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THREE TYPES OF CALCIUM-DEPENDENT CHANNEL IN RAT LACRIMAL GLANDS

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SUMMARY

1. Isolated cells from rat lacrimal glands were studied with patch-clamp techniques. Whole-cell and cell-attached recordings were obtained while the cells were stimulated by application of carbamylcholine or of the Ca ionophore, A23187. The results were compared with recordings of Ca-dependent channels obtained in isolated patches.

2. Whole-cell recordings revealed two types of carbamylcholine-induced current. At low levels of stimulation, a specific class of Ca-dependent K channels was selectively activated ('BK channels'). With more intense stimulation an inward current, I_i, was obtained at the cell resting potential. I_i rose rather abruptly after a long delay. In several cells, I_i currents presented spontaneous oscillations.

3. Both K and I_i current responses to carbamylcholine were due to activation of muscarinic receptors. Both responses were elicited by a rise of the intracellular Ca concentration. The immediate source of Ca was intracellular.

4. Replacement of intracellular K with either Na or Cs blocked BK channels entirely, thus allowing the study of I_i currents free from K currents. I_i responses to carbamylcholine were, however, less frequently obtained in Na- or Cs-dialysed cells than in K-dialysed cells.

5. In symmetrical NaCl solutions, I_i inverted at 0 mV. When replacing part of the intracellular or extracellular Cl with glutamate the reversal potential, E_i, was found to vary in the same direction as the equilibrium potential for Cl ions, E_{Cl}. In some experiments, E_i was close to E_{Cl} but in others E_i deviated strongly from E_{Cl}. These experiments suggested that I_i was mainly due to a Cl-selective conductance, and that another conductance type was contributing to I_i in variable proportions. It was found that, in K-free solutions, I_i had a reversal potential very close to E_{Cl}.

6. Noise analysis showed that the Cl channels involved in I_i current had a unit conductance of about 1–2 pS in symmetrical NaCl solutions. At −60 mV, the mean channel open time derived from noise power spectra was about 200 ms.

7. The activation of the Ca-dependent Cl channels was increased by depolarization. Voltage jumps elicited slow exponential relaxations. At −60 mV, the time constants of the relaxations were in the range 100–250 ms.

8. Cell-attached recordings suggested that internal Ca activated three types of

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channel, depending on the Ca concentration: BK channels, 2–4 pS channels and 25 pS channels. Inside-out and outside-out patch conditions allowed a rough estimate to be made of the Ca concentration needed to activate each class of channel. The 25 pS channels appeared to be cation selective and not to discriminate between Na and K.

9. Taken together, the results from whole-cell and from patch experiments allow the characterization of three types of Ca-dependent channel, one selective for K (BK channels), one selective for Cl, and one selective for cations. In addition, the results of point 5 above indicate the presence of a fourth type of Ca-dependent conductance, which is presumably cation selective and sensitive to external K ions.

10. Possible roles of the different classes of Ca-dependent channel in fluid secretion are discussed.

INTRODUCTION

Exocrine glands such as exocrine pancreas, salivary glands and lacrimal glands secrete both proteins and fluid. The exact mechanism underlying water and salt secretion is far from being understood, but it is probably relevant that secretory agents induce permeability changes in acinar cell membranes (for reviews, see Petersen, 1980; Ginsborg & House, 1980). Similar permeability changes may be elicited by intracellular injection of Ca ions in mammalian salivary glands (Iwatsuki & Petersen, 1978*a*) and in pancreatic acini (Iwatsuki & Petersen, 1977*a*). Likewise, application of a Ca ionophore elicits ion fluxes in mammalian salivary gland cells similar to those obtained with secretory agents (Selinger, Eimerl & Schramm, 1974). Thus, it appears that secretory agents (such as acetylcholine or noradrenaline) use intracellular Ca as a second messenger to elicit the electrical responses (Petersen, 1980; Ginsborg & House, 1980).

The ions involved in the electrical responses have not been unambiguously identified. In many cases, an increase in K permeability has been demonstrated. The best-characterized example is found in insect salivary glands as reviewed by Ginsborg & House (1980). In most preparations, however, voltage responses are complex and fail to present a unique reversal potential. Thus, it is likely that at least two permeability systems are involved, one selective to K ions, and the other possibly involving Na ions (see the above reviews).

