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MUSCARINIC RESPONSE IN RAT LACRIMAL GLANDS

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SUMMARY

A large variety of responses has been uncovered by recent investigations of conductance changes elicited by muscarinic agonists. In exocrine glands, the permeability to K^+, Cl^- and Na^+ ions is increased, and internal Ca^{2+} serves as a second messenger. Patch-clamp analysis of the secreting cells has revealed three types of Ca^{2+}-dependent channels, which are respectively selective for K^+, for Cl^-, and for monovalent cations. The channels differ in their sensitivity to the internal Ca^{2+} concentration, Ca_i. K^+-selective channels are partially activated at rest, with Ca_i approx. 10 nmol l^{-1}; Cl^--selective channels are activated between 100 nmol l^{-1} and 1 μmol l^{-1}; activation of cationic channels requires micromolar Ca_i levels.

Cell-attached recordings, performed either on isolated cells or on cell clusters, show an activation of all three channel types upon application of acetylcholine. In whole-cell recordings, mostly K^+- and Cl^--selective channels are activated. The cell currents display slow oscillations linked to variations of Ca_i. Whole-cell currents rise after a delay of approx. 1 s, and decay with a time constant of approx. 0·7 s upon removal of acetylcholine. They do not depend on extracellular Ca^{2+}.

The recent demonstration that Ca^{2+}-dependent currents can also be obtained when dialysing the cells with inositoltrisphosphate or with GTPγS, a non-hydrolysable analogue of guanosine triphosphate, opens promising leads to an analysis of intracellular events regulated by acetylcholine.

INTRODUCTION

Conductance changes elicited by muscarinic agonists are one of the best studied examples of 'slow synaptic responses', which are characterized both by slow kinetics and by the intervention of several molecular processes between agonist–receptor binding and cell response (reviewed by Purves, 1978; Kehoe & Marty, 1980; Hartzell, 1981). It was shown 30 years ago that electrical effects of vagal stimulation on heart muscle appear with a marked delay (Del Castillo & Katz, 1955). Direct application of muscarinic agonists to heart muscle cells or to parasympathetic

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ganglion cells similarly produces delayed conductance changes with a lag of at least 100 ms (Purves, 1976; Hartzell, Kuffler, Stickgold & Yoshikami, 1977). Such effects mark a striking contrast with the fast nicotinic transmission found in vertebrate neuromuscular junctions and sympathetic ganglia, where the synaptic delay is less than 1 ms (Katz, 1969). Activation of muscarinic agonists also generally leads to an alteration of the cell metabolism, which is manifested at the level of the plasma membrane, where the turnover of phosphatidylinositol derivatives is increased (Berridge & Irvine, 1984), and in the cytoplasm, where the level of intracellular solutes such as Ca^{2+} or cyclic GMP is changed (Purves, 1978).

Recently, a great deal of effort has been devoted to understanding the conductance changes elicited by muscarinic agonists in various vertebrate preparations. Four broad types of response were found. In certain bullfrog sympathetic neurones, called B neurones, the main action of muscarinic agonists is to inhibit a special category of K^+ channels responsible for the 'M current' (Brown & Adams, 1980; Adams, Brown & Constanti, 1982*a*,*b*). The same effect has also been observed in sympathetic neurones of vertebrates (Brown & Selyanko, 1985), in neurones of the central nervous system (Halliwell & Adams, 1982; Nowak & Macdonald, 1983) and in smooth muscle cells (Sims, Singer & Walsh, 1985). In heart muscle cells, on the other hand, muscarinic agonists increase the opening probability of another class of K^+ channels which are already present at rest (Sakmann, Noma & Trautwein, 1983). These channels have faster kinetics than M current channels. Furthermore, the heart channels are activated by membrane hyperpolarization, whereas M currents are enhanced by depolarization. It is possible that the same channel as that described by Sakmann, Noma & Trautwein regulates the K^+ conductance increase found in C neurones of certain bullfrog sympathetic ganglia (Dodd & Horn, 1983) as well as some recently described inhibitory responses of central neurones (McCormick & Prince, 1986; Egan & North, 1986).

A third effect of muscarinic agonists, also observed in heart muscle cells, is to inhibit Ca^{2+} currents. As a matter of fact, the long-held view that acetylcholine (ACh) decreases the Ca^{2+} currents observable at rest is now questioned by a recent study using isolated, dialysed heart muscle cells, which suggests that at least part of the current shift previously attributed to a decrease of Ca^{2+} currents is instead due to an increase of K^+ currents (Iijima, Irisawa & Kameyama, 1985). Nevertheless, after activation of Ca^{2+} currents with beta-adrenergic agonists, ACh clearly decreases the Ca^{2+} currents back to their resting level under conditions where K^+ currents are effectively blocked (Breitwieser & Szabo, 1985).

It is not known whether the action of muscarinic agonists on the M current involves a second messenger mechanism. Experiments on cell-attached patches showed that the activation of the K^+ current in the heart does not require any soluble cytoplasmic substance (Soejima & Noma, 1984). By analogy with other systems, it seems likely that the reversion of adrenergic activation of Ca^{2+} currents in the heart is due to a decrease of intracellular cyclic AMP, but direct evidence is still lacking. In the fourth main type of muscarinic response, on the other hand, there is a very strong case in favour of the involvement of a second messenger substance, in this case

calcium. The best documented example is that of exocrine glands, where the calcium rise evoked by ACh leads to an increase of membrane conductance, to exocytosis and to intercellular uncoupling (Petersen, 1980). The conductance effect was recently shown to result from the activation of several classes of Ca^{2+}-dependent channels, and it is thought to be the main factor regulating extrusion of electrolyte (Petersen & Maruyama, 1984; Marty, Tan & Trautmann, 1984).

Finally, yet other types of responses have been described, which do not readily fall into the above categories. In some neurones, activation of muscarinic receptors leads to an *inhibition* of Ca^{2+}-dependent currents (North & Tokimasa, 1983; Cole & Nicoll, 1984; Pennefather, Lancaster, Adams & Nicoll, 1985). In smooth muscle cells, one of the actions of muscarinic agonists is to increase a cation-selective conductance pathway (Benham, Bolton & Lang, 1985). However, these effects have yet to be fully characterized.

