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Membrane mechanism associated with muscarinic receptor activation in single cells freshly dispersed from the rat anococcygeus muscle

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1 The mechanism of action of carbachol was studied on freshly dispersed cells of the rat anococcygeus using microelectrodes and patch pipettes.

2 Micro-ionophoretic application of carbachol evoked reproducible depolarizations which were reduced or blocked by atropine (10^{-7}–10^{-6} M). The time courses of the responses to noradrenaline and carbachol were similar.

3 The reversal potential of the carbachol-induced response was −3.8 mV and similar to the value (−6.2 mV) found for noradrenaline.

4 During the response to carbachol the membrane conductance was increased. At depolarized membrane potentials carbachol evoked biphasic membrane responses suggesting an increase in two separate ionic conductances.

5 With patch pipettes in the whole-cell configuration under voltage-clamp, carbachol produced an inward current at a holding potential of −50 mV. The inward current was associated with an increase in membrane conductance with an equilibrium potential of about 0 mV.

6 It is suggested that muscarinic receptors and adrenoceptors in the rat anococcygeus may activate similar membrane conductances. The most prominent mechanism is an increase in chloride ion conductance.

Introduction

Contraction of smooth muscle evoked by muscarinic receptor activation is generally accompanied by membrane depolarization (see Bolton, 1979). In early studies using whole tissue preparations of guinea-pig ileum, it was argued that carbachol increases membrane conductance by opening up ion channels that are permeable mainly to sodium and potassium (Bolton, 1972).

There are many difficulties in studying membrane mechanisms in whole tissue preparations because of the syncytial nature of smooth muscle. An advance in studying smooth muscle mechanisms has been brought about by the development of dissociation techniques to produce single isolated cells. Using isolated cells freshly dispersed from rabbit jejunum, Benham *et al.* (1985) showed that the depolarization to acetylcholine was produced by an inward current. These authors favoured the notion that muscarinic receptor activation produced a non-selective cation conductance increase. In contrast, in freshly dissociated gastric smooth muscle cells of the toad, Sims *et al.* (1985) have demonstrated that the depolarization to acetylcholine is caused by a decrease in membrane conductance through a suppression of a voltage-dependent potassium conductance. The discrepancy in these observations may be due to differences in animal species or smooth muscle types used. However, in the studies on freshly isolated cells patch pipettes were used by Benham *et al.* (1985), whereas microelectrodes were used by Sims *et al.* (1985). It is possible that diffusible cytoplasmic constituents may be washed out of the cell when recording with the relatively large-tipped patch pipettes. Therefore it may be that receptor-mediated responses observed in freshly dispersed single cells depend to some extent on the recording technique employed. In the present study we have investigated the action of carbachol on isolated cells of the rat anococcygeus muscle using both patch pipettes and microelectrodes. Gillespie (1972) demonstrated that muscarinic receptor activa-

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tion produces contraction of the rat anococcygeus muscle. Moreover, we have recently demonstrated that agonist-induced responses can be recorded from cells freshly dissociated from the rat anococcygous muscle (Byrne & Large, 1987). In the present experiments, muscarinic receptor activation produced a depolarization which was generated by an inward current that occurred as a consequence of an increase in membrane conductance. In the rat anococcygeus muscle, the muscarinic receptor mechanism resembles strongly the increase in membrane chloride conductance produced by α-adrenoceptor activation in dispersed single cells of this tissue (Byrne & Large, 1987).

Methods

Single smooth muscle cells were separated from the rat anococcygeus muscle by incubating the tissue in low calcium physiological salt solution containing collagenase and elastase (Byrne & Large, 1987). Microelectrodes were filled with 1.0 M KCl and had resistances of 80–150 MΩ. A single microelectrode was used for both recording membrane potential and passing current using the bridge circuit of an Axoclamp-2A microelectrode clamp amplifier (Axon Instruments, Inc.). Cells were impaled by gently pressing the microelectrode against the cell surface and briefly increasing the amount of capacitance neutralization. Whole-cell membrane currents were measured using standard patchpipette techniques and a List EPC-7 patch clamp amplifier (Hamill *et al.*, 1981). Patch pipettes had resistances of 2–5 MΩ. Drugs were applied either by ionophoresis or by inclusion in the bathing solution and experiments were carried out at room temperature (20–23°C). The concentration of carbachol and noradrenaline in the pipettes was 0.2 M. The ionophoretic pipette was placed within 5 μm of the cell. For both microelectrode and patchpipette studies the normal external salt solution contained (mM): NaCl 126, KCl 6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 10 and glucose 11. The normal patchpipette solution contained (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, glucose 11 and EGTA 1.0. Both external and pipette solutions were buffered with NaOH to pH 7.2. In a few experiments with patchpipettes K-free conditions were used by omitting KCl from the external solution. In the K-free pipette solution, KCl was replaced by an equimolar amount of NaCl. Also 10 mM tetraethylammonium chloride was added to the K-free external solution to prevent the movement of ions through K channels.