Recently, specific ionic channels have been identified in exocrine gland cells by using patch-clamp recording techniques. Ca-dependent channels permeable to both Na and K ions have been found in pancreas acinar cells (Maruyama & Petersen, 1982*a*); Ca-dependent K channels were found in mouse and rat salivary glands (Maruyama, Gallacher & Petersen, 1983), in rat lacrimal glands (Trautmann & Marty, 1984) and in pig exocrine pancreas (Maruyama, Petersen, Flanagan & Pearson, 1983).

The first class of channels was shown to be activated by cholecystokinin in pancreatic cells (Maruyama & Petersen, 1982*b*). Recently, it was demonstrated that the second class of channels (called 'BK channels', for *B*ig unitary conductance *K* channels) is activated by acetylcholine or carbamylcholine in rat lacrimal glands (Trautmann & Marty, 1984). In the latter study, it was shown that whereas low carbamylcholine doses exclusively activate BK channels, larger agonist concentrations also induce an inward current flowing through an independent permeability system. In the present work, this inward current is examined in detail. We found to

our surprise that, provided that the stimulus is not too strong, the inward current flows mostly through Cl-selective channels. These channels are voltage- and Ca-sensitive. They present an unusually small unitary conductance. Two additional Ca-dependent permeability systems also contribute to the inward current. The first type presents a very small current noise, and depends for its activation on extracellular K. The second type resembles the Ca-dependent cation-selective channel described in other preparations (Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982), including exocrine pancreas (Maruyama & Petersen, 1982*a*, *b*).

METHODS

Cell dissociation

Cells were isolated from freshly dissected rat exorbital lacrimal glands as described previously (Kanagasuntheram & Randle, 1976; Herzog, Sies & Miller, 1976).

Isolated cells were placed on culture dishes (Falcon) and kept in culture medium (M 199) in an incubator until use. The cells were found to adhere readily to the dishes provided that the density was not too great, and that serum was not added to the medium.

Since cells loose their sensitivity to cholinergic agonists in a matter of hours (see Results), we always used them within 12 h after plating.

Electrical recording

Standard patch-clamp methods were used (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Drugs were applied with a fast microperfusion technique (Krishtal & Pidoplichko, 1980; Fenwick, Marty & Neher, 1982). The effective exchange time at the onset of a drug application was estimated to be around 0·2 s.

All experiments were carried out at room temperature (20–25 °C).

Noise analysis

Variance analysis of whole-cell currents elicited by the Ca ionophore was complicated by slow variations of the mean current. Samples (1–10 s long) of non-stationary current were fitted by eye with tilted straight lines. Mean variance over each segment was calculated as

$$v = 1/N \sum_{1}^{N} (x_i - a_i)^2,$$

where N is the number of points in a segment, x_i is the experimental value of point number i, and a_i is the corresponding ordinate of the model straight line. Provided that the channels have a low probability of opening (a hypothesis consistent with the shape of the variance–mean current curve), v is an approximation of the variance corresponding to the mean current I calculated over the noise segment. Under the same assumption of low opening probability, the ratio v/I gives an estimate of the elementary current for a homogeneous population of channels (see Neher & Stevens, 1977).

The above procedure resulted in an underestimate of the variance both at low and at high frequencies. The variance loss was estimated using noise power spectra calculated in favourable cases where the sample duration could be set at 10 s. The spectra were fitted to a single Lorentzian curve with a cut-off frequency f_c. From the value of f_c (around 1 Hz), it appeared that the power loss at high frequencies was negligible. At low frequencies, the procedure used to calculate v effectively reduced the variance by a factor of 2 at a frequency f_s close to the inverse of the sample length (i.e. 0·1–1 Hz). This was corrected for according to

$$v/v_{th} = 1 - 2/\pi \,.\, \arctan\,(f_s/f_c), \tag{1}$$

where v_{th} is the corrected variance value. The validity of this correction was tested as shown in Fig. 1. Variance analysis was performed on samples of variable durations from a record of ionophore-induced current. As expected, the calculated elementary current decreased as the sample length was reduced. A noise power spectrum obtained from the same data (Fig. 10 below) yielded an f_c value of 0·69 Hz, which was used to draw the curve corresponding to eqn. (1) in Fig. 1. The

agreement between experimental points and theoretical curve indicates that the correction of eqn. (1) is adequate.