The present paper focuses on the Ca^{2+}-dependent currents found in exocrine glands. The main features of the channels and of their regulation by muscarinic agonists are reviewed. New results concerning the effects of ACh on the channels, the kinetics of the response and the influence of extracellular Ca^{2+} are presented.

Ca^{2+}-DEPENDENT K^+ CHANNELS IN EXOCRINE GLANDS

Three types of Ca^{2+}-dependent channels are found in exocrine glands (reviewed in Petersen & Maruyama, 1984; Marty *et al.* 1984).

Single-channel activity of the first type of channel, which we called the 'BK channel' for Big K^+ channel, is illustrated in Fig. 1. The records were obtained in an outside-out patch from a cell isolated from a rat lacrimal gland. The internal side of the membrane was bathed with a high-K^+ solution containing 10 nmol l^{-1} Ca^{2+}. The patch contained two channels, which displayed a marked sensitivity to membrane potential. Single-channel currents are remarkably large (Fig. 1B).

BK channels are found in salivary and lacrimal glands of rodents, and in pig exocrine pancreas (Petersen & Maruyama, 1984). Inside-out patch experiments demonstrate that BK channels are activated by very low concentrations of Ca^{2+}, between 10 and 100 nmol l^{-1} (Maruyama, Gallacher & Petersen, 1983; Findlay, 1984). Above 1 μmol l^{-1}, Ca^{2+} blocks the channels in a voltage-dependent manner (Marty *et al.* 1984).

Single-channel currents due to BK channels may readily be identified in cell-attached experiments, owing to their characteristic single-channel conductance and voltage dependence. Comparison of the open state probability with results from inside-out patches indicates that the resting level of calcium is very low, of the order of 10 nmol l^{-1} (Findlay, 1984). Upon application of ACh in the bath, a clear increase of the channel open state probability is observed (Fig. 2). The effects of ACh on the cell resting potential could be simultaneously estimated by following the size of the BK unitary currents. There was no general rule concerning the evolution of the resting potential. In the experiment shown, it seemed rather stable, at a value which was estimated at -60 mV by comparison with isolated patch results. In other cases,

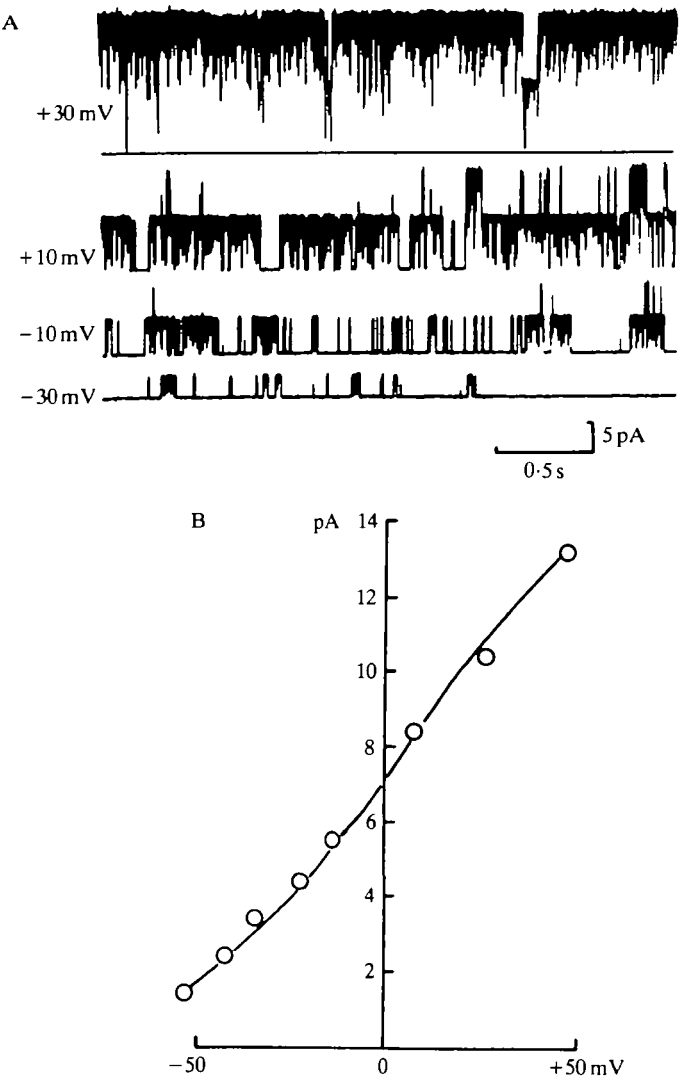

Fig. 1. BK channels in an outside-out patch. (A) Sample recordings at four different membrane potentials, as indicated on the left of each trace. Only two channels were present in this patch. (B) Single channel I/V curve from the patch in (A). These recordings, as well as those presented in the other figures, were obtained from cells of rat lacrimal glands according to previously described methods (Marty, Tan & Trautmann, 1984). Briefly, a mixture of single cells and of cell clusters was obtained by successive incubations in trypsin, in divalent free solution, and in collagenase. After dissociation, cells were placed in an incubator at 37°C for 1–6 h before use. The normal bath solution for patch-clamp recording contained (in mmol l^{-1}): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; Hepes-NaOH pH 7·2, 5. Recordings were performed at room temperature. In this experiment the pipette (internal) medium contained (in mmol l^{-1}): KCl, 140; $MgCl_2$, 2; EGTA, 5·5; $CaCl_2$, 0·5 (free Ca^{2+} concentration, 10 nmol l^{-1}); Hepes-KOH pH 7·2, 5.