Drugs used: atropine sulphate (Sigma), carbamylcholine chloride (carbachol, Sigma), noradrenaline bitartrate (Sigma) and phentolamine mesylate (Ciba).

The values given in the text are the mean ± s.e.mean of n values.

Results

General observations using microelectrodes

The value of the resting membrane potential (E_m) of single freshly dispersed cells measured with microelectrodes was somewhat less negative than the value found in whole tissue preparations. In isolated cells values of acceptable impalements ranged between −30 and −40 mV compared to a mean value of about −60 mV found in whole tissue preparations (e.g. Byrne & Large, 1984). At least in part the lower value of membrane potential found in isolated cells was likely to be due to a leakage conductance caused by microelectrode impalement. The input resistance of single isolated cells, calculated from the voltage response to small hyperpolarizing currents, was 338 ± 86 MΩ ($n = 17$). Using patch pipettes in the whole-cell mode of recording the input resistance was always in excess of one GΩ.

The membrane potential was set between −50 mV and −65 mV by passing a small amount of inward current and the responses to agonists were observed. In some early experiments the actions of noradrenaline were studied for comparison with (a) responses to noradrenaline in whole tissue, and (b) responses to carbachol. Figure 1 illustrates depolarizations to ionophoretically-applied noradrenaline (Figure 1a and b) and carbachol (Figure 1c and d). A dose-effect relationship to both drugs was revealed as the amplitude of the depolarization was increased by increasing the duration of the ionophoretic charge. In many cells noradrenaline and carbachol depolarized the cells to about 0 mV if a sufficiently large ionophoretic pulse was used.

In the course of these experiments two interesting observations were made in comparison with patch pipette studies. Firstly, it was found that with acceptable microelectrode impalements (input resistance >100 MΩ) all cells (23 tested) were depolarized by ionophoretically-applied noradrenaline (10nA for 10–500 ms), whereas with patchpipettes many cells did not respond to noradrenaline (Byrne & Large, 1987). A similar pattern was found with carbachol in that most cells (20/26) were depolarized when microelectrodes were used but a much smaller proportion (<20%) responded to carbachol if patchpipettes were used. Secondly, with repeated application it was possible to obtain depolarizations of constant amplitude to both ionophoretically-applied noradrenaline and carbachol using microelectrodes. With patch pipettes, however, reproducible responses were found in only a few cells (Byrne & Large, 1987 and present study) and rarely could more than 6 responses (often fewer) be obtained before the cells became unresponsive. These results suggest that agonist-induced mechanisms may be compromised when