Some patch-current records (e.g. those illustrated in Fig. 12 below) were analysed using Sigworth's (1980) 'ensemble analysis' method. Briefly, averages of successive current responses to voltage jumps were obtained, and the deviations of individual traces around the mean were analysed. The mean variance corresponding to these variations was plotted as a function of the mean current, and the resulting plot was fitted with a parabola, from which the elementary current amplitude and the number of channels present in the patch were calculated.

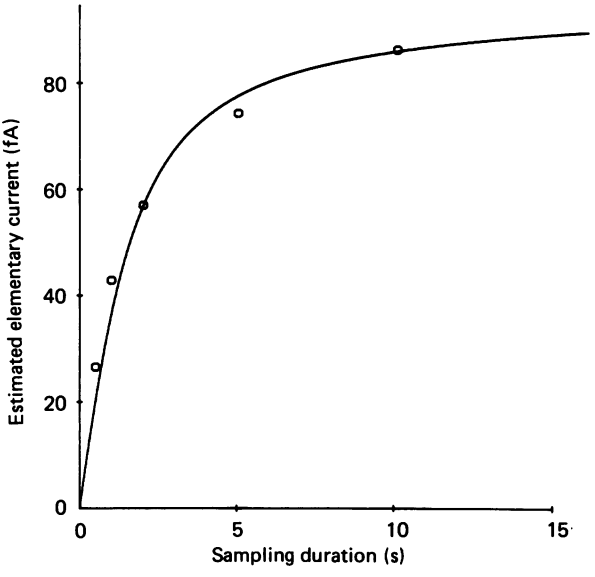

Fig. 1. Effect of sample length on calculated elementary current. The points represent elementary current values derived from current–variance plots on the same data, but with variable sample lengths (0·5, 1, 2, 5 and 10 s). The curve gives the theoretical relation between the estimated elementary current and the sample length, according to eqn. (1). The cut-off frequency of the spectrum established on the same data was 0·69 Hz (Fig. 10, below).

Solutions

Unless otherwise stated, the external solution contained (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1·2; HEPES–NaOH (pH 7·2), 5. The internal solution used in the study of whole-cell potassium currents contained (in mM): KCl, 140; EGTA (ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid), 0·5; $MgCl_2$, 2; HEPES–KOH (pH 7·2), 5. The EGTA concentration of this solution was empirically determined to meet two requirements. On one hand, some intracellular Ca buffering was needed in order to obtain stable currents in unstimulated cells. On the other hand, the Ca-buffering capacity had to be low so that a significant increase of the intracellular Ca concentration, $[Ca]_i$, could be elicited when stimulating the cell with carbamylcholine or with the Ca ionophore. Blockage of the K currents was generally achieved with an internal solution containing (in mM): NaCl, 140; EGTA, 0·5; $MgCl_2$, 2; HEPES–NaOH, 5.

Experiments on isolated patches used similar internal solutions. Between 10 nM and 1 μM-Ca, a Ca–EGTA buffer was employed (total EGTA concentration around 2 mM), and the free-Ca concentration was calculated supposing an apparent Ca–EGTA dissociation constant of 10^{-7} M (Caldwell, 1970). Between 1 and 10 μM-Ca, HEDTA (*N*′-(2-hydroxyethyl) ethylenediamine-*N*,*N*,*N*′-triacetic acid) was preferred to EGTA, because of its higher apparent dissociation constant (2·5 μM). In HEDTA-buffered solutions, Mg was omitted, in order to avoid complications due to Mg–HEDTA binding. Above 10 μM, Ca was not buffered.

In all cases where the pipette and bath solutions were different, a junction potential arose at the pipette tip. This potential was measured as described before (Fenwick *et al.* 1982) and was corrected for. The Ca ionophore, A23187, was purchased from Sigma and was stored as a stock solution at 2 mM in methanol.

