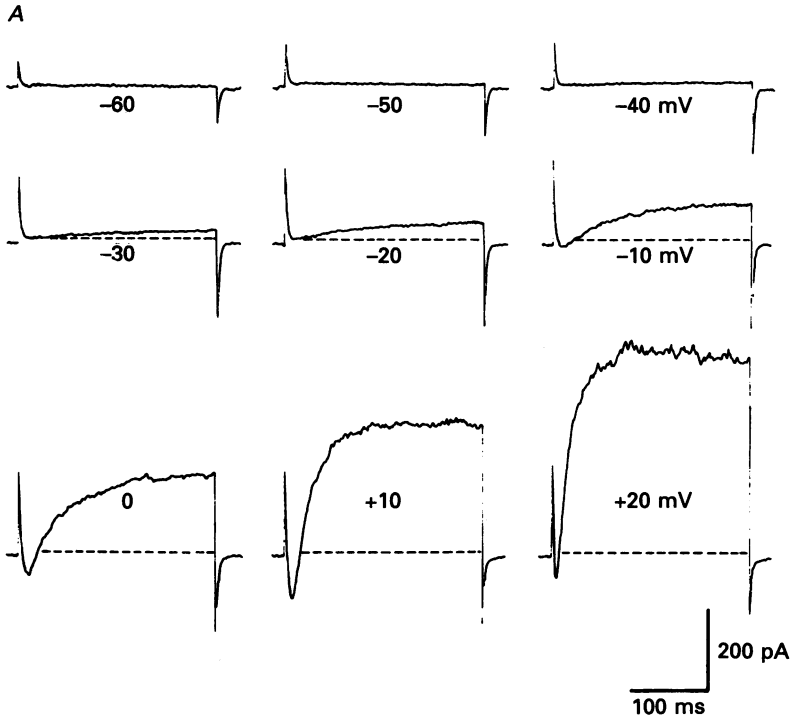
Electrophysiological measurements. A portion of isolated cells was transferred to the experimental set-up by pipetting 100 μl of the storing medium into a thermostatically controlled experimental chamber containing physiological salt solution (PSS). The chamber was mounted on an inverted microscope through which the cells were viewed by means of a TV system. Following a time period to allow the cells to attach to the glass bottom, they were continuously superfused with PSS at 25–28 °C. After 30 min ACh was added to the solution within the chamber (bath concentration 10^{-7} mol/l), and the contractile responses of a group of selected cells were stored on a videotape. Usually 50–70 % of the cells showed contractile responses to ACh (shortening to less than 80 % of their initial length, followed by partial relaxation after withdrawal of the agonist). We chose only cells for voltage clamp experiments which contracted to this test application of the agonist.

Experiments were performed in the 'whole-cell mode' of the patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using a clamp system designed in our laboratory functionally similar to commercially available units (List, Darmstadt, models EPC5 and EPC7). The head stage of the current-to-voltage converter was equipped with a low-noise operational amplifier (Burr Brown, type OPA 128 CM) and a 100 MΩ low-noise feedback resistor. Settling time of the voltage clamp circuit was less than 500 μs. Generation of command pulses was performed by an interval pulse generator (WPI Corporation, model 830/31) and external voltage divider. Patch pipettes were pulled from Pyrex glass (Jencons H 15/10) and had resistances of 3–4·5 MΩ when filled with standard intracellular solution. Seal resistances determined before rupturing the membrane patch within the tip of the electrode were usually between 10 and 20 GΩ.

In order to obtain an estimate of the surface area of typical cells chosen for experiments, their membrane capacity was calculated from current transients produced by hyperpolarizing or small depolarizing voltage clamp pulses without series (pipette) resistance (R_S) compensation. Taking the difference of the time constants of the current transients before and after rupturing the membrane and dividing by R_S gave a value of $16 \pm 2{\cdot}5$ pF ($n = 8$). On the assumption of 1 μF/cm^2 for specific membrane capacitance this indicates a surface area of $1{\cdot}6 \times 10^{-5}$ cm^2. A value about 30 % smaller than this was obtained by evaluating video pictures of the same cells approximating their shape by a cylinder (length 82 ± 7 μm, diameter 5 ± 1 μm).

Junction potentials between different pipette and bathing solutions were measured by changing the extracellular solution with the pipette solution in a two-compartment chamber using a KCl–agar bridge. Estimated membrane potentials were corrected appropriately.

Current recordings and voltage clamp protocols were recorded on videotape using a PCM recorder (Sony, PCM-501 ES, DC to 8 kHz). During the experiments, current and voltage were also continuously monitored on an oscilloscope and a chart pen recorder (Gould, 2400). For the evaluation of fast activating currents, recordings were first transferred to an FM tape-recorder (Racal, Thermionic store 4, frequency response 20 kHz) and subsequently played back at reduced



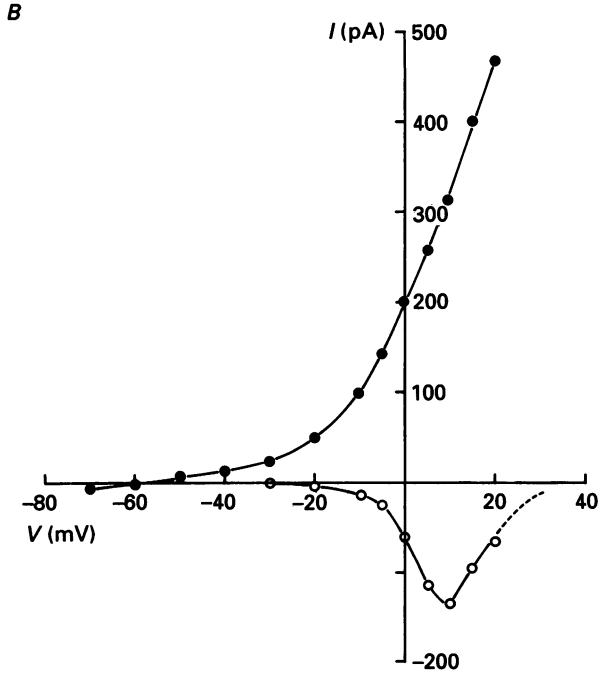


Fig. 1. For legend see facing page.

speed onto the pen recorder. Data evaluated on a scale of several seconds (steady-state currents) were taken from chart records obtained either during the experiment or after replaying the PCM recordings on the pen recorder at a higher paper speed. Further data acquisition and calculations were performed on a PC AT using the software package pClamp 5.0 (Axon Instruments Corporation) and Symphony 1.2 (Lotus Development Corporation). Data were not corrected by linear leak compensation.

Solutions and reagents for electrophysiological measurements. The control extracellular solution (PSS) had the following composition (mmol/l): NaCl, 125; KCl, 4·8; $MgCl_2$, 1·2; KH_2PO_4, 1·2; glucose, 11; $CaCl_2$, 2·5; HEPES, 10; pH 7·3.

The control pipette solution contained (mmol/l): NaCl, 5; KCl, 120; $MgCl_2$, 1·2; K_2HPO_4, 1·2; glucose, 11; HEPES, 10; EGTA, 1·2; oxalacetic acid, 5; sodium pyruvate, 2; sodium succinate, 5; pH 7·3. The EGTA concentration was chosen to be high enough to prevent complete or transient loss of the giga-seal caused by ACh-induced contraction as was often observed with lower concentrations. At the same time it was aimed to keep the intracellular Ca^{2+} buffering capacity low in order not to miss possible effects on Ca^{2+}-activated K^+ currents. In an early stage of experiments, in which voltage-activated Ca^{2+} currents were recorded, the EGTA concentration in the pipette solution was 10 mmol/l and 1 mmol/l $CaCl_2$ was also included. For suppression of outward K^+ currents KCl in the pipette solution was replaced by CsCl in some experiments. External solutions of different K^+ concentrations were prepared by equimolar substitution of NaCl by KCl. In some cases the external Na^+ concentration was reduced by isotonic replacement of NaCl by sucrose. In order to determine contribution to the membrane current of current through Cl^- channels, KCl was replaced by potassium gluconate and NaCl by sodium isethionate in some recordings.

Reagents used were acetylcholine (ACh; Dispersa Baeschlin), atropine (Fresenius) and tetraethylammonium chloride (TEA; Merck).