Fig. 2. Response to acetylcholine (ACh) in a cell-attached patch. Repetitive, 60-mV hyperpolarizing voltage jumps were given from the cell resting potential. Rare openings of BK channels gave unitary outward currents of 0·8 pA in the control. Upon application of ACh (0·5 μmol l^{-1}), the mean number of BK channels activated at the cell potential went from 0·01 to 0·30 (as estimated on 10 consecutive periods at this potential representing a total of 14 s in the control and of 14 s during ACh application and immediately afterwards). This effect was totally reversible; the mean number of BK channels dropped back to 0·04 in the next seven consecutive periods at the cell resting potential (after which the voltage pulse protocol was changed), and subsequently to a very low value comparable to that observed before ACh. Effects of ACh on BK activity became noticeable 2·5 s after the onset of drug application, and they outlasted the application by 25 s. There was no marked effect on the cell membrane potential, as the size of the unitary currents remained approximately constant. Cell-attached recordings were obtained from superficial cells of compact cell clusters (20–50 cells), presumably from the basolateral membrane. The pipette recording solution contained a K^+-free saline in order to avoid any possible contribution of BK channels to inward currents at hyperpolarized potentials.

a hyperpolarization, or a depolarization followed by a hyperpolarization, were observed. Thus, the increase of BK channel activity is not a direct consequence of a membrane potential change. It is most probably due to an increase of intracellular Ca^{2+} ions.

In rat lacrimal glands, BK channels account entirely for the conductance of the secreting cells at rest. Due to the voltage dependence of the channels, cell I/V curves display a pronounced outward rectification (Trautmann & Marty, 1984). This cellular current, as well as the unitary currents shown in Fig. 1, may be blocked by bath application of 2 mmol l^{-1} tetraethylammonium (Trautmann & Marty, 1984).

Ca^{2+}-DEPENDENT Cl^- CHANNELS

Ca^{2+}-dependent Cl^- channels of low unit conductance were demonstrated in rat lacrimal glands (Marty *et al.* 1984). Owing to the small value of this conductance, the channels were mostly studied using noise analysis rather than direct, single-channel measurements. In cell-attached patches, application of the Ca^{2+} ionophore, A 23187, to the bath solution resulted in a noisy inward current response with occasional single-channel steps of about 0·4 pA at an estimated membrane potential of −115 mV (Marty *et al.* 1984). We now report on new cell-attached experiments which address two questions. First, can the inward current response be stimulated by ACh as well

as by the Ca^{2+} ionophore? And, if so, is this response only present in isolated cells, where luminal and basolateral membranes are exposed, or does it also occur when recording from cell clusters, which presumably expose only their basolateral membrane to patch-clamp pipettes?

At least two types of inward unitary currents which were likely to be due to Cl^- channels were observed at hyperpolarized potentials. The first type consisted of well-defined single events of very small amplitude, around 0·1 pA near −100 mV (Fig. 3). The second type of event had a larger amplitude, of 0·3–0·6 pA near −100 mV, and was accompanied by large, open-channel noise (Fig. 3). In many instances, the larger events seemed to break down into smaller steps, suggesting that the two types of events may have represented two gating modes of a common channel population. (Similar results were previously obtained with Ca^{2+} ionophore applications: Marty *et al.* 1984, p. 312.) The identification of the single-channel events of Fig. 3 as being due to Ca^{2+}-dependent Cl^- channels rests on three arguments. First, the single-channel steps are very similar to the events previously described when stimulating with the Ca^{2+} ionophore. Second, their size varied qualitatively as expected from Cl^- channels, with a reversal potential somewhat above the cell resting potential.

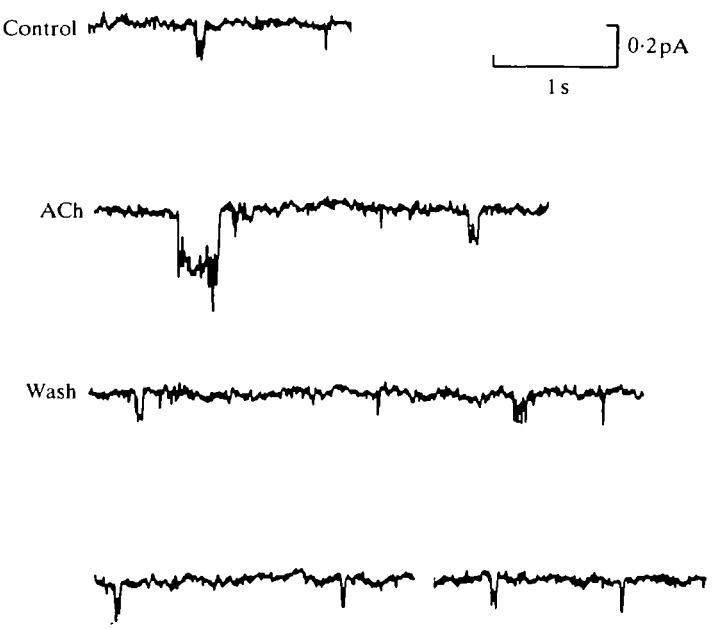

Fig. 3. Cell-attached recordings of presumptive Ca^{2+}-dependent Cl^- channels. Recordings were obtained before, during and after a 10-s application of 0·5 μmol l^{-1} acetylcholine (ACh). The patch was maintained 40 mV more negative than the cell potential (approximate membrane potential: −90 mV). Unit events of 0·1 pA were obtained; in addition, a noisy event with a main amplitude of 0·3 pA was observed during ACh application. Due to the low frequency of the 0·1 pA events, it was not possible to ascertain whether they were under the control of ACh. Assuming that the single-channel events would revert at −30 mV (a reasonable estimate of E_{Cl}), the unit conductance is estimated at 1·5 pS. Same methods as in Fig. 2; 50 Hz low-pass filter.

(Note, however, that a clear reversal was obtained only with the larger type of channel.) Third, whole-cell recordings indicate that only Ca^{2+}-dependent Cl^- channels have a single-channel conductance compatible with the single-channel observations.