RESULTS

(*1*) *Inward and outward currents induced by carbamylcholine*

It was shown in a previous work that low doses of carbamylcholine (CCh; 0·5 μM) exclusively activate K currents in our whole-cell recording conditions, whereas larger concentrations (≥ 1 μM) also induce an inward current, called I_i hereafter, at holding potentials of −50 to −80 mV (Trautmann & Marty, 1984). It was mentioned that the responses were blocked by atropine and by internal EGTA, but that they remained in the absence of external Ca. We found in the present experiments that atropine eliminated entirely both outward and inward currents at a concentration of 10 nM. Thus, both types of current depend on the activation of muscarinic receptors. Secondly, we confirmed that the *two* types of current were blocked by increasing the concentration of EGTA in the cell from 0·5 to 5 mM. In one preparation, 2 μM-CCh elicited large (≥ 200 pA) I_i currents at −60 mV in three out of four control cells; all control cells displayed large CCh-induced outward currents at 0 mV. Out of three cells dialysed with a 5 mM-EGTA internal solution, one displayed only a weak outward current response at 0 mV (but none at −60 mV), and the other two failed to respond to CCh entirely. It therefore appears that both inward and outward currents depend on intracellular Ca. An example of a response to 2 μM-CCh obtained with the standard internal solution is shown in Fig. 2*A*. Also shown is the lack of response of a cell dialysed with the 5 mM-EGTA solution (Fig. 2*B*). Finally, Fig. 2*C* illustrates normal inward and outward current responses obtained after replacement of external Ca ions with EGTA, showing that the Ca ions involved in the activation of both response types come from intracellular stores.

It was previously shown that in a given cell, the responses decreased sharply when the same CCh concentration was applied repeatedly, even if rather long rest intervals were given between applications (Trautmann & Marty, 1984). This effect was confirmed in the present investigation, particularly with the inward current. In many cases, I_i was totally absent in the second or third CCh trial, even though the previous application had evoked a large (≥ 200 pA) inward current. Some activation of the outward current was still observed at this stage. The decrease of the response to CCh imposed limitations on the extent of quantitative analysis which could be performed on a given cell. A second difficulty was that the cells lost their sensitivity to CCh in our culture conditions after a relatively short period of time, in the range of 5–20 h. Finally, some cell preparations failed to respond to CCh altogether, presumably as a result of damage during the dissociation. Nevertheless, the responses obtained in freshly dissociated cells of a healthy preparation were quite consistent. Thus, in the series presented in Table 1, only one out of twenty control cells failed to respond to CCh.

To study the properties of I_i without complications arising from the simultaneous activation of the K current, the latter was blocked by replacing K with either Na or Cs in the recording pipette (Trautmann & Marty, 1984). It was found that Na-

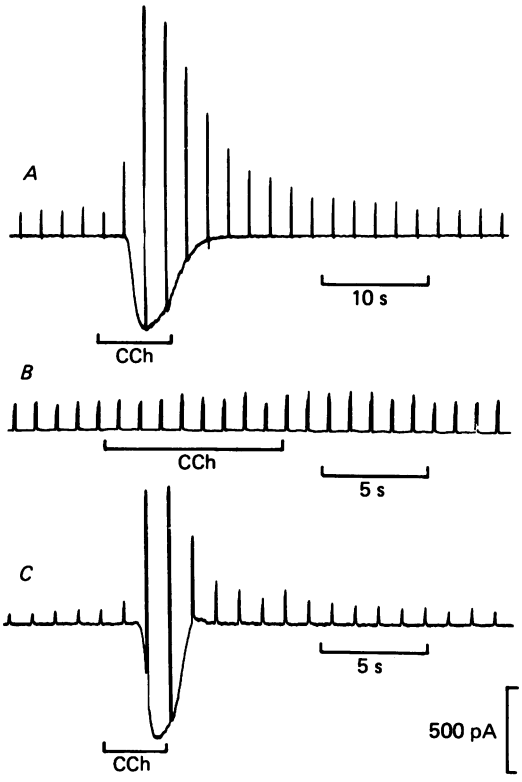

Fig. 2. Inward and outward current responses to carbamylcholine. Responses to the application of 2 μM-CCh, from three different cells. *A*, typical response with standard internal solution (high K, 0·5 mM-EGTA). The zero-current level is indistinguishable from that measured at the holding potential, $V_H = -60$ mV, before the CCh application. Positive pulses to 0 mV were applied at 0·5 Hz. The test pulse gave rise to an outward current relaxation which, at 0 mV, can only be attributed to the voltage-dependent activation of K channels. (In this and in other cells, hyperpolarizing jumps of 40–60 mV only elicited small, ohmic current responses of 20–50 pA. The leakage current is therefore negligible on the current scale used. Of the various Ca-sensitive conductances to be described below, all but the K-selective one have a reversal potential of 0 mV under the present ionic conditions.) Following application of CCh, the outward current relaxation grew markedly. In addition, a 600 pA inward current was observed at −60 mV. *B*, lack of response in a cell dialysed with a high-K solution containing 5 mM-EGTA, 60 mV pulses were applied. $V_H = -60$ mV. *C*, normal response in an external solution containing no Ca and 1 mM-EGTA. Both bath and microperfusion solutions contained EGTA instead of Ca. Standard internal solution (0·5 mM-EGTA). 40 mV pulses from $V_H = -45$ mV.