RESULTS

Some features of activated membrane currents

The first report of activated currents recorded in cells freshly isolated from the muscularis of the guinea-pig stomach was published by Mitra & Morad (1985). These cells, enzymatically dispersed from the corpus region with another technique, did not show contractile responses to muscarinic agonist, but exhibited an inward Ca^{2+} current followed by a K^+ current which appeared to be at least partly activated by internal Ca^{2+}. Although we did not initially aim to provide a detailed analysis of voltage and time parameters of these currents, we include some data in order to contribute to the picture of activating currents existing in the organ under investigation. Qualitatively, Ca^{2+} and K^+ currents in the fundus appear similar to those described for corpus cells. Figure 1*A* shows current traces from an experiment in which the membrane voltage was stepped to different values from the holding potential of −70 mV. At potentials above −30 mV, the initial capacitive surge was followed by a transient net inward current that peaked in about 10 ms, then declined and reversed to become an outward current. From the family of traces shown in these panels the two current–voltage relations in Fig. 1*B* were constructed.

There are several experimental observations indicating that the net current is made up of two major components, an initial Ca^{2+} inward current (I_{Ca}), and an outward current carried by K^+ (I_K), which follows and partly overlaps I_{Ca}. Firstly,

Fig. 1. *A*, current traces in response to depolarizing clamp pulses of 250 ms duration from a holding potential of −70 mV to the potential values given in each panel. *B*, current–voltage relations constructed from the results illustrated in *A*; ○, peak inward current; ●, current at the end of the clamp pulse. Dashed lines indicate zero current.

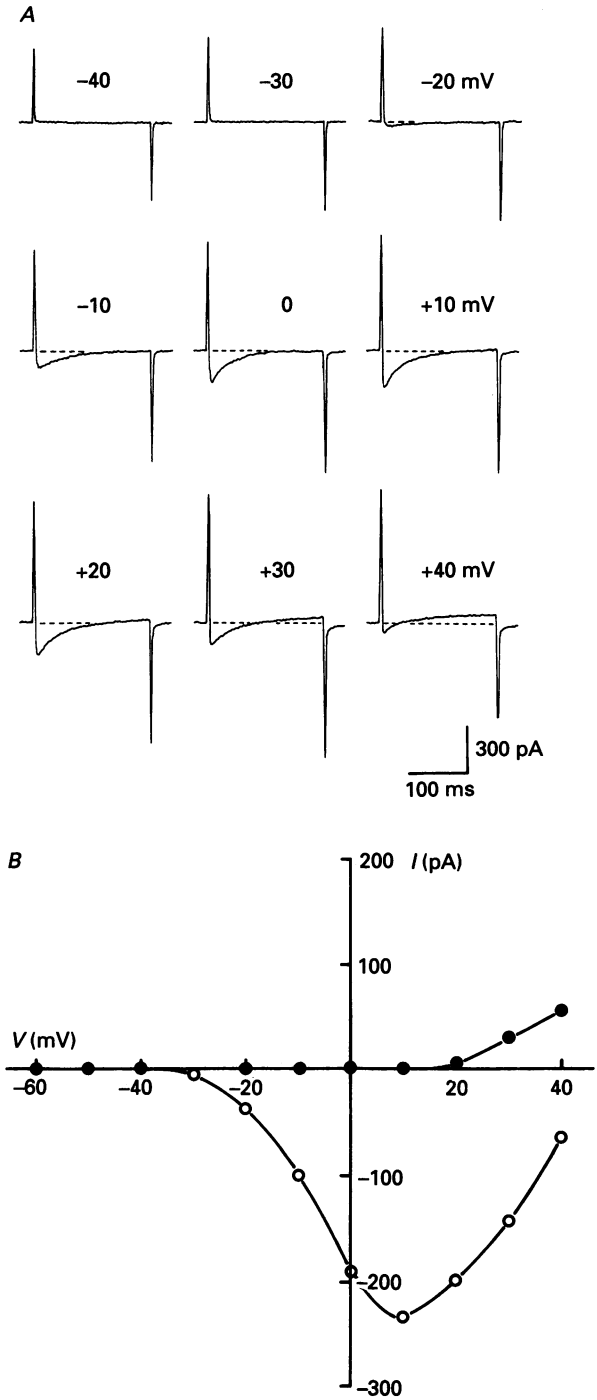


Fig. 2. *A*, current traces evoked by voltage clamp pulses using a pipette in which KCl was replaced by CsCl; holding potential −70 mV, clamp potentials given in each panel. *B*, peak inward current (○) and current at the end of the clamp pulse (●) determined from current traces in *A* plotted against membrane potential. Dashed lines indicate zero current.

the outward current was largely suppressed upon replacing KCl in the pipette solution by CsCl (Fig. 2). The current–voltage relation describing the peak inward current under this condition has much in common with that reported for corpus cells by Mitra & Morad (1985). Secondly, the inward current could be blocked by Cd^{2+} (0·5 mmol/l) and strongly reduced by the Ca^{2+} entry blocker nifedipine (10^{-5} mol/l), whereas it was augmented by the calcium agonist Bay K 8644. In accordance with observations in other isolated smooth muscle cells, procedures which reduced Ca^{2+} inward current also reduced outward current suggesting that at least part of the outward current is caused by opening of Ca^{2+}-activated K^+ channels (e.g. Klöckner & Isenberg, 1985; Mitra & Morad, 1985).

Decrease of steady membrane conductance by acetylcholine

The voltage clamp procedure performed to determine the effect of ACh on the steady-state current–voltage relation is illustrated in Fig. 3. The cell was usually held at a relatively depolarized membrane potential (here −20 mV), and clamp steps of 10 s duration to different levels were applied in control solution and during continuous application of the agonist. Shortly before and after changing the perfusion medium, the membrane potential was either kept at a constant level as in the example of Fig. 3 or voltage jumps of constant size were applied as in the experiment depicted in Fig. 5 below. The external K^+ concentration in the present case was 20 mmol/l. One effect of ACh, apparent from the current recording in Fig. 3, was the small reduction of the steady outward current at the holding potential. Information about the membrane conductance change underlying this effect is obtained by evaluating the current excursions associated with the voltage clamp steps under control conditions and in the presence of the agonist. Plotting the current level at the end of the command pulse against the clamp potential yields the current–voltage curves in Fig. 4*A*. A general feature of these curves is that they show outward rectification and, as will be described later, can be well fitted on the basis of the constant-field equation (Goldman, 1943; Hodgkin & Katz, 1949) in the range of membrane potential investigated. ACh produced a decrease of the membrane permeability. In the example of Fig. 4*A*, the current–voltage curves determined in the absence and in the presence of ACh cross each other at −39 mV (E_{rev}), a value 8 mV more positive than the calculated K^+ equilibrium potential (E_K). Comparing in Fig. 3 the time course of the current flowing at $V = -40$ mV (which is near E_{rev}) under control conditions and in the presence of ACh, it appears that the current, suppressed by ACh, was initially inward during the pulse. This indicates that E_{rev} was more positive still and there was a small (~ 5 mV) negative shift in E_{rev} during the pulse. The effect of 3×10^{-6} ACh was reversibly blocked by 5×10^{-5} mol/l atropine (Fig. 4*B*).