In the patch illustrated in Fig. 3, activity was so infrequent that it was impossible to ascertain whether ACh had any effect on the channels. In other examples, a clear channel activation was obtained, but it was then difficult to distinguish single steps as overlaps were the rule (Fig. 4). Such a response is observable both near −60 mV and near −120 mV in the experiments of Fig. 2 and Fig. 4A. All responses had a delay of about 1 s, and they outlasted the ACh response by 10–30 s, after which the currents returned to control values. The recordings of Fig. 4A and 4B show examples of single steps of about 0·1 pA which seem to merge into larger events.

Ca^{2+}-dependent channels require a Ca_i level in excess of 100 nmol l^{-1} for their activation and they are fully closed in a resting cell (Trautmann & Marty, 1984; Marty *et al.* 1984; Evans & Marty, 1986*b*). Cellular currents due to these channels have been studied in cells dialysed with a K^+-free solution buffered with *N*-hydroxyethylenediamine triacetic acid (HEDTA) (Evans & Marty, 1986*b*). The currents are activated by depolarization, and display slow exponential relaxations in response to voltage jumps. They are blocked by high concentrations of furosemide (Evans, Marty, Tan & Trautmann, 1986).

Ca^{2+}-DEPENDENT CATION CHANNELS

A third type of Ca^{2+}-dependent channel was described in pancreatic acinar cells by Maruyama & Petersen, who showed that it is activated by internal Ca^{2+} upon stimulation by cholecystokinin (Maruyama & Petersen, 1982*a*,*b*). This channel has a unit conductance near 25 pS (Fig. 5), is selective for monovalent cations and is largely independent of voltage. In lacrimal glands, activation of this channel requires micromolar concentrations of Ca^{2+} (Marty *et al.* 1984). It is therefore questionable whether ACh is actually able to raise the internal Ca^{2+} concentration to a high enough level to activate these channels. In fact, cell-attached experiments often failed to show such activation. That the channels were nevertheless present in some of these patches was indicated by the observation of single channels with a very low opening probability throughout the experiment. In some other cases (e.g. in Fig. 4C), ACh did open the channels, but the effects were limited to a single opening, or they were poorly reversible. These experiments are consistent with our previous suggestion that the cationic channels participate in the ACh response (Marty *et al.* 1984), but further work will be needed to ascertain the exact experimental conditions needed for their activation.

Ca^{2+}-DEPENDENT CHANNELS AND CELL POLARITY

It would be obviously important to know whether any of the three types of channels are preferentially localized on the basolateral or on the luminal membrane.

Since K^+- and cation-selective channels may be recorded on the superficial membrane of cell clusters which have retained an acinar structure, it is likely that these two channel types belong to the basolateral membrane (Petersen & Maruyama, 1984). As the experiments illustrated by Figs 2, 3 and 4A were performed on such aggregates, the same also applies to Ca^{2+}-dependent Cl^- channels. However, it

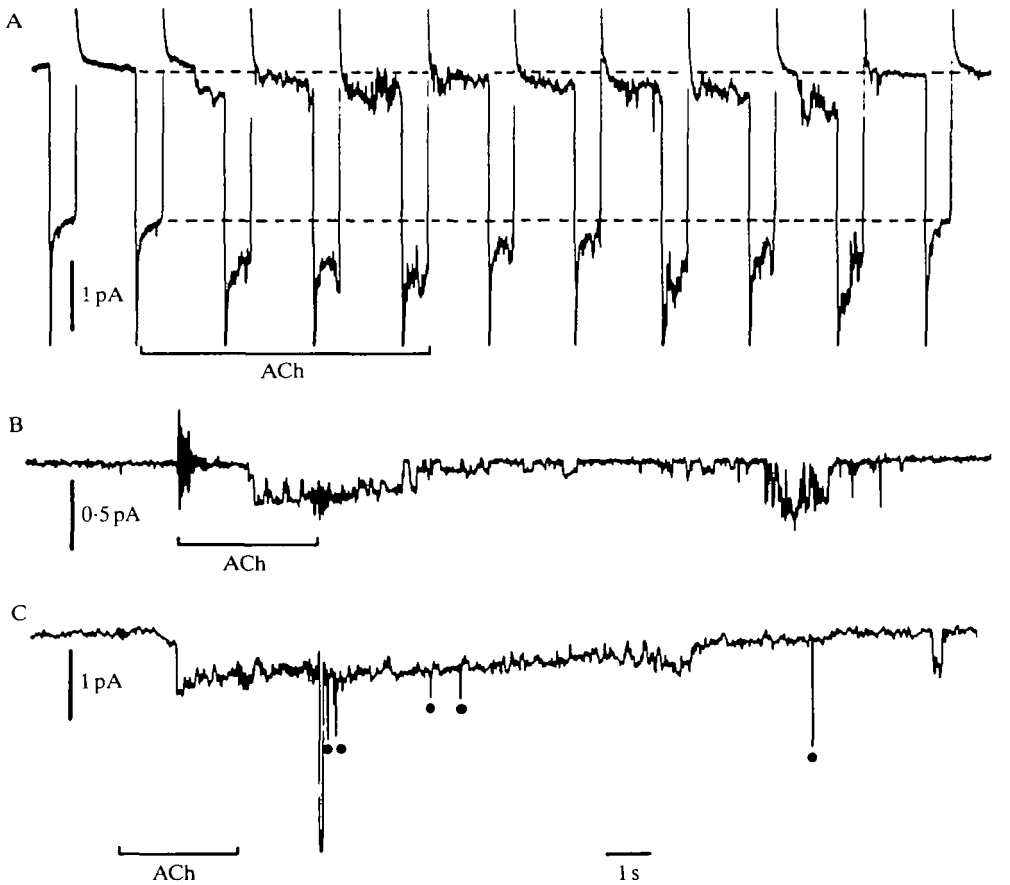

Fig. 4. Activation of inward current by acetylcholine (ACh) in cell-attached patches. (A) In this patch, repetitive, 60-mV hyperpolarizing voltage jumps were given from the cell resting potential. Application of 0·5 μmol l^{-1} ACh (bar) elicited a noisy inward current both at the cell potential and at the hyperpolarized test potential. The effects were observed 1·2 s after application onset, and they outlasted the application by 11 s. Experimental conditions as in Fig. 2, except that this patch did not contain any BK channel (as judged by the absence of large, outward current events when depolarizing voltage pulses were given). (B) Cell-attached patch on a single cell, maintained 40 mV below the cell potential. Application of ACh (2 μmol l^{-1}) elicits single-channel currents of approx. 0·1 pA that occasionally seem to merge into larger events (approx. 0·25 pA). (C) Same experimental conditions as in B. In this patch, application of ACh elicited 2·5 pA inward current events in addition to the noisy inward current response. Five of these openings (●) appear as smaller deflections on the figure due to heavy (40 Hz) low-pass filtering. The 2·5 pA events are most probably due to Ca^{2+}-dependent cationic channels.