TABLE 1. Responsiveness of cells dialysed with K, Na, or Cs, to the application of CCh

Intracellular cation	K	Na	Cs
Percentage of responses	95	30	20
No. of cells	20	11	10

CCh was applied usually at 2 μM (except for four cells where either 1 or 5 μM was tested). Control cells (dialysed with the K-rich solution) were considered responsive if they displayed both a large outward current (at the test potential) and a large ($\geqslant$ 100 pA) inward current at the holding potential. The same criterion was used for Na- and Cs-containing cells, except that outward currents were then absent. At least 1 min was allowed between establishment of the whole-cell recording and application of CCh to insure good dialysis of the cell interior with the pipette solution.

or Cs-containing cells responded less readily than K-containing cells to the application of CCh: many cells failed to respond, whereas large inward currents were found in the control recordings taken in the same preparations (Table 1). The implications of these observations are that Na and Cs presumably inhibit the Ca-release process (see Discussion). The few cells which did respond displayed CCh-evoked currents which

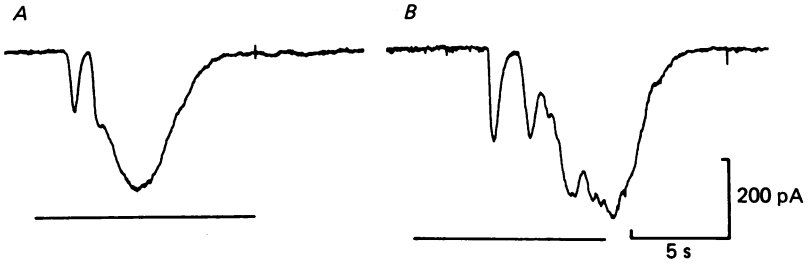

Fig. 3. 'Waves' of inward current responses to carbamylcholine. *A*, inward current response in a Cs-dialysed cell. $V_H = -80$ mV. Before CCh application, the cell presented a high resistance (3 GΩ) at least up to 0 mV, indicating that BK channels were entirely blocked. In the presence of 2 μM-CCh (bar below record), an inward current developed in two successive peaks. The current was back to the base-line level before the end of the CCh application. *B*, example of a complicated 'wave' pattern in a K-dialysed cell. $V_H = -80$ mV. Response to 2 μM-CCh.

were very similar to those observed with K-dialysed cells at the holding potentials used (between −80 and −60 mV). However, no CCh-induced current was observed at 0 mV in Na- or Cs-dialysed cells, indicating that the K channels involved in the control response at this potential were entirely blocked. In many of the responsive Na- or Cs-dialysed cells, successive 'waves' of inward current were observed at the holding potential. Such waves had a highly variable appearance. In several cases, the inward current subsided almost entirely after a prominent wave lasting 1–2 s, and rose again smoothly later on. In other cells, waves were numerous and irregular. Similar fluctuations were also occasionally observed in K-dialysed cells. Examples of wave patterns with an isolated initial maximum and with numerous irregular peaks are illustrated in Fig. 3*A* and *B* respectively. The cells were dialysed with Cs in the first record and with K in the second.

The responses of Figs. 2–3 displayed additional features which were noted previously (Trautmann & Marty, 1984): (1) the inward current presented a delay of about 2 s, (2) activation of the outward current (in K-dialysed cells) presented a shorter delay (Fig. 2*A*), (3) the inward current rose and fell rather abruptly. It often went back to base line before the end of the CCh application (Fig. 3*A*), (4) in K-dialysed cells, activation of the outward current outlasted that of the inward current (Fig. 2*A*).