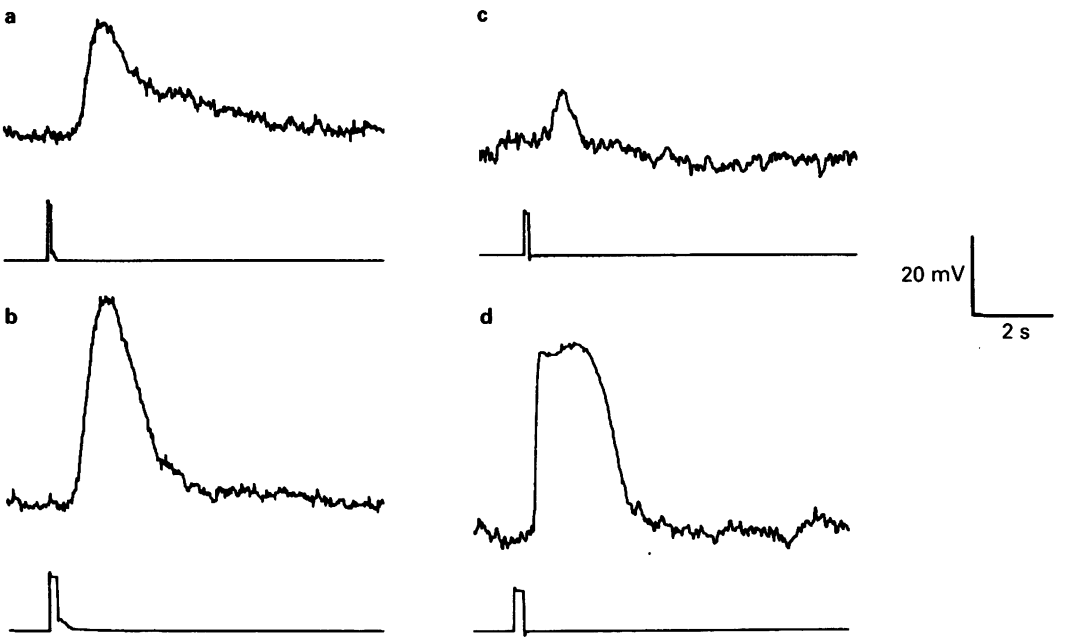

Figure 1 Comparison of depolarizations to noradrenaline (a and b) and carbachol (c and d) using intracellular microelectrode recording. Membrane potential (E_m): −55 mV for (a and b); −61 mV for (c and d). Parameters of ionophoretic pulse (lower traces): 10 nA for 100 ms (a and c) or 200 ms (b and d).

Table 1 Comparison of the characteristics of the responses to ionophoretically-applied noradrenaline and carbachol using microelectrodes

	Membrane Potential (mV)	*Amplitude of response* (mV)	*Latency** (ms)	*Rise time†* (ms)	*Half-decay time* (ms)
Depolarization					
Noradrenaline ($n = 15$)	−56.2 ± 2.0	29.7 ± 4.8	515 ± 50	540 ± 44	918 ± 121
Carbachol ($n = 16$)	−56.4 ± 2.0	36.4 ± 3.2	691 ± 61	825 ± 88	1027 ± 92
Hyperpolarization					
Carbachol ($n = 10$)	+40.5 ± 4.5	40.9 ± 6.3	715 ± 86	785 ± 61	3000 ± 357

*Latency is the period between the beginning of the ionophoretic pulse and the start of the response.
†Rise time is measured from the onset to the peak of the response. Parameters of ionophoresis: 10 nA for 10–1000 ms.

patch pipettes are used in the whole-cell configuration.

The characteristics of the time course of the depolarizations to ionophoretically-applied noradrenaline and carbachol studied with microelectrodes are shown in Table 1. The value of the latency of the depolarizations to noradrenaline in whole tissue (471 ms, Table 1, Byrne & Large, 1984) is similar to the mean value (515 ms, Table 1) found in isolated cells in the present study. The depolarizations to carbachol are also characterized by a latency (691 ms, Table 1) between the start of the ionophoretic pulse and the onset of the response. Moreover, it can be seen from Table 1 that there is a similarity in the time course of the depolarizations to noradrenaline and carbachol.

The α-adrenoceptor antagonist, phentolamine (10^{-8} M) blocked depolarizations to noradrenaline but