should be stressed that in all cases, the glands were subjected to partial dissociation using enzymatic and mechanical treatment. Even though care was taken to select aggregates with a compact appearance, the possibility exists that these treatments may have damaged the tight junctions and thus allowed luminal membrane proteins to diffuse to the basolateral domain. To sum up, the present evidence suggests, but does not prove conclusively, that all three types of Ca^{2+}-dependent channels are located on the basolateral membrane. This is in line with the model of electrolyte secretion of Marty *et al.* (1984), which assumes that the basolateral membrane

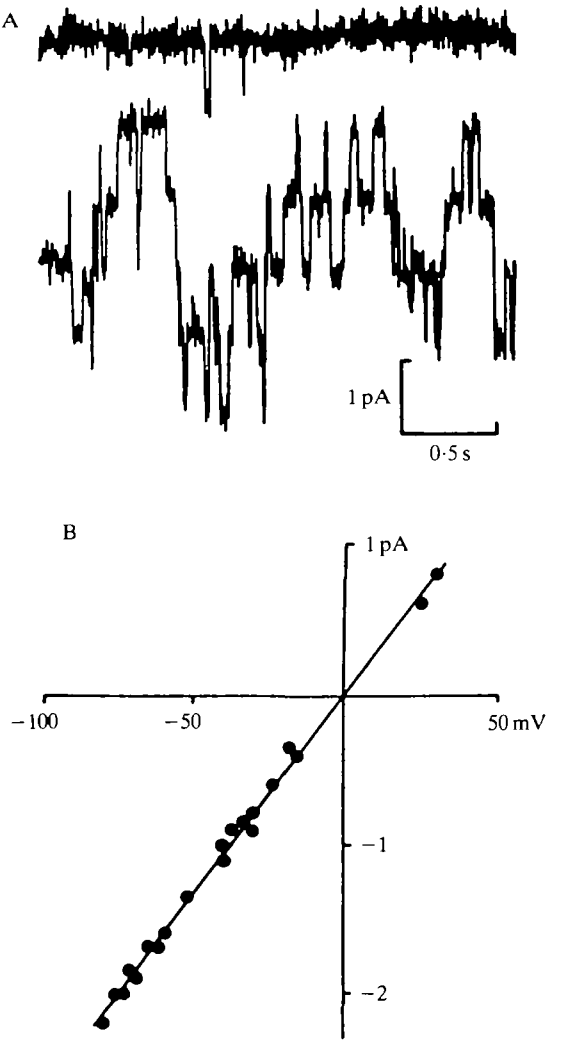

Fig. 5. Ca^{2+}-dependent cation-selective channels. Inside-out patch with 140 mmol l^{-1} NaCl on both sides. (A) Single-channel currents recorded at −40 mV. Upper record: internal face of the membrane exposed to a Ca^{2+}-free solution containing 1 mmol l^{-1} EGTA. Lower record: 1 mmol l^{-1} Ca^{2+} solution. (B) I/V curve of the single-channel currents shown in A. Elementary conductance: 27 pS. Reproduced with permission from Marty, Tan & Trautmann, 1984.

possesses the three kinds of channel. There is no direct information on the presence of channels on the luminal membrane.

The results of cell-attached experiments on isolated cells and on cell clusters are summarized in Table 1. Most patches showed a noisy inward current response, probably due to Cl^- channels. A few patches also displayed activation of BK channels and/or of cationic channels. No difference appears between isolated cells and cell clusters. The failure of 4/5 of the patches to show the BK response is most probably due to the comparatively low density of BK channels (one in 5–15 μm^2, Trautmann & Marty, 1984), since the corresponding patches also failed to show any BK channel opening when the membrane was depolarized before ACh application. (Rather small pipettes, with an input resistance of about 5 MΩ in normal saline, were selected in these experiments, and a minimum amount of suction was employed, such that the patch areas were probably of a few μm^2.)

EFFECTS OF ACETYLCHOLINE ON WHOLE-CELL CURRENTS

At low concentrations (typically 0·1 $\mu mol\,l^{-1}$ ACh, or 0·5 $\mu mol\,l^{-1}$ carbamylcholine, CCh) muscarinic agonists increase the voltage-dependent K^+ current (Trautmann & Marty, 1984; Marty *et al.* 1984). This effect results from an increase in the opening probability of BK channels, rather than from recruitment of new channels (Trautmann & Marty, 1984). It can be blocked by increasing the internal Ca^{2+}-buffering capacity (Trautmann & Marty, 1984; Marty *et al.* 1984). On the other hand, removal of all external Ca ions does not abolish the current (Trautmann & Marty, 1984; Marty *et al.* 1984). These experiments suggest that ACh activates Ca^{2+}-dependent channels by releasing Ca^{2+} from internal stores.

Whereas low agonist concentrations only activate BK channels, stronger stimulations result in the additional development of a Cl^--selective conductance (Trautmann & Marty, 1984; Marty *et al.* 1984; Findlay & Petersen, 1985). This is due to the activation of the low unitary conductance, Ca^{2+}-dependent Cl^- channels described above.