Nature of the steady conductance change produced by acetylcholine

Figure 4 compares the effect of ACh on the current–voltage relations at 10 and 20 mmol/l external K^+. With increasing K^+ concentration, the point of intersection of the current–voltage curves determined in the absence and presence of the agonist (E_{rev}) was shifted to the right. This observation and the closeness of E_{rev} and E_K

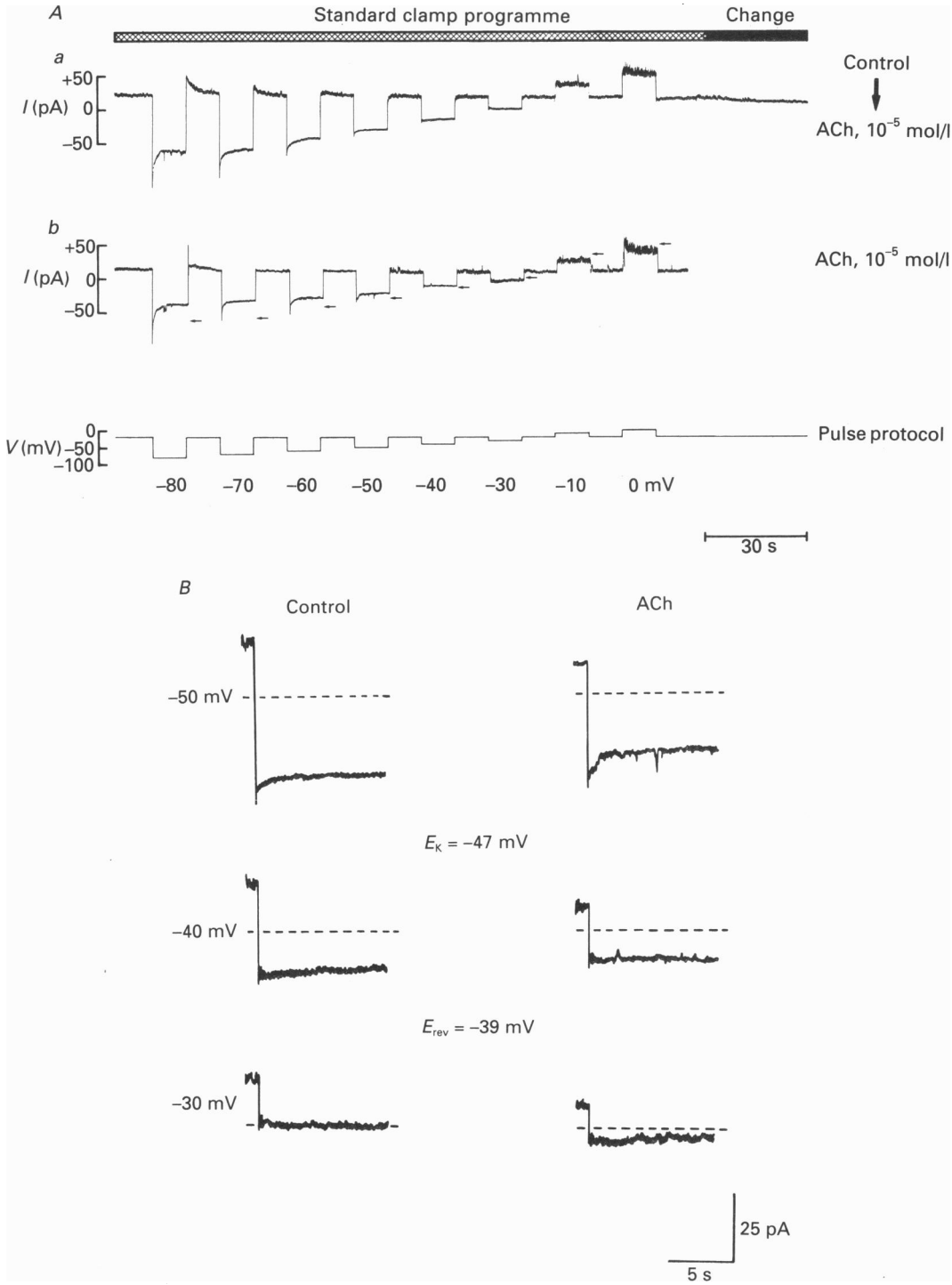


Fig. 3. For legend see facing page.

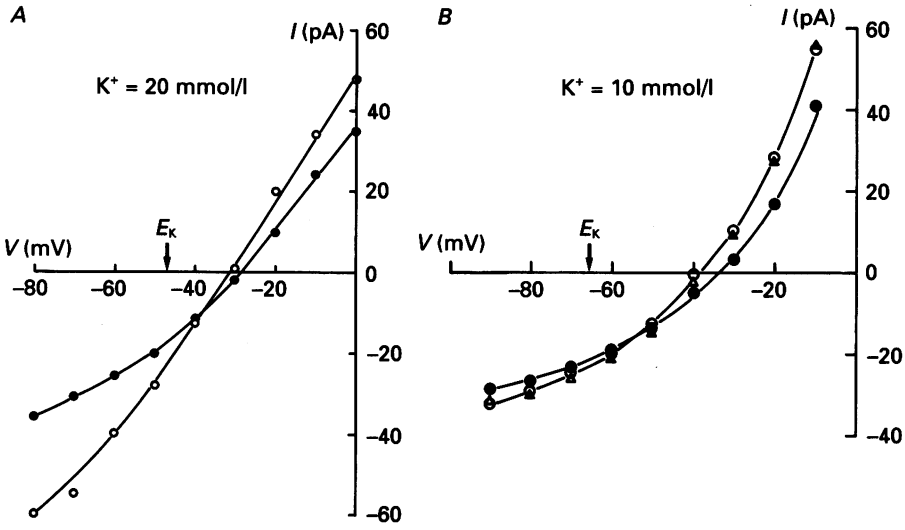


Fig. 4. *A*, current–voltage relations obtained by evaluation of the recording shown in Fig. 3. Currents measured at the end of the command pulses were plotted against membrane potential values. ○, control conditions; ●, application of ACh (10^{-5} mol/l). *B*, current–voltage curves of another cell determined first in the absence (○), then in the presence (●) of ACh (3×10^{-6} mol/l), and finally after adding atropine (5×10^{-5} mol/l) to the ACh-containing solution (△).

indicate a major contribution of K^+ to the current suppressed by ACh. On the other hand, the fact that at each concentration E_{rev} was more positive than E_K suggests that either ACh acts on channels which are predominantly selective to K^+ but also allow the passage of some other ion species, or that, in addition to a K^+ conductance, another conductance is also affected by ACh. Deviation of E_{rev} values from

Fig. 3. Voltage clamp study performed to determine the effect of ACh on the membrane current–voltage relation. The membrane was held at a relatively depolarized level (here −20 mV), and a series of voltage jumps of 4–10 s duration (here 10 s) to different levels was applied before and after changing to a perfusion medium containing ACh. A continuous voltage clamp recording from one cell is shown in *A*. (A time segment of 5 s has been omitted between the current records depicted in *Aa* and *Ab* for the purpose of positioning the voltage and current deflections one above the other.) The voltage protocol is displayed in the bottom panel of *A* with the time period of changing the bathing fluid on its right end as indicated at the top of the figure. Command voltages are given underneath. *Aa*, membrane current recorded before and at the beginning of ACh application. *Ab*, membrane current recorded in the presence of ACh (10^{-5} mol/l). Arrows in panel *Ab* indicate current levels determined at the end of the corresponding pulses in panel *Aa*. *B* shows the current traces upon stepping to command potentials of −50, −40 and −30 mV (indicated on the left) at an expanded voltage and time scaling. Dashed lines mark zero current level. Note STOC (see p. 271) of inward direction at −50 mV and of outward direction at −40 mV occurring in the presence of ACh. $[K^+]_o = 20$ mmol/l and $E_K = -47$ mV in this experiment.