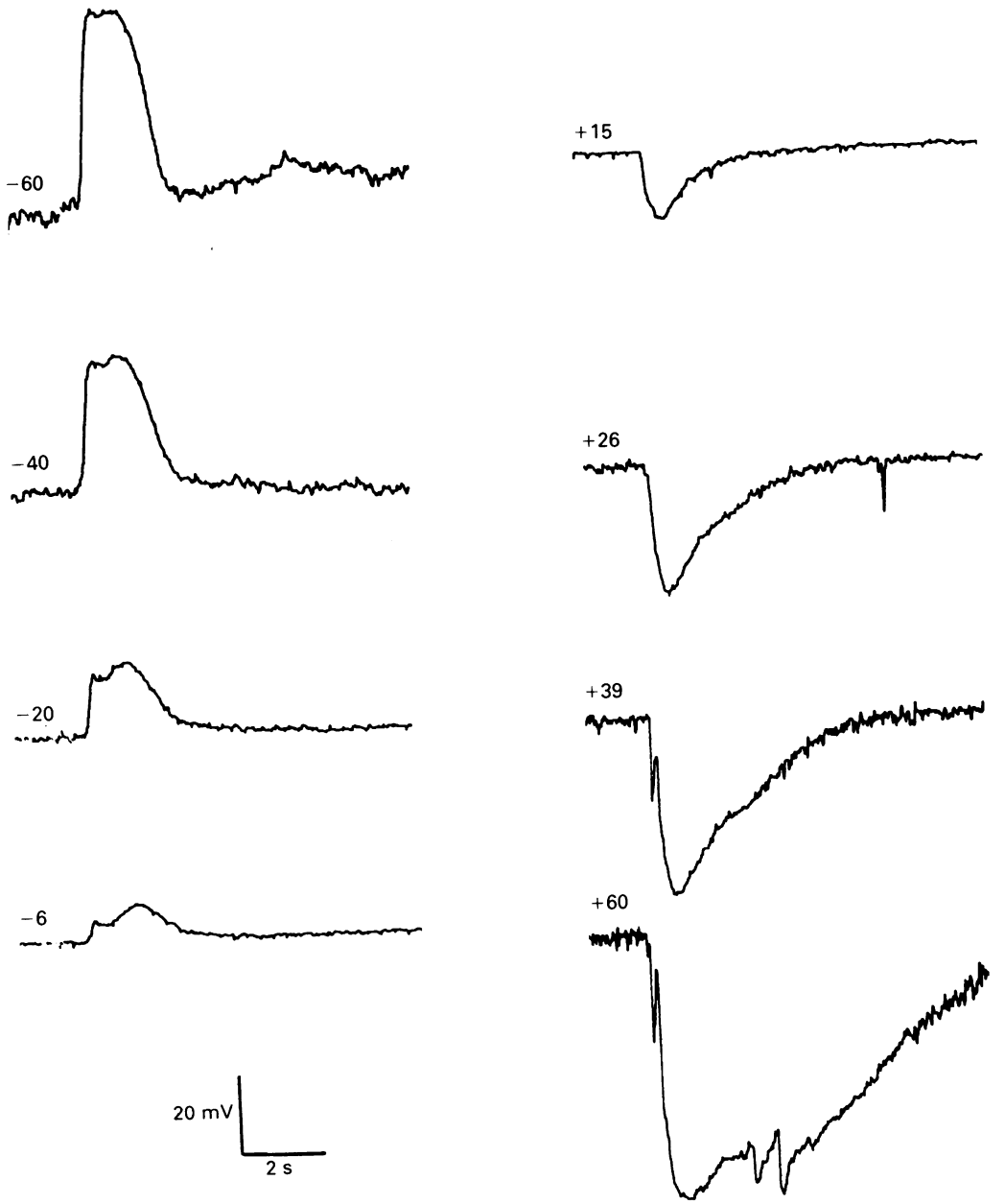

Figure 2 Influence of membrane potential on carbachol-induced response using current clamp. Values of holding potential in mV are given beside each trace. Ionophoretic pulse: 10 nA for 150 ms. Note the spontaneous hyperpolarizations that occur at positive membrane potentials, e.g. +26 mV and during the response at +60 mV.

not those to carbachol. In contrast responses to carbachol were attenuated or blocked in the presence of 10^{-7}–10^{-6} M atropine. It is concluded that the depolarizations to carbachol are mediated by muscarinic receptors.

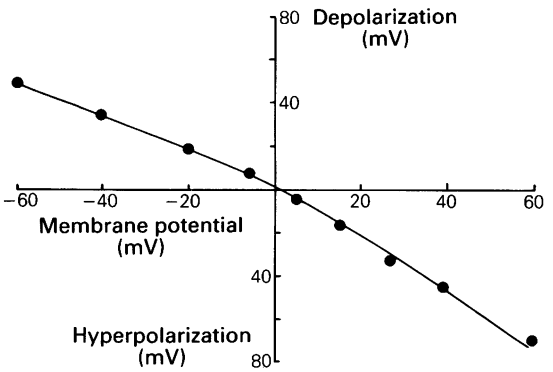

Figure 3 Amplitude of carbachol-induced response as a function of membrane potential. Data taken from cell shown in Figure 2.