Cl^--selective channels may also be activated by stimulating the cell with the Ca^{2+} ionophore A 23187, or by dialysing the cell with a buffered high-Ca^{2+} solution

Table 1. *Activation of Ca^{2+}-dependent channels in cell-attached patches*

	BK channels activation	Cl^- channels activation	Cationic channels activation
Isolated cells	2/10	9/10	3/10
Clusters	2/9	7/9	3/9

Cell-attached recordings were obtained either from isolated cells or from compact cell clusters. Identification of the various channels was as explained for the experiments of Figs 2–4. Very little single-channel activity was present at rest. Patches which lacked BK channel responses to acetylcholine also failed to show BK channel openings when the cells were depolarized before agonist application, suggesting that they did not contain any BK channel.

ACh (0·5, 1 or 2 $\mu mol\,l^{-1}$) was applied for 2- to 10-s periods.

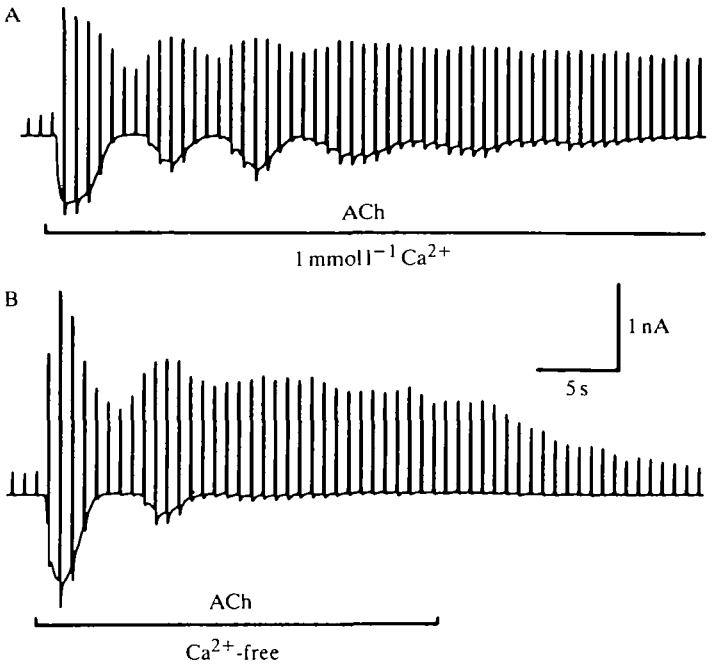

Fig. 6. Whole-cell responses to acetylcholine (ACh). Depolarizing voltage pulses were given repetitively from a holding potential of −60 mV. (A) Application of ACh (1 μmol l^{-1}) in normal saline. (B) Application of ACh (1 μmol l^{-1}) in a Ca^{2+}-free solution containing 0·5 mmol l^{-1} EGTA. The bath solution contained 1 mmol l^{-1} Ca^{2+}. Single cells were used for recording. The pipette (intracellular) solution contained (in mmol l^{-1}): KCl, 140; $MgCl_2$, 2; EGTA, 0·5; Hepes-KOH pH 7·2, 5. ACh was applied to the bath with a microperfusion system which had an exchange time of about 0·1 s at the application onset. The return to baseline was much longer, probably because of slow washout of ACh. (No bulk bath perfusion was used.)

(Marty *et al.* 1984; Evans & Marty, 1986*b*). Such studies showed that the Ca^{2+}-dependent Cl^- channel has a low unitary conductance (1–2 pS) and that it is activated by membrane depolarization. It is totally inactive at 10^{-7} mol l^{-1} Ca^{2+}, and entirely activated at 10^{-6} mol l^{-1} Ca^{2+} or above (Evans & Marty, 1986*b*).

OSCILLATIONS OF Ca^{2+}-DEPENDENT CURRENTS

Some of the features of the electrical response to ACh are shown in Figs 6–8. Under standard recording conditions, the holding current measured at −60 mV is very small; depolarizing pulses to 0 mV elicit outward relaxations due to BK channels (Trautmann & Marty, 1984). Upon application of ACh, activation of BK channels leads to an increase of the outward current observed at 0 mV, and activation of Ca^{2+}-dependent Cl^- channels leads to inward current observable at −60 mV. [The current at 0 mV is entirely carried by K^+-selective channels, as Cl^-- and cation-selective channels have their reversal potential at 0 mV under the conditions of Fig. 6. At −60 mV, on the contrary, contributions from K^+ currents are small due to the proximity of the K^+ equilibrium potential (−84 mV). The main current carriers are

Cl^- ions at this potential, since cation-selective channels have a high threshold for activation by Ca^{2+}.] Both Cl^- and K^+ current responses display damped oscillations with a period of 5–10 s (Fig. 6A). The oscillations are particularly marked at −60 mV, where six periods may be recognized on the example shown. The holding current comes back to zero between successive waves, and it eventually subsides entirely in spite of the continued presence of ACh. At 0 mV, synchronous waves are observed, but their relative amplitude is less pronounced than at −60 mV. After 40 s of ACh application, the outward current is more than three times larger than before stimulation, and shows only a very slow decline. Even after 2 min of continuous agonist application, a substantial residual activation was observed (not shown).

Inward tails which are observed after voltage jumps are also due to Cl^- currents. Comparison with whole-cell results with fixed internal Ca^{2+} solutions (Evans & Marty, 1986*b*) suggests that, during such waves, the internal Ca^{2+} level rises from about 0·1 μmol l^{-1} to nearly 1 μmol l^{-1}. At the end of the stimulation, the inward current dropped to a very low level, indicating that Ca_i had decreased to 0·1 μmol l^{-1} or below. The fact that K^+ channels were still activated suggests that Ca_i was in fact close to 0·1 μmol l^{-1}.

LACK OF EFFECT OF EXTERNAL Ca^{2+}

In the experiment of Fig. 6B, ACh (1 μmol l^{-1}, as in Fig. 6A) was applied in a Ca^{2+}-free solution containing 0·5 mmol l^{-1} EGTA. As may be seen, a large K^+ and Cl^- response was obtained. The differences between Fig. 6A and B are well within the range of cell-to-cell variations observed with either experimental condition. These results confirm our previous observations that external Ca^{2+} does not markedly affect the response (Trautmann & Marty, 1984; Marty *et al.* 1984).