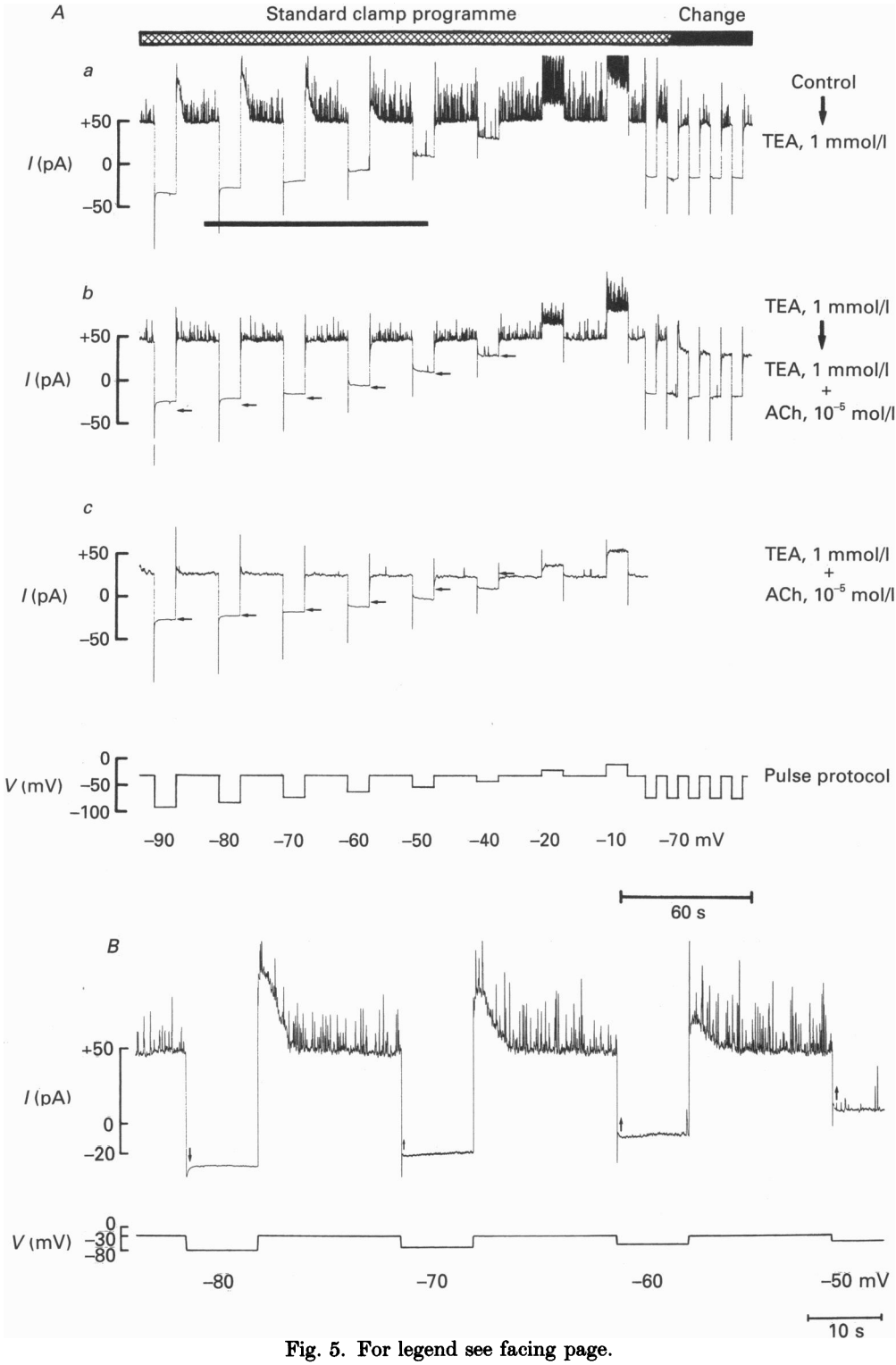


Fig. 5. For legend see facing page.

calculated E_K values at different K^+ concentrations were $18{\cdot}2 \pm 2{\cdot}1$ mV ($n = 8$) at $[K^+]_o = 6$ mmol/l, $11{\cdot}8 \pm 1{\cdot}8$ mV ($n = 4$) at $[K^+]_o = 10$ mmol/l, and $5{\cdot}1 \pm 1{\cdot}9$ mV ($n = 6$) at $[K^+]_o = 20$ mmol/l.

Arguments supporting a simultaneous action of ACh on two different conductances arise from a series of experiments, one of which is depicted in Figs 5, 6 and 7. In these experiments, the standard voltage clamp procedure was applied to the same cell under four conditions. A first run under control conditions (Fig. 5*Aa*) was followed by a second run performed in the presence of 1 mmol/l TEA (Fig. 5*Ab*) and a third run during which the cell was continuously superfused by the control medium containing both 1 mmol/l TEA and 10^{-5} mol/l ACh (Fig. 5*Ac*). Finally, the TEA concentration in the bathing solution was increased from 1 to 10 mmol/l (Fig. 6).

The original aim of testing the effect of the 'K^+ channel blocker' TEA on the current–voltage relation was to gain evidence validating the deviation from E_K of the reversal potential of the current suppressed by ACh. Evaluating first the currents flowing at the end of the command pulses (5 s), it turns out that the current suppressed by 1 mmol/l TEA reversed at a command voltage of about -50 mV (curve 1 in Fig. 7). In contrast, the current additionally suppressed by ACh in the presence of 1 mmol/l TEA had an apparent reversal potential near the calculated E_K (-79 mV) (curve 2 in Fig. 7).

Information about the reversal potential of the current suppressed by increasing the TEA concentration to 10 mmol/l in the presence of 10^{-5} mol/l ACh is provided by Fig. 6, which presents the continuation of the recordings shown in Fig. 5*Ac*. In this situation, TEA strongly reduced outward currents evoked by pulses to $+10$ mV as well as the holding current. However, at the same time the steady-state values of currents observed upon stepping the command potential to -70 mV were shifted in an inward direction. This indicates the suppression of an outward current with a reversal potential more negative than -70 mV, i.e. again close to the calculated E_K and significantly different from the reversal potential of the current initially suppressed by 1 mmol/l TEA.

Judged from the effects on steady-state (late) currents, the combined effects of ACh and TEA suggest that both substances suppress, in addition to a K^+ conductance, another yet undefined conductance, which is apparently completely blocked at a TEA concentration of 1 mmol/l. Such complete blockade would explain

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Fig. 5. Voltage clamp study illustrating the combined effect of TEA and ACh. Experimental procedure and representation of recordings as in Fig. 3*A*, except $[K^+]_o = 6$ mmol/l and $E_K = -79$ mV. Panels *Aa*, *Ab* and *Ac* show current responses evoked consecutively in the same cell. The voltage clamp protocol is displayed at the bottom. Holding potential was -30mV, and pulses of 5 s duration of constant amplitude to -70 mV were applied during the time periods of changing the perfusion fluids (indicated on the right). *Aa*, membrane current under control conditions (PSS) and initially after changing to a solution containing TEA (1 mmol/l). *Ab*, membrane current in the presence of 1 mmol/l TEA and initially after adding ACh (10^{-5} mol/l) to the bathing solution. *Ac*, membrane current in the presence of both TEA and ACh. Horizontal arrows in panel *Ab* indicate current levels determined at the end of the corresponding pulses in panel *Aa*, arrows in panel *Ac* those determined at the end of the pulses in panel *Ab*. Panel 5*B* shows the section of recording marked in Fig. 5*Aa* at an expanded time and voltage scaling. Vertical arrows indicate direction and size of the initial components of tail currents.

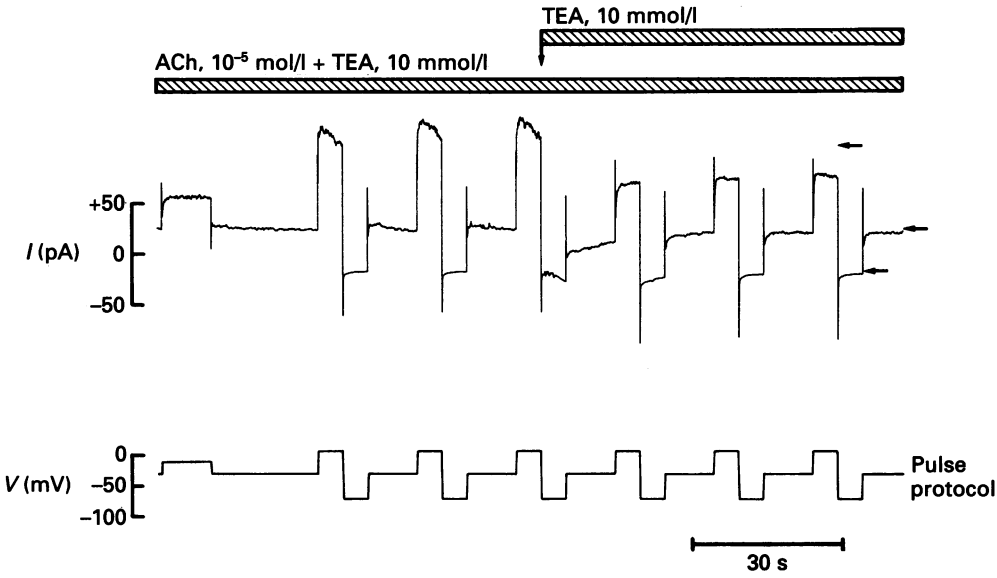

Fig. 6. Effect on membrane current of increasing the TEA concentration from 1 to 10 mmol/l in the presence of 10^{-5} mmol/l ACh. Continuation of the record shown in Fig. 5 (last pulse in Fig. 5*Ac* and first pulse in Fig. 6 are the same). Holding potential −30 mV and command pulses to +10 and −70 mV as indicated in the lower panel. Arrows on the right of the upper panel indicate holding current and currents at the end of the command pulses before adding TEA (10 mmol/l) to the bathing fluid.