Muscarinic receptor-activated conductance mechanism studied with microelectrodes

Figure 2 shows the result of an experiment in which the influence of membrane potential on the amplitude of the carbachol-induced response was studied. It can be seen that conditioning membrane depolarization reduced the amplitude of the depolarization to carbachol and that the response reversed to hyperpolarization at between −6 and +15 mV. The peak amplitude of the carbachol response is plotted against membrane potential in Figure 3; the interpolated reversal potential is +3 mV. In eight experiments with carbachol the reversal potential (E_r) was -3.8 ± 2.4 mV and with noradrenaline $E_r = -6.2 \pm 2.4$ mV ($n = 5$). These values of E_r for carbachol and noradrenaline were not statistically significantly different.

It can be seen from Figure 3 that the relationship between the carbachol-induced response and membrane potential was not linear. The hyperpolarizations were disproportionately larger at positive membrane potentials than depolarizations with the same potential driving force (membrane potential − reversal potential). Thus, in Figure 2 the amplitude of the response at +60 mV was 1.4 times greater than the response at −60 mV (the driving force is roughly the same at both potentials). This might be explained by an increase in input resistance on depolarization but this is unlikely and depolarization often decreased input resistance. Alternatively the non-linearity observed in Figure 3 can be explained if the conductance increase produced by carbachol were voltage-dependent. This latter explanation is supported by the observation that the decay of the response became very prolonged at positive membrane potentials (note the decay of the hyperpolarization at +60 mV in Figure 2 and see half-decay times in Table 1). Both of these results can be explained if the probability of channel opening were increased by membrane depolarization, as occurs with noradrenaline (Byrne & Large, 1987).

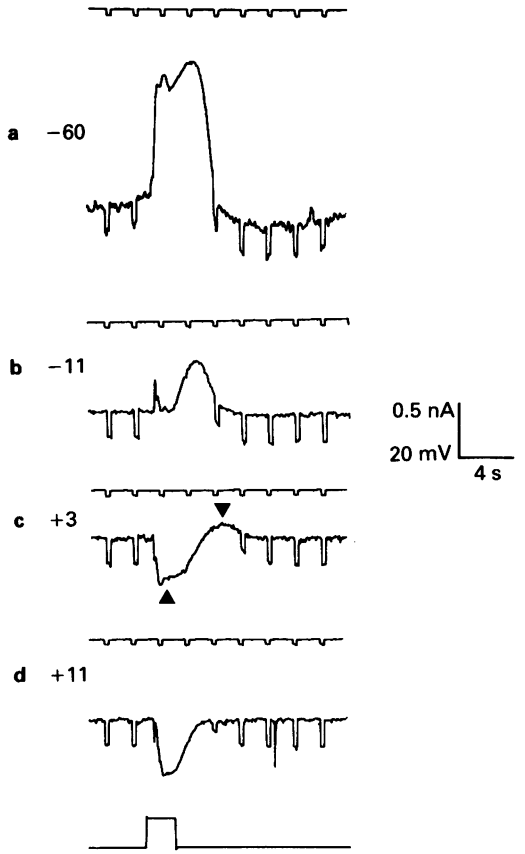

Figure 4 Effect of carbachol on membrane conductance. Electrotonic potentials were recorded to 50 pA hyperpolarizing current pulses (upper traces) at various membrane potentials indicated in mV to the left of the records. Pulse duration was 300 ms delivered every 2 s. Ionophoretic pulse (bottom record): 10 nA for 2 s. In (c) the arrowheads indicate the two components of the biphasic response and the electrotonic potentials were abolished during both components. Also note that in (b, c and d) the membrane resistance was greatly reduced at stages of the response when there was little apparent change in membrane potential.

Some of the responses in Figure 2 (e.g. at −20 mV and −6 mV) appeared to be biphasic. This phenomenon became more pronounced when long ionophoretic pulses of carbachol were used at depolarized potentials (e.g. Figure 4c). Figure 4 illustrates the responses to a 2 s ionophoretic pulse of carbachol at various membrane potentials. Membrane resistance was monitored by recording electronic potentials to