(2) *Application of a Ca ionophore*

Application of the Ca ionophore A23187 results in an increase of membrane permeability to K ions (Selinger *et al.* 1974). We applied the same compound to isolated lacrimal gland cells and found large inward and outward current responses. Some experiments were performed by diluting the Ca ionophore in standard saline. Alternatively, the Ca concentration was raised to 10 mM (instead of 1 mM) in the test

solution containing the ionophore. The latter procedure was introduced with the aim of reducing the ionophore dose and thus improving the reversibility of the responses. In fact, it was rather difficult to obtain reversible ionophore responses with either 1 or 10 mM-Ca. All experiments were performed with the usual 0·5 mM-internal EGTA. Fig. 4 illustrates the response to the application of 0·1 μM-A23187 in a

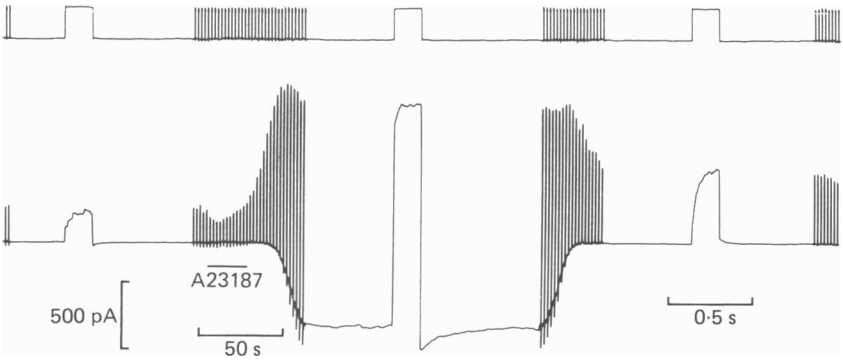

Fig. 4. Response to the application of Ca ionophore. Cell dialysed with standard internal solution (see Methods). $V_H = -60$ mV, test potential -10 mV. The Ca ionophore (0·1 μM) was applied together with 10 mM-Ca during 25 s. The outward current relaxation was somewhat reduced at first by the high Ca. After washing off the Ca ionophore solution, the outward current relaxation increased markedly. A large inward current developed at the V_H after an even longer delay. After prolonged wash, both inward current and outward current returned to the control levels (not shown).

K-dialysed cell. During application of the test solution (containing 10 mM-Ca), the amplitude of the outward current relaxations induced by 60 mV depolarizing pulses was decreased. This effect was observed without any delay whenever a 10 mM-Ca solution was applied, even in the absence of ionophore. Control experiments suggested that it was due to a 6–8 mV shift of the effective membrane potential. This shift was attributed to a reduction of the size of the (negative) surface potential present on the external face of the membrane. The A23187 response proper slowly developed mainly after returning to the standard saline, as shown in Fig. 4, where the amplitude of outward current relaxations starts to increase about 15 s after the onset of the ionophore application. (No such response was observed in the control experiments where 10 mM-Ca was applied without ionophore.)

The outward current relaxations induced by A23187 were in all respects similar to those elicited by low concentrations of CCh. Noise characteristics and activation kinetics of the A23187-induced outward current strongly suggested that this current was due to activation of the K channels involved in the cholinergic response ('BK channels': see Trautmann & Marty, 1984). As in the cholinergic response, the amplitude of the relaxations decreased near the peak of large responses, presumably because a large fraction of BK channels was activated at both holding and test potentials (Fig. 4; compare with Trautmann & Marty, 1984, Fig. 4).

Strong ionophore stimulations also evoked an inward current at the holding potential, which presented many similarities with the CCh-induced inward current illustrated in Figs. 2 and 3. As will become apparent in later sections, both types of inward current responses are due to the same ionic channels, and are therefore both

termed I_i. The fact that I_i may be activated by the ionophore provides additional evidence that the I_i current is due to a rise in the intracellular Ca concentration, $[Ca]_i$. Since the outward current rises sooner and decays later than the inward current (Figs. 2 and 4), and since low doses of either CCh (see Trautmann & Marty, 1984) or ionophore (results not shown) only elicit the outward current, it appears that this current is activated by lower Ca concentrations than the inward current.

Ionophore-activated I_i current rose after a long delay, ranging from a few seconds to several tens of seconds (this delay was about 35 s in Fig. 4). No 'waves' were associated with this current. Nevertheless, it was observed on many occasions that the current amplitude was difficult to modulate; once the ionophore had been applied long enough to obtain some I_i current, the response was usually seen to develop to several hundred pA. Also, rather abrupt increases were occasionally observed during the rise of I_i currents. These observations, as well as the 'waves' of Fig. 3, suggest the presence of a positive feed-back mechanism in the regulation of $[Ca]_i$.