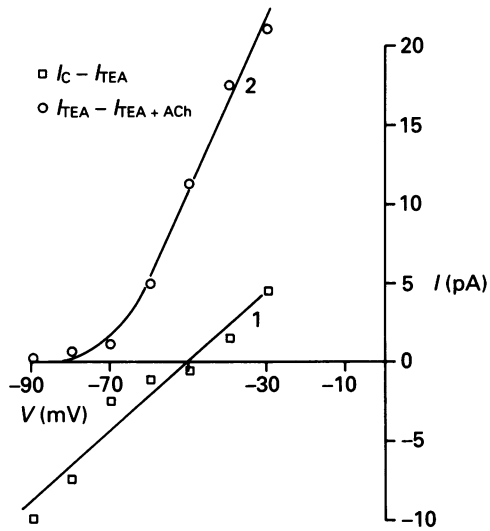

Fig. 7. Current–voltage relations illustrating the current suppressed by 1 mmol/l TEA (curve 1, □) and the current suppressed in the presence of 1 mmol/l TEA by 10^{-5} mol/l ACh (curve 2, ○). Evaluation of the recordings of Fig. 5. Currents plotted were obtained by subtracting the currents flowing at the end of the command pulses. Definition of symbols in inset: I_C, current under control conditions; I_{TEA}, current in the presence of TEA; $I_{TEA+ACh}$, current in the presence of both TEA and ACh.

why subsequent application of ACh to the cells as well as increasing the TEA concentration in the bathing solution, affected solely the K^+ conductance. It should be noted, that the blockade of the potassium conductance by TEA was rapid, whereas the action on the 'non-potassium' conductance often took more than 1 min to become fully established (compare Fig. 8). The rapid effect of TEA on the potassium conductance is indicated by the fact that, initially after TEA administration, the shift in the holding current was accompanied by a slight shift to inward currents evoked by command pulses to −70 mV (Fig. 5*Aa*, right).

Time-dependent components of membrane current and effect of acetylcholine

Two features of time-dependent variations of the membrane currents deserve consideration, because they concern possible errors in relating the potassium equilibrium potential (E_K) to the command voltage (V). First, as can be seen in Figs 3 and 5, current fluctuations of varying amplitude occurred. They were particularly pronounced when the membrane potential was clamped to depolarized levels where they had the appearance of spontaneous transient outward currents (STOCs). STOCs have also been observed in single smooth muscle cells of rabbit jejunum and rabbit ear artery by Benham *et al.* (1985) and Benham & Bolton (1986). They were interpreted to represent potassium currents through Ca^{2+}-activated K^+ channels which arise from cyclic discharging of Ca^{2+} from intracellular stores. This is particularly likely under conditions of elevated internal calcium, as may be expected to occur during depolarization at moderate EGTA concentrations in the pipette solution. Application of TEA (1 mmol/l) reduced these currents in both frequency and amplitude, and in most cases this inhibition was further increased by ACh (Fig. 5). Both of these observations are in accordance with reports by Benham *et al.* (1985) and Benham & Bolton (1986).

Important in the present context is that the reversal of STOCs from inward to outward direction provides an indication of E_K independent of its calculated value. In Fig. 3 STOCs reverse between −50 and −40 mV (see 4th and 5th pulses in panel *Ab* and corresponding current traces in panel *B*). The calculated E_K in this case is −47 mV. At a command voltage of −40 mV where a STOC of outward direction appears, the current in the presence of ACh was less inward than under control conditions, whereas at −30 mV the current in the presence of ACh was more inward than its control. This supports the existence of a true deviation from E_K of the reversal potential of the current suppressed by ACh, as estimated from the current–voltage curves in Fig. 4*A* (−39 mV). A similar argument holds for the deviation from E_K of the reversal potential of the current suppressed by 1 mmol/l TEA in the experiment of Fig. 5 (−50 mV). At this potential, the current traces in Fig. 5 show distinct transients of outward direction (5th pulse in panels *Aa* and *Ab*).

Another indication of E_K which is independent of its calculated value may be derived from the reversal of tail currents upon repolarizing the cell membrane after depolarization. Provided these currents are not contaminated by other time-dependent currents, they reflect the deactivation of K^+ currents activated during the preceding depolarization. As can be seen in the recordings of Fig. 5, a section of which has been expanded in Fig. 5*B*, the appearance of the tail currents was rather complex. Comparison with Fig. 3 shows that there was also some variability between

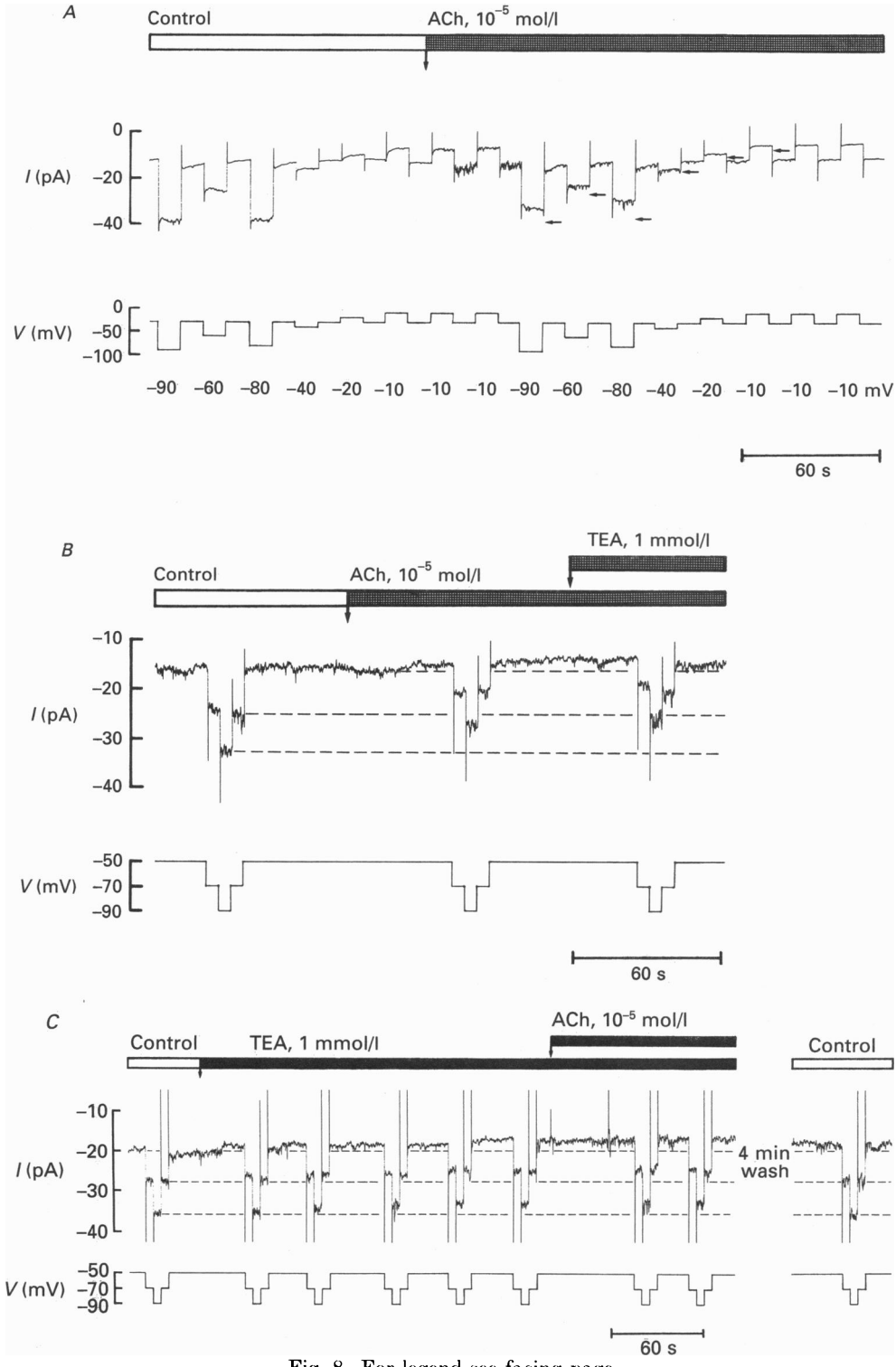

Fig. 8. For legend see facing page.