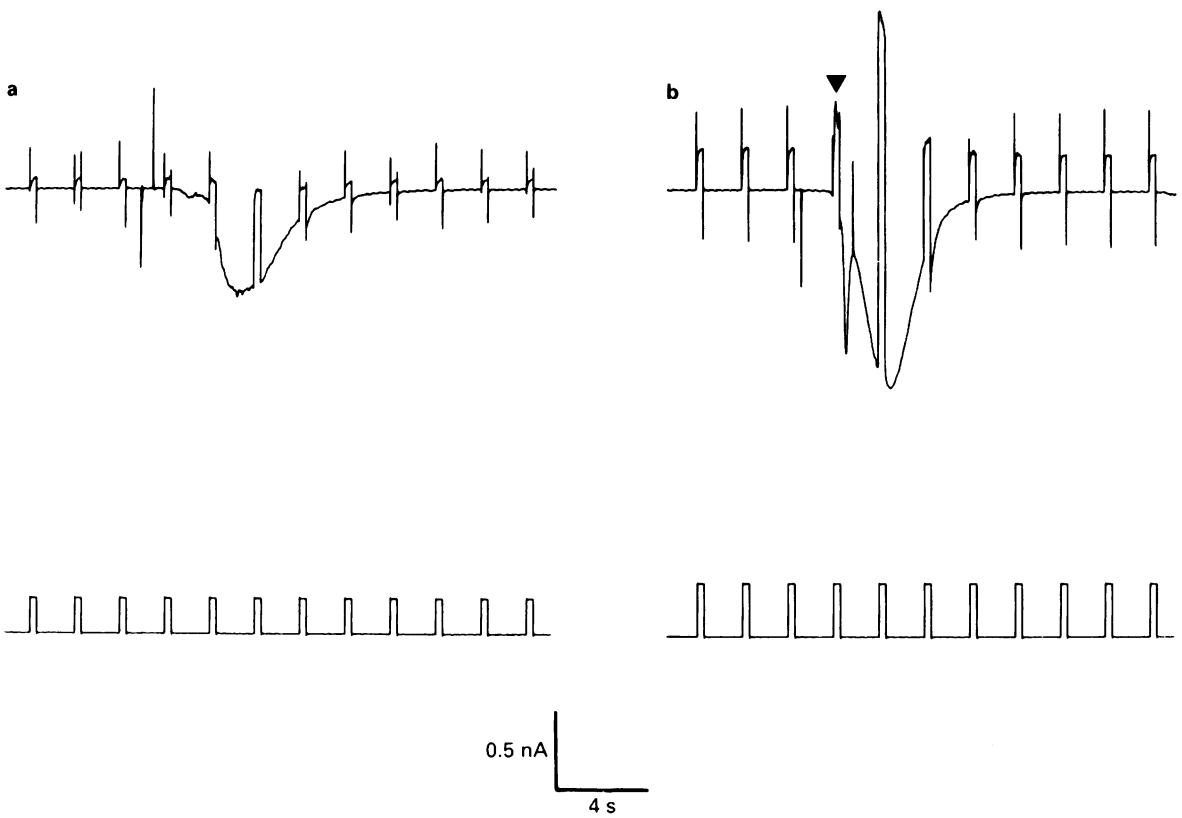

Figure 5 Effect of ionophoretically-applied carbachol studied under voltage-clamp with patchpipettes. Upper and lower traces are respectively current and voltage records. Membrane potential was held at −50 mV and stepped to either −10 mV (a) or +10 mV (b) for 300 ms every 2 s. Normal K-containing solutions (see Methods) were used in this experiment. The ionophoretic pulse was 50 nA for 0.5 s (a) and 2 s (b). The ionophoretic artefacts can be seen quite clearly in the current records. Note in (b) that the outward current evoked by the depolarizing step (indicated by arrowhead) was roughly doubled at the onset of the inward current at −50 mV.

small hyperpolarizing current pulses. At −60 mV the electronic potentials were abolished during the carbachol-induced depolarization (Figure 4a). This increase in membrane conductance is not due to the depolarization *per se* because when the cell was held close to the reversal potential the amplitude of the electronic potential was still reduced or abolished during the carbachol-induced response (Figure 4b–d), even when there was little change in membrane potential. It should be noted that the membrane conductance is increased during both components of the carbachol-induced response (Figure 4c). This suggests that stimulation of muscarinic receptors can activate (at least) two conductance mechanisms. Similar biphasic responses were evoked by noradrenaline.