Due to the slow time course of ionophore-induced I_i responses and to the lack of waves in the records, it was more convenient to perform noise and voltage-jump relaxation analysis on the ionophore-induced current than on the CCh-induced current. In addition, Na-dialysed cells, which responded badly to the application of CCh (Table 1), presented large inward current responses to the application of the Ca-ionophore. It was thus much easier to study the inward current free from currents carried by BK channels when stimulating the cell with the ionophore than with CCh. The next sections describe quantitative studies of the response to the ionophore in Na-dialysed cells.

(3) Ionic selectivity

The I_i current induced by the ionophore in Na-dialysed cells inverts near 0 mV (Fig. 7 below). The same reversal potential was also found for the CCh-induced currents of Na- or Cs-dialysed cells. Such a value is expected either if the channels present a cationic selectivity without discriminating between Na and Cs ions, or if the channels are Cl-selective.

To test the latter possibility, I_i currents were measured under conditions where cations (mostly Na) were at equilibrium at 0 mV whereas the Cl equilibrium potential, E_{Cl}, was shifted by replacing a fraction of either the internal or the external Cl by glutamate or isethionate. These experiments were all performed on currents induced by A23187 in Na-dialysed cells. Voltage jumps to various test potentials were applied first in control conditions, and then again during an ionophore-induced response. I_i currents were obtained by subtracting the control traces from the records during the response. Instantaneous I–V curves were constructed by measuring I_i within 5 ms after the voltage jumps. The intersect between the instantaneous I–V curve and the voltage axis was taken as the reversal potential of the I_i current, E_i. Two examples of instantaneous I–V curves obtained in this manner are shown in Fig. 5. In the first cell 90 % of the internal Cl was replaced by glutamate. The ionophore-induced current presented a reversal potential of -46 mV, close to E_{Cl} (-55 mV). The other I–V curve is from an experiment where 75 % of the external Cl was replaced by isethionate. I_i reversed here at $+29$ mV, again close to the value of E_{Cl} ($+35$ mV).

These results indicate that Cl-selective channels contribute to a large extent to the I_i current. This conclusion is further supported by the curvatures of the instantaneous

I–V curves shown in Fig. 5. The curvatures are not very pronounced, but they are both in the direction expected if Cl ions carry the I_i current; the conductance is lower when Cl crosses the membrane from the compartment of lower Cl concentration to the compartment of higher Cl concentration.

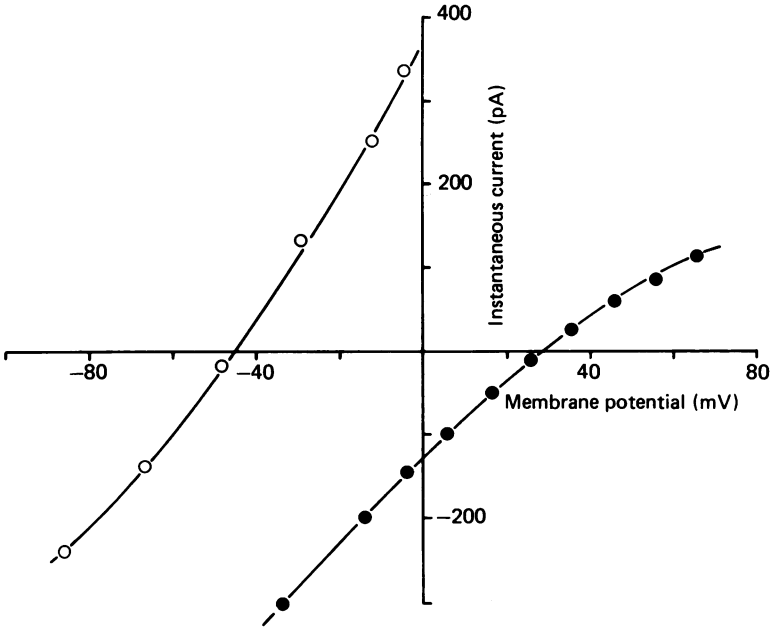

Fig. 5. Rectification of instantaneous I_i current in asymmetric Cl solutions. Instantaneous I_i current was measured within 5 ms after stepping the potential to various levels. Control currents obtained before ionophore application were subtracted. An appropriate scaling of the currents was applied to compensate for the variations of the holding I_i current during the run. Na-filled pipettes. Open circles, cell containing 17 mM-Cl (plus 127 mM-glutamate) in a 149 mM-Cl bath solution. Filled circles, cell containing 144 mM-Cl, bathed in 37 mM-Cl (plus 112 mM-isethionate). The reversal potentials were -46 mV ($E_{Cl} = -55$ mV) and $+29$ mV ($E_{Cl} = +35$ mV) respectively. Tip diffusion potentials (see Methods) and series resistance artifacts were corrected for.