different cells. At present the complex time course of these currents is not fully understood. In Fig. 5 E_K was −79 mV and the tail current is inward at −80 mV. At −70 mV there is a small initial outward component before the current becomes almost flat showing a slight tendency of a slowly declining inward current (Fig. 5*B*). The initial outward tail component becomes more pronounced with stepping to less negative command potentials. In Fig. 6, which represents the continuation of Fig. 5, tail currents upon repolarization are still inward at −70 mV. In the experiments of Fig. 3, in which E_K was −47 mV, slow components of tail currents reverse positive to this value at about −40 mV. However, it is also discernable in the expanded record in Fig. 3*B* that the tail current at −40 mV shows a small initial outward component. Thus, some features of the tail currents would suggest the existence of some systematic error such that their reversal potential is positive to calculated E_K. On the other hand, as already mentioned on p. 269 in connection with Fig. 6, 10 mmol/l TEA inhibited potassium currents but it did not affect the amplitude of the tail currents upon repolarizing command pulses to −70 mV. This might indicate the inclusion in the tail currents of inward current which is not carried by potassium and possibly dependent on the level of the pre-potential. An inward current of these characteristics would explain the opposite direction of the initial tail current component at −70 mV in Figs 5 and 6, because the pre-potentials in both cases were very different (−30 mV in Fig. 5 and +10 mV in Fig. 6). It is also noteworthy in this context that the noise on the tail currents in Fig. 5*B* is almost absent at −80 mV but increases with increasing deviation of the command voltage from this value. Assuming that this current noise reflects opening and closing of K^+ channels, this would suggest that the calculated E_K (−79 mV) does not significantly deviate from that experienced by the cell membrane. Taken together, the reversal of STOCs from inward to outward direction supports the existence of a deviation from E_K both of the reversal potential of the current suppressed by 10^{-5} mol/l ACh and of the current suppressed by 1 mmol/l TEA, when these substances are applied separately. However, this remains uncertain following determination of reversal potentials of tail currents.

Experiments in K^+-free solution

In order to test the interpretation derived from the results described above of two steady conductances being affected by ACh, experiments were carried out to gain information about the identity of the 'non-potassium' conductance. In these experiments, K^+ in the external and in the pipette solutions was completely replaced by Na^+.

Figure 8 shows the effect of ACh on current deflections evoked by command pulses

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Fig. 8. Voltage clamp recordings illustrating the effects of ACh and TEA in three different cells (*A*, *B* and *C*) when K^+ in the pipette and in the bathing solution was completely replaced by Na^+. In each case the voltage protocol is shown underneath the membrane current. In *B* and *C*, ACh and TEA were added cumulatively to the bathing solution, but the order of application was different. Panel *C* right, control after 4 min washing. EGTA concentration in the pipette solution was 1·2 mmol/l in *A* and 5 mmol/l in *B* and *C*. Arrows in panel *A* and dashed lines in panels *B* and *C* indicate reference values of currents under control conditions.

in three different cells. In the experiments of Fig. 8*B* and *C* the EGTA concentration in the pipette solution was 5 mmol/l (usually 1·2 mmol/l) and the holding potential was set to −50 mV. Under the conditions chosen, the equilibrium potentials of all ion species expected to contribute to the membrane current were more positive than the

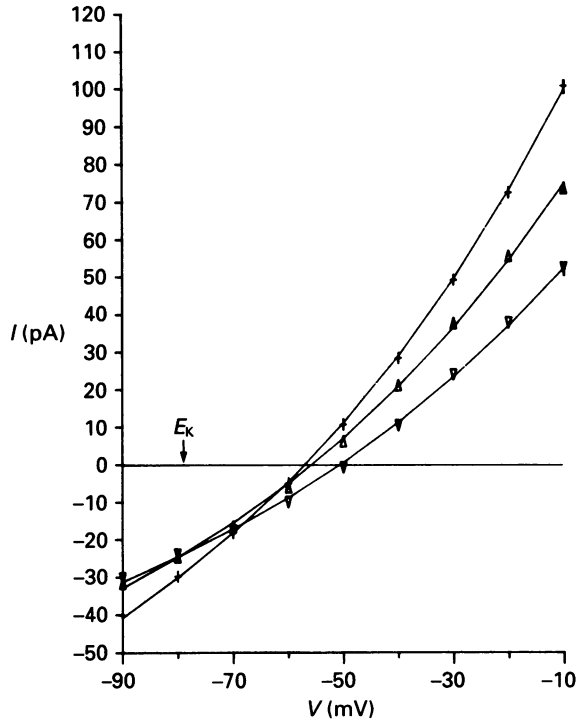

Fig. 9. Current–voltage relations determined consecutively in the same cell under control conditions (PSS, +), in the presence of 1 mmol/l TEA (△), and after adding ACh (bath concentration 10^{-5} mol/l) to the bathing solution (TEA + ACh, ▽). Mean values obtained from experiments on three different cells conducted according to the protocol of Fig. 5 are plotted. They were scaled with respect to the mean permeabilities under control conditions (see text). After this scaling, standard errors of the means were smaller than the size of the symbols. The continuous lines are theoretical curves fitted to the data on the basis of the constant-field equation.

clamp potentials. Therefore the increase in Fig. 8*A* of the first current deflection upon repolarizing the cell membrane from −10 to −30 mV compared with its control immediately before ACh application indicates an increase in membrane conductance. However, this increase was transient and followed by a persisting conductance decrease, as is most evident in Fig. 8*A* when comparing the later current deflections evoked by command pulses to very negative potentials in the presence of ACh with their respective controls. Another transient feature following ACh application was an increase of the noise level on the current trace which is consistent with a transient increase of a membrane conductance. Similar effects were also observed in some cells under more physiological conditions. A slight transient shift of the holding current in an inward direction is also seen in the experiment of

Fig. 8*B* with elevated intracellular EGTA concentration. However, if at all present, this effect of ACh appears to be less pronounced than in Fig. 8A. Figure 8*B* also illustrates the decrease in membrane conductance induced by ACh, indicated by the fact that the current flowing on command pulses to −70 and −90 mV are less inward in the presence of the agonist than under control conditions. Furthermore, it is seen that TEA added to the bathing solution in the presence of ACh produced a slight reduction in membrane current which was, however, very small or absent compared with the preceding effect of ACh. When TEA (1 mmol/l) was applied in the absence of ACh it caused a reduction of membrane conductance which was similar in magnitude to that induced solely by ACh but took more time to develop (Fig. 8*C*). These effects of ACh and TEA did not depend on whether Cl^- was present as a charge carrier or not (replacement of Cl^- by gluconate in the pipette and by isethionate in the bathing solution). We therefore conclude that the 'non-potassium' conductance affected by ACh and TEA represents a sodium conductance under these conditions.