However, it was reported that the whole-cell response of pig pancreatic cells to cholecystokinin, which has many similarities with the muscarinic response studied here, strongly depends on extracellular Ca ions (Maruyama & Petersen, 1984). To retest this point a different experimental approach was taken (Fig. 7). ACh was applied for a long period with a double microperfusion system (see figure legend). The same ACh concentration was placed in each microperfusion solution. One solution contained 1 mmol l^{-1} Ca^{2+} and the other contained 0·5 mmol l^{-1} EGTA instead. When the external solution was abruptly changed from one solution to the other, no discontinuity was observed in the evolution of the response (Fig. 7). We conclude from these experiments that external Ca^{2+} does not affect the muscarinic responses in our recording conditions. The reason for the difference from the response to cholecystokinin in the pancreas remains to be elucidated.

The results of Figs 6 and 7 contrast with those obtained with radiolabelled Rb^+ efflux, where muscarinic agonists induce an early component which does not depend on extracellular Ca^{2+} and a late component which does (Putney, 1979; Parod, Leslie & Putney, 1980). From such evidence, it was concluded that the decline of the Rb^+ efflux in Ca^{2+}-free solution is due to an exhaustion of Ca^{2+} stores, which need external Ca^{2+} to be replenished. Direct comparison with the present experiments is

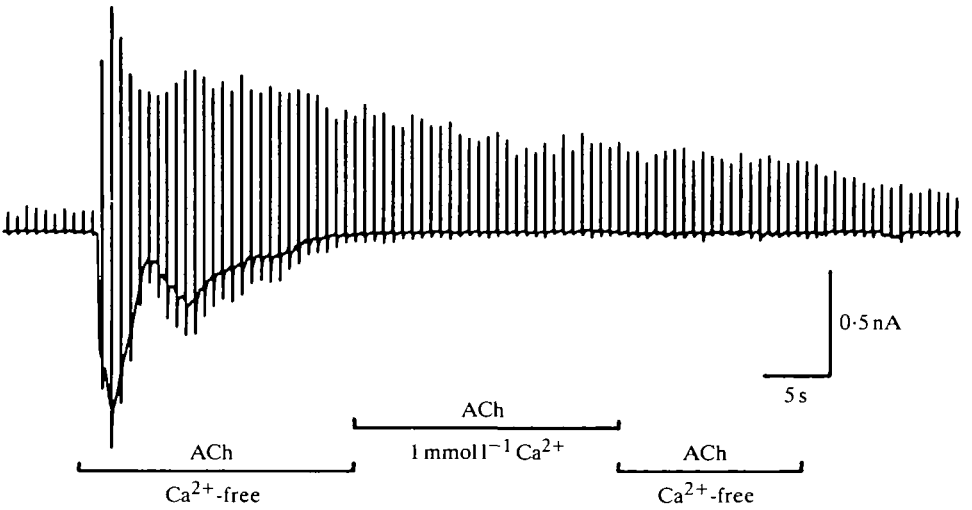

Fig. 7. Rapid readmission of Ca^{2+} during a long acetylcholine (ACh) application. ACh (1 μmol l^{-1}) was applied first in a Ca^{2+}-free saline containing 0·5 mmol l^{-1} ACh, then in normal saline containing 1 mmol l^{-1} Ca^{2+}, and finally again in Ca^{2+}-free saline. There is no discontinuity of the response when switching from one solution to the other. Whole-cell recording as in Fig. 6. Voltage jumps from −60 mV to 0 mV. Two microperfusion systems were positioned close to the cell. One was switched off as the other was switched on. The effective exchange time, as estimated with tetraethylammonium in one of the microperfusion systems, was about 0·2 s.

difficult since the time span of flux studies is much longer than that of a whole-cell recording. Nevertheless, the decline of the currents in Figs 6 and 7 is probably not due to depletion of Ca^{2+} stores, since dialysis of the cell with inositoltrisphosphate elicits a response which lasts for many minutes (Evans & Marty, 1986*a*). On the other hand, unknown intracellular factors necessary for the effect of external Ca^{2+} may be lost in the whole-cell recording experiments.

KINETICS OF RESPONSE ONSET AND RETURN TO BASELINE

The microperfusion system used in the present study allows very rapid changes of the bath solution. After a step application of ACh, a lag of about 1 s was always observed before the rise of Ca^{2+}-dependent currents (Figs 6–8; Trautmann & Marty, 1984; Marty *et al.* 1984). The delay is longer for Cl^- currents than for K^+ currents. If several applications of ACh are performed on the same cell, the delay is found to increase (Trautmann & Marty, 1984; Marty *et al.* 1984). The time required for the new external solution to equilibrate around the cell is of the order of 0·1 s, as estimated in control experiments using tetraethylammonium (TEA) as a blocker of BK currents. Thus, the delay is a genuine property of the response, and it must be attributed to the processes occurring in the cell membrane and cytoplasm between agonist binding and the final activation of ion channels by internal Ca^{2+}.

It was also of interest to study the return of the current to the baseline after removal of ACh in order to gain insight into the last events governing Ca^{2+} release. This could not be achieved with a simple microperfusion system, and two separate microperfusions were used instead (Fig. 8). The results indicate that the Ca^{2+}-induced currents return to the baseline with a time constant of 0·7 s. Again, this is longer than the delay for effective solution exchange as assayed in control experiments using TEA. The results of Fig. 8 suggest that the termination of the response is more rapid than its onset, provided that the agonist is removed quickly enough. The time of decay of the current is too short to represent the diffusion time of a water-soluble Ca^{2+}-releasing substance such as inositoltrisphosphate (IP_3, see following section) out of the cell. Possible rate-limiting steps for this decay are the elimination of IP_3 by endogenous phosphatases or the rate of inactivation of the phosphodiesterase (phospholipase C) which produces IP_3.