The data from Figures 2–4 show that carbachol increases membrane conductance and this mechanism was investigated further using voltage-clamp. Whole-cell patch pipette recording was used for these studies as the high resistance of the microelectrodes limited the performance of the switching single electrode voltage-clamp.

Membrane currents induced by carbachol studied with patchpipettes

With the whole-cell mode of recording under voltage-clamp, the membrane potential was set at a holding potential of −50 mV by passing a small amount of inward current. In addition depolarizing voltage steps to either −10 mV (Figure 5a) or +10 mV (Figure 5b) were applied every 2 s to monitor membrane conductance. It can be seen that before carbachol application during the depolarizing steps to the command potentials of −10 mV and +10 mV (Figure 5a and b) an outward current develops following the instantaneous

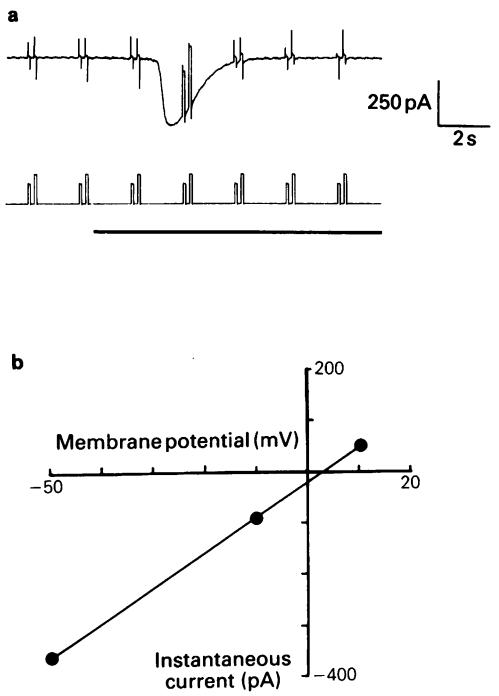

Figure 6 Voltage-current relationship of the carbachol-induced response. (a) Membrane potential (lower trace) was held at -50 mV and stepped for 100 ms to -10 and $+10$ mV. Bath-applied carbachol (10^{-4} M) was present throughout the period indicated by the solid horizontal bar. K-free solutions. (b) Carbachol-induced membrane current plotted as a function of the clamp potential. The current was calculated by subtracting the value of the current in the absence of carbachol from the value in the presence of carbachol.

current step. These outward relaxations were not observed if K-free external and pipette solutions were used. (e.g. Figure 6a). Therefore, it is likely that the outward currents to depolarizing voltage jumps represent the activation of potassium currents. At the holding potential of -50 mV the ionophoretic application of carbachol evoked an inward current (Figure 5a and b), which was associated with an increase in the amplitude of the current steps indicating an increase in membrane conductance. An estimate of the increase in conductance and the direction of the membrane current evoked by carbachol was made by subtracting the values of the instantaneous current evoked by the depolarizing steps in the absence of carbachol from the values measured in the presence of carbachol. The values of the instantaneous current were measured within 10 ms of the depolarizing step. During the depolarizing steps it can be seen that the carbachol-induced current is inward at a command potential of -10 mV (Figure 5a) but outward at $+10$ mV (Figure 5b). Assuming a linear relationship between the instantaneous current and membrane potential (see Figure 6b) the carbachol equilibrium potential was -1.7 ± 3.2 mV ($n = 5$). In K-containing solutions the calculated equilibrium potentials (E) for Na, K and Cl were respectively $+82$, -77 and -2 mV.

In normal K-solutions depolarizing voltage steps often elicited an increased outward current during the early part of the carbachol-induced response (e.g. see arrowhead in Figure 5b). Also, sometimes the beginning of the response appeared to be multiphasic (see Figure 5b) which suggests that more than one conductance mechanism was activated by carbachol. Since these effects were not observed when K-free solutions were used, it is probable that carbachol evokes a small transient increase in potassium conductance. We investigated the responses in K-free solutions in order to gain further information regarding the conductance change produced by carbachol. Our data with these solutions are limited as so few cells responded to carbachol when K was omitted from intra- and extracellular solutions. Figure 6a shows that bath-applied carbachol (10^{-4} M) produced an inward current of about 370 pA at the holding potential of -50 mV which declined in the continued presence of the drug. Figure 6b illustrates that the relationship between the amplitude of the instantaneous current and clamp potential current was linear and the reversal potential was $+3$ mV. In three cells the reversal potential of the carbachol-induced response was 0.3 ± 1.5 mV. In K-free solutions the calculated values of E_{Na} and E_{Cl} were 0 mV and -2 mV, respectively.