Fit of current–voltage relations to the constant-field equation

As already mentioned, the steady current–voltage relations within the range of membrane potentials investigated showed a shape predicted by the Goldman–Hodgkin–Katz constant-field equation. Figure 9 summarizes current–voltage relations constructed from the results of the series of experiments conducted according to the protocol of Fig. 5. As can be seen, the average deviation from E_K of the current suppressed by 1 mmol/l TEA amounts to about 20 mV. At the same time Fig. 9 shows that simulation of the effect of ACh as a simultaneous reduction in the permeability P_K of the cell membrane to a potassium current (I_K) and the permeability P_{Na} to a sodium current (I_{Na}), both of which normally contribute to the resting current, provides an excellent fit to the experimental data. In order to account for the different surface areas (or absolute number of channels) of the different cells in the experiments of Fig. 5, currents carried by the various ion species were scaled with respect to the mean permeability of each species. These values were calculated by fitting each current–voltage curve obtained under control conditions according to the constant-field equation and then referred to a standard surface area of $1{\cdot}6 \times 10^{-5}$ cm^2 (see Methods). For the calculations it was assumed that the total current I was made up of I_K and I_{Na} and a component carried by Cl^- (I_{Cl}):

$$I = I_K + I_{Na} + I_{Cl},$$

such that

$$I_K = P_K \frac{VF^2}{RT} \frac{[K^+]_o - [K^+]_i \exp(VF/RT)}{1 - \exp(VF/RT)},$$

$$I_{Na} = P_{Na} \frac{VF^2}{RT} \frac{[Na^+]_o - [Na^+]_i \exp(VF/RT)}{1 - \exp(VF/RT)},$$

$$I_{Cl} = P_{Cl} \frac{VF^2}{RT} \frac{[Cl^-]_o - [Cl^-]_i \exp(VF/RT)}{1 - \exp(VF/RT)},$$

where V is the membrane potential, P_{Cl} the membrane permeability to Cl^-, and F, R and T have their usual meanings.

The permeability coefficients obtained by a procedure optimizing a least-squares

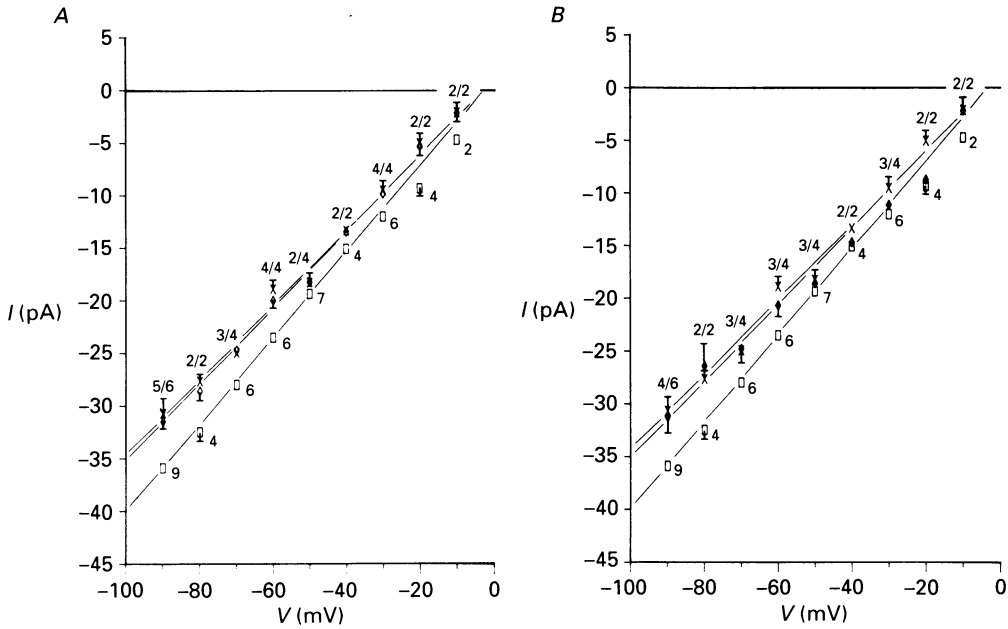

Fig. 10. Effect of ACh and TEA on current–voltage relations when intra- and extracellular K^+ was replaced by Na^+. Evaluation of experiments performed on nine cells according to the protocols of Fig. 9. Data points of each cell (minimum: three points per cell) under control conditions were first fitted on the basis of the constant-field equation. From the nine curves so obtained a mean curve was calculated which is identical in *A* and *B* (continuous line connecting control values: □). P_{Na} values of all currents were scaled with respect to the mean of P_{Na} under control conditions. Six out of the nine cells were exposed to ACh (10^{-5} mol/l) plus TEA (1 mmol/l) by cumulative application. *A*, mean currents of cells which were first or solely exposed to ACh (◇); *B*, mean currents of cells which were first or solely exposed to TEA (◆). For illustration of the smallness of the additional effect of either substance currents measured in the presence of ACh plus TEA are included in both *A* and *B* (×). Data points obtained in the presence of the drugs were also fitted to the constant-field equation (continuous lines). Numbers at symbols indicate number of data points. Numbers left of the slashes in *A* refer to symbols indicating the effect of ACh and in *B* to symbols indicating the effect of TEA; numbers right of the slashes in both *A* and *B* refer to the combined effect of the two substances. S.E. of mean indicated by bars when exceeding the size of symbols. Note that current–voltage curves do not pass the origin because of slight asymmetry of intra- and extracellular Na^+ concentration.

TABLE 1. Permeability values (mean ± S.E. of mean; $n = 3$) obtained from the results of Fig. 9

	Control	TEA	TEA + ACh
P_K (10^{-10} cm/s)	66·42	50·16 ± 3·17	36·41 ± 2·08
P_{Na} (10^{-10} cm/s)	3·71	2·92 ± 0·10	2·92 ± 0·10
P_{Cl} (10^{-10} cm/s)	0·75	0·75	0·75

curve fit of the current–voltage data measured under the three experimental conditions are given in Table 1. The introduction of I_{Cl} into the calculations improved the fit between experimental data and theoretical curves slightly.

Figure 10 summarizes results of experiments performed with K^+-free solution on either side of the cell membrane. The continuous lines in Fig. 10 are likewise

theoretical curves calculated from the Goldman–Hodgkin–Katz constant-field equation. In order to account for different surface areas of the membrane a scaling procedure was applied which is described in the legend of Fig. 10, taking also into account that Cl^- was substituted by impermeant anions in two of the nine experiments. The permeability coefficients obtained from this series of experiments are given in Table 2.

TABLE 2. Permeability values (mean ± S.E. of mean) obtained from the results of Fig. 10

	Control ($n = 9$)	ACh ($n = 5$)	TEA ($n = 4$)	ACh + TEA ($n = 6$)
P_K (10^{-10} cm/s)	0	0	0	0
P_{Na} (10^{-10} cm/s)	4·16	3·55 ± 0·09	3·56 ± 0·23	3·46 ± 0·16
P_{Cl} (10^{-10} cm/s)	0·65	0·65	0·65	0·65

The effects of ACh and TEA on P_{Na} are small but become significant for changes of I_{Na} at larger driving forces than 50 mV (Fig. 10). Under physiological conditions the driving force is always larger than this value. The close agreement between the corresponding permeability values obtained by fitting the two different sets of experiments supports the proposals that the non-potassium permeability suppressed by ACh is also blocked by TEA, that the underlying channel population is permeable to Na^+, and that a complete block of this population is achieved by each of the two substances at the concentrations used.

Lack of evidence for Ca^{2+} entry through receptor-operated channels

A series of experiments was addressed to the question whether Ca^{2+} entry via receptor-operated channels (ROCs), a mechanism proposed to act directly as a link between receptor occupation by agonists and contraction (see Bolton, 1979), might have escaped our measurements. Evidence that ionic channels associated with muscarinic receptors of smooth muscle may admit Ca^{2+} was provided by experiments on multicellular preparations of Bolton & Kitamura (1983). In isolated arterial smooth muscle cells, Benham & Tsien (1987) reported the activation of a receptor-operated Ca^{2+} current by ATP. Studies illustrating the opening of a non-selective cationic conductance by ACh in isolated visceral smooth muscle cells left open the possibility of Ca^{2+} participating in the activated current (Benham *et al.* 1985) or of additional activation of receptor-operated Ca^{2+} influx (Inoue *et al.* 1987). In our experiments Ca^{2+} entry could be missed if it was masked by the conductance decrease described. Such a possibility might be especially envisaged to occur at physiological concentrations with E_{Na} and E_{Ca} being both positive, but effects of ACh on a Na^+ and a Ca^{2+} current being of opposite direction. Experiments were carried out in which $[Na^+]_o$ was reduced by replacing NaCl in the external solution with sucrose, to make $E_{Na} = E_K$ ($[K^+]_o = 10$ mmol/l, $[K^+]_i = 123$ mmol/l; $E_K = -63$ mV). At this combined equilibrium potential, the activation of a receptor-operated Ca^{2+} current by ACh would be detectable, because the driving force on I_K and I_{Na} is zero. Experiments in five cells using this approach generally gave current–voltage

relations with much smaller K^+ permeabilities compared with those determined in PSS (about 5-fold). At potentials more negative then $E_K = E_{Na}$, the membrane current was less dependent on the membrane potential than predicted by the constant-field equation, i.e. the current–voltage curves became rather flat in this range. Whatever the reason for these alterations, the results gave no indication of an inward Ca^{2+} current which is directly gated by receptor binding of ACh and capable of contributing to the current at potentials near −60 mV.