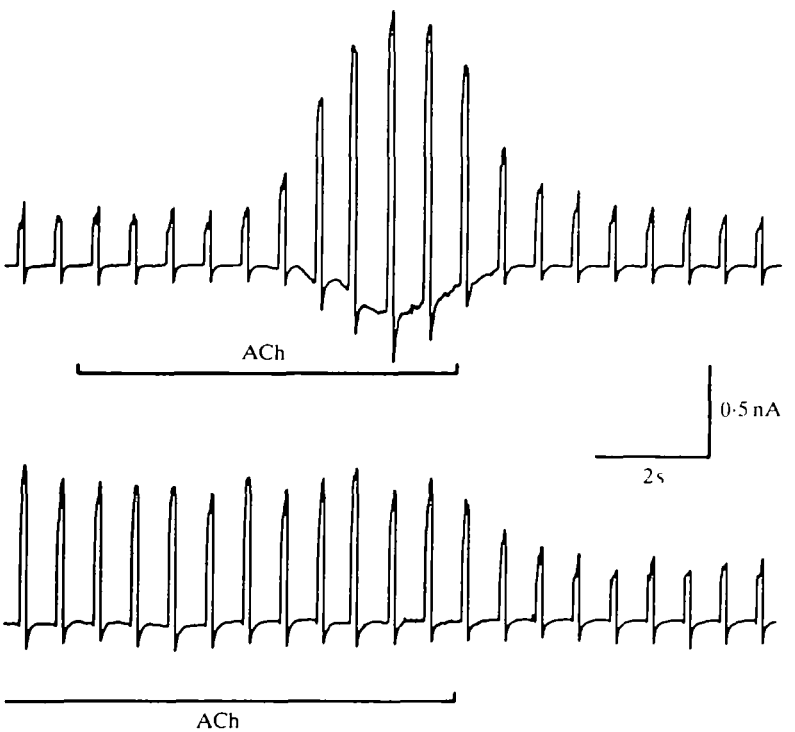

Fig. 8. Return of the response to baseline. Upper trace: response to a short application of acetylcholine (ACh) (1 μmol l^{-1}). The response starts after a 3 s delay, and returns to baseline within 1 s upon washout. 40 s after the end of the upper trace, ACh was applied again for 25 s. The lower trace shows the cell current during the end of this application and the return of the trace to baseline. The K^+ currents returned to control approximately as an exponential with a time constant of 0·7 s. Whole-cell recording as in Figs 5 and 6. Two microperfusion systems were used to ensure a rapid removal of ACh. In this figure, inward tails observable after voltage jumps were partly due to uncompensated capacitive transients.

MECHANISM OF Ca^{2+} RELEASE

Previous work on permeabilized pancreatic cells and on isolated cellular fragments suggests that the endoplasmic reticulum is the store from which Ca^{2+} is released, and that 1,4,5-inositoltrisphosphate (IP_3) is the agent directly responsible for Ca^{2+} release (Streb, Irvine, Berridge & Schulz, 1983; Prentki *et al.* 1984). When testing the effects of IP_3 in our preparation, we found a rapid and sustained activation of Ca^{2+}-dependent channels (Evans & Marty, 1986*a*). Furthermore, we examined the effects of various nucleotides on the ACh response and found that dialysis with GTPγS, a non-hydrolysable analogue of guanosine triphosphate, is able to potentiate the effects of ACh (Evans & Marty, 1986*a*). In addition, GTPγS alone was able to elicit occasional waves of Ca^{2+}-dependent currents. These results confirm the role of IP_3 as a Ca^{2+} releasing factor in the present preparation, and they provide evidence that a GTP-binding protein is involved in the production of IP_3. The latter suggestion is in line with earlier findings on insect salivary glands and in blood platelets (Litosch, Wallis & Fain, 1985; Cockcroft & Gomperts, 1985).

It was shown recently that in the presence of GppNHp, another non-hydrolysable analogue of GTP, the muscarinic activation of K^+ currents in the heart becomes irreversible (Breitwieser & Szabo, 1985). The same is true of the decrease of Ca^{2+} currents elicited by ACh in the same preparation. It was furthermore shown that the K^+ current response is potentiated by GTP and blocked by pertussis toxin, an inhibitor of one of the G proteins characterized so far (Pfaffinger *et al.* 1985). Taken together, the results obtained in the heart and in exocrine glands suggest that all muscarinic responses may involve the activation of a G protein as an early step occurring next to the agonist–receptor binding. Some basic differences may, however, be noted between the two preparations. In the heart, non-hydrolysable analogues of GTP are ineffective in the absence of muscarinic agonists, whereas GTPγS elicits Ca^{2+}-dependent currents by itself in exocrine glands. Second, GppNHp uncouples the receptor from the response in heart muscle cells, whereas Ca^{2+}-dependent currents may still be regulated by ACh in the presence of GTPγS in exocrine glands. The exact molecular origin of these differences has yet to be characterized.

CONCLUSION

During the past 4 years, application of new electrophysiological methods has led to a detailed description of muscarinic responses in exocrine glands. The work was first devoted to characterization of the molecular components of the conductance modulated by ACh. Attention is now shifting to the kinetic properties of the response and to the chain of events taking place in the membrane, in the cytosol and in the endoplasmic reticulum, which links the agonist–receptor interaction to Ca^{2+} release. Application of patch-clamp techniques to these problems will undoubtedly be difficult since the accessible measurements (cell currents) are the final consequence of a complex set of reactions, which cannot easily be manipulated individually.

Fortunately, a reasonable working hypothesis may be put forward for this chain of events since it is now realized that Ca^{2+} release is the final consequence of a receptor–protein G interaction and of the subsequent production of IP_3 by phospholipase C. As expected from this broad scheme, it is possible to stimulate Ca^{2+}-dependent currents by dialysing the cell with GTPγS or with IP_3. These new developments show that it is possible to enter the system at various levels, and they indicate what could be a step-by-step approach to the intracellular events put into play by muscarinic agonists.

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