The results obtained for five different E_{Cl} values are shown in Fig. 6. Each filled circle represents an estimate of E_i in one cell. As may be seen, E_i varies roughly as E_{Cl}. However, E_i was found in all cases to have a value that lay between 0 and E_{Cl}, and the results presented a large scatter.

It was unattractive to assign the difference between E_i and E_{Cl} to a partial permeability of the channels for cations, because of the large cell-to-cell variability. In addition, it was found in many cases that E_i was shifted towards 0 mV as the response developed (in such cases, it is the early E_i value which is represented in Fig. 6). These observations suggested that the discrepancy was due to contamination by a separate type of cation-selective conductance. In some cells, a pronounced noise increase accompanied the departure of E_i from E_{Cl}, and the contaminating conductance was probably due to the 25 pS channel observed in isolated patches (see below, Fig. 13). In most cases, however, the elementary conductance value calculated from

noise analysis (see below, Fig. 9) was in the range 1–2 pS, even when marked differences between E_{Cl} and E_i were obtained. Yet another Ca-dependent permeability seems necessary to account for these observations. This permeability was not studied in detail, but two lines of evidence indicated that it was much reduced by replacing with Na the 5 mM-K normally present in the bath solution. First, experiments

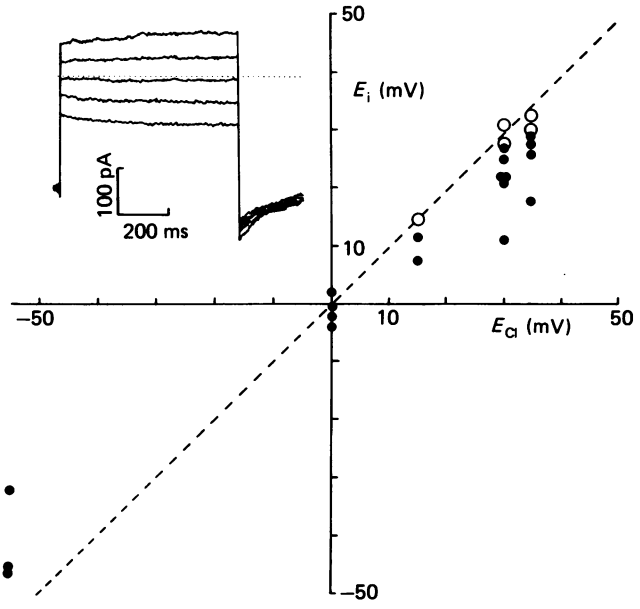

Fig. 6. Reversal potential of I_i current (E_i) as a function of E_{Cl}. Filled circles, points obtained with normal external K concentration (5 mM). Open circles, points obtained in K-free solutions. Dashed line, prediction for a purely Cl-selective conductance. Insert, responses to voltage jumps of ionophore-induced current. Test potentials ranged between +7 and +47 mv in 10 mV steps. Outer solution, K-free, low-Cl, high-glutamate solution (E_{Cl} = +30 mV). Reversal potential E_i = +29 mV. One of the traces has been scaled up by 10% to compensate for a slow drift of the current at the holding potential (−33 mV). The currents shown are differences between traces obtained after ionophore application (0·4 μM, in 1 mM-Ca) and control traces.

performed under such conditions gave E_i values very close to E_{Cl} (open circles in Fig. 6). Secondly, in another series of experiments, the effect of K-free saline was tested on cells dialysed with a NaCl solution containing 10 or 100 μM-Ca. The cell resistance, which is in the GΩ range if the internal Ca is low, was found to fall under these conditions below 100 MΩ within the first few minutes of whole-cell recording. The admission of a K-free solution elicited a marked and reversible increase of the cell resistance (by 30–190%; three experiments).

The K-free experiments strongly suggest that the deviations between E_i and E_{Cl} are due to contamination by a separate Ca-dependent permeability system. This conductance has a low noise level; it is presumably cation selective (since it pulls E_i towards E_{Na}); and it is sensitive to external K ions. In K-free solutions, deviations between E_i and E_{Cl} are less than 10%. This allows an upper limit