Discussion

In isolated cells freshly dispersed from the rat anococcygeus muscle, muscarinic receptor activation produces depolarization. This response is generated by an inward current which occurs as a consequence of an increase in membrane conductance. In this respect our data agree with the studies on mammalian intestinal muscle (Bolton, 1972; Benham *et al.*, 1985) but are at variance with the results of Sims *et al.* (1985) on amphibian gastric muscle. No evidence was found for a conductance decrease to carbachol whether microelectrodes or patch pipettes were used. This suggests that the differences in the mechanisms associated with muscarinic receptor activation that have been observed in isolated smooth muscle cells are not due to differences in the recording techniques used. A more plausible explanation is that excitatory muscarinic receptors in smooth muscle may be linked to a variety of mechanisms depending on the species and/or type

of smooth muscle. However, it is worth noting that the use of patch pipettes did seem to limit the ability to record agonist-induced responses. It was possible to record responses to noradrenaline and carbachol in nearly all cells when recording with micro-electrodes. This was not so when using patch pipettes. Moreover, when using patch pipettes, the amplitude of the responses rapidly declined with repeated application; this did not occur with micro-electrodes. With micro-electrodes the amplitude of the responses to brief pulses of ionophoretically-applied drugs remained constant as long as a stable impalement was maintained. These data suggest that intracellular constituents may be washed out with patch pipettes. The results are in agreement with the belief that intracellular biochemical mediators link muscarinic and adrenoceptor binding to the effector response (e.g. the opening of membrane ion channels).

In the present work there was a striking resemblance in the responses associated with α-adrenoceptor and muscarinic receptor activation. With micro-electrode recording the time course and the reversal potential of the responses to noradrenaline and carbachol were similar. Using patch pipettes the equilibrium potential of the carbachol-induced membrane current in either K-containing or K-free solutions was close to 0 mV which is also similar to the value found for noradrenaline (Byrne & Large, 1987). With the values of the equilibrium potentials of Na, K and Cl in these solutions it is probable that carbachol opens up channels that are permeable either to sodium and potassium or to chloride. Previously it has been shown that in isolated cells of the rat anococcygeus muscle α_1-adrenoceptor activation produces a large increase in chloride conductance with a smaller transient increase in potassium conductance (Byrne & Large, 1987). The increase in chloride conductance is presumably the major membrane mechanism underlying the depolarization to ionophoretically-applied noradrenaline in single cells and whole tissues (Byrne & Large, 1985). The close resemblance between the responses to carbachol and noradrenaline indicates that muscarinic receptor activation in the anococcygeus muscle also produces an increase in chloride conductance. There is also evidence that, like noradrenaline, carbachol can activate more than one conductance mechanism. Experimental support for two separate ionic mechanisms is shown in Figure 4 and in voltage-clamp experiments an initial increased outward (potassium) current to depolarizing steps is sometimes seen at the beginning of the inward current (Figure 5b). This also occurs with α-adrenoceptor activation (Byrne & Large, 1987). Overall there is strong evidence that, in the rat anococcygeus muscle, activation of α-adrenoceptors and muscarinic receptors may trigger similar (same?) membrane mechanisms.

It is interesting that in rat lacrimal glands, muscarinic receptor stimulation evokes an increase in chloride and potassium conductance which is mediated by an increase in intracellular calcium concentration (Marty *et al.*, 1984). In the rat anococcygeus muscle the calcium ionophore A23187 and caffeine, which releases calcium from intracellular stores, increase membrane chloride conductance (Byrne & Large, 1987). With aequorin luminescence, it has been shown that activation of both α-adrenoceptors and muscarinic receptors increases the intracellular calcium concentration in vascular smooth muscle (Morgan & Morgan, 1984; Bradley & Morgan, 1987). A working hypothesis is that, in the rat anococcygeus muscle, activation of both muscarinic receptors and adrenoceptors by respectively carbachol and noradrenaline may independently release calcium from internal stores, which in turn opens up membrane chloride and potassium channels by an action on the inner surface of the cell membrane.

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