DISCUSSION

One motivation for initiating the present study was the apparent existence of two qualitatively different membrane mechanisms underlying cholinergic activation of visceral smooth muscle. The discrepant mechanisms both arose from analysis in single freshly dispersed cells and are both in accordance with the depolarization generally observed in multicellular preparations. The mechanism determined in cells isolated from mammalian intestinal tissue is an increase in the membrane conductance for a current carried by Na^+ and K^+ (and/or Ca^{2+}) (rabbit jejunum: Benham *et al.* 1985; guinea-pig ileum: Inoue *et al.* 1987). On the other hand, it was shown in single cells from the toad stomach that one of the primary cholinergic effects is a suppression of a K^+ current termed M-current (Sims *et al.* 1985), a mechanism described previously for certain neurons (Kobayashi & Libet, 1970; Brown & Adams, 1980).

Qualitatively, our results bear resemblance to those reported for toad stomach in showing that the main effect of ACh is a suppression of a K^+ current. However, some noticeable differences are also evident. The K^+ current does not exhibit the properties defining the M-current; i.e. a non-inactivating K^+ current activated relatively slowly between −60 mV and −10 mV (Brown & Adams, 1980). Like the resting membrane current, the steady K^+ current suppressed by ACh in gastric fundus cells of the guinea-pig showed a voltage dependence obeying the Goldman–Hodgkin–Katz constant-field equation. Consistent with this, ACh produced a reduction in K^+ current at potentials not only above, but also more negative than −60 mV, i.e. in a potential range in which M-channels are shut. This effect was particularly evident at elevated external K^+ concentrations, when the driving force for K^+ currents at very negative potentials was high (Fig. 4*A*). Furthermore, although the current evoked by command pulses showed relaxations, these were not significantly affected by ACh as observed in toad stomach.

Another possible difference between cholinergic actions on guinea-pig gastric fundus cells and toad stomach cells is a suppressive effect of ACh on a small Na^+ current. The underlying conductance was completely blocked both by ACh at a concentration of 3×10^{-6} mol/l and by 1 mmol/l TEA. Since TEA was generally employed to decrease current noise in the experiments on toad stomach cells (Sims *et al.* 1985), it may be that this additional effect of ACh escaped detection. One possible interpretation of the multiple blocking effect of TEA is that the Na^+ channels blocked by ACh share some common structural homologies with the TEA-sensitive K^+ channels. An interpretation of this kind was recently discussed by Tang,

Presser & Morad (1988) in connection with blocking actions of amiloride on low-threshold Ca^{2+} channels as well as on special TTX-insensitive Na^+ channels.

Major arguments suggesting the suppression of a second (non-potassium) conductance by ACh were (a) the deviation from the calculated E_K of the reversal potential of the current suppressed by ACh (E_{rev}) and (b) the shift of E_{rev} to more negative values and its coincidence with E_K after application of 1 mmol/l TEA. Deviation of measured E_{rev} values from calculated E_K in spite of a suppression of a pure potassium current could principally be due to some error in the measurement of voltage or (and) in E_K as seen by the cell membrane. Inspection of the reversal of spontaneous transient outward currents (STOCs) argued against such kind of methodical errors in the present study, whereas tail currents upon repolarizing command pulses did not clarify this point. A shift of E_{rev}, as observed in the presence of 1 mmol/l TEA, is reminiscent of results in bull-frog sympathetic ganglia where muscarinic receptor activation shuts off an M-current and at the same time opens a cationic conductance (Tsuji, Minota & Kuba, 1987; Tsuji & Kuba, 1988). There in different cells and under different conditions one or the other effect may predominate. Assuming the reversal potential of the current suppressed by 1 mmol/l TEA in our experiments to represent the 'true' E_K, a reversal potential of an ACh-induced current more negative than this value would suggest a simultaneous activation of an inward current and the shutting off of a potassium current. This interpretation, however, is inconsistent with the steady reduction of sodium current by ACh and TEA in K^+-free solution. Moreover, barium ions, which blocked the M-current and induced appearance or potentiation of muscarinic activation of the cationic conductance in ganglia (Tsuji *et al.* 1987), failed to produce similar effects in gastric fundus cells (results of six experiments not included in the Results section).

Considering the present results and those reported earlier, the diversity of membrane actions of ACh on visceral smooth muscle cells seems puzzling and raises the question whether the differences are methodical (e.g. due to different cell isolation procedures or pipette solutions) or represent biological differences related to specialized physiological functions in different animal species and/or organs. Applying the whole-cell patch clamp technique with internal perfusion of the cells, our methodical approach has more in common with previous experiments performed in mammalian intestinal smooth muscle cells than with the studies in toad stomach cells, which were performed with a microelectrode and therefore avoid dialysis of the cells. Interestingly, despite the difference in the cholinergic action on the steady-state membrane conductance, the suppressive effects of TEA and ACh on spontaneous transient outward currents occurring at depolarized potential levels (Fig. 5) are very similar to those observed in rabbit jejunum cells by Benham *et al.* (1985) and Benham & Bolton (1986). As mentioned in connection with the results of Fig. 8, there was in some cells an indication of a transient increase of a cationic conductance. However, if present, this effect was negligible in magnitude compared with reports from rabbit jejunum (Benham *et al.* 1985) and guinea-pig ileum (Inoue *et al.* 1987; Inoue & Isenberg, 1989). Whereas in most previous studies ACh was applied for a short time (e.g. as a single shot by pressure or ionophoretic ejection), our analysis focuses on long-term effects of the agonist.

Two other considerations are of interest in judging the results of the present study.

First, the relatively small decrease in Na^+ permeability produced by ACh leads to the question how many channels might be affected by the agonist. Based on the results obtained in K^+-free solution, the conductance decrease (Δg_{Na}) calculated from the difference in permeability coefficients obtained in the absence and in the presence of ACh amounts to approximately 0·02 pS/μm^2. Assuming 1 pS as a lower limit of channel conductance – a value recently measured by DiFrancesco (1986) for rather unselective pacemaker channels in cardiac sino-atrial node cells – one arrives at an estimate of 0·02 channels/μm^2. This value is small compared with densities of most types of channels, but is of the same order of magnitude or even larger than the density of a Na^+- and K^+-permeable channel recently studied in guinea-pig ileum cells by Inoue *et al.* (1987).

The second consideration concerns the question whether the effects of ACh on steady-state permeabilities described are in accordance with electrophysiological observations in whole-tissue preparations. As mentioned initially, the electrical response of guinea-pig gastric fundus preparations to ACh is a depolarization combined with an increase in spike frequency, both effects lasting as long as the agonist is present in the bathing solution. Microelectrode measurements by Wagner (1976) have shown that ACh at a concentration of 10^{-6} mol/l produces a depolarization of about 5 mV in this preparation and increases the spike frequency by a factor of two (from 1 to 2 Hz).

From experiments in bull-frog sympathetic neurons (Adams, Brown & Constanti, 1982) it is known that the physiologically predominant effect of a reduction in steady K^+ conductance is the tendency to produce repetitive spikes, rather than any concomitant depolarization. Related results come from experiments on squid axon and theoretical simulations based on the Hodgkin–Huxley model, which show that a reduction of the steady K^+ conductance ($\bar{g}_K$) beyond a certain value induces automatic spike generation whose frequency is $\bar{g}_K$ dependent (Lammel & Mandrek, 1982; E. Lammel, unpublished calculations). A tentative suggestion, therefore, to provide some physiological relevance to the combined action of ACh on P_K and P_{Na}, is that the decrease in P_K is a main factor producing an increase in spike frequency, while at the same time the decrease in P_{Na}, which is most effective at a large driving force for I_{Na}, counteracts depolarization, thus reducing the degree of possible inactivation of I_{Ca}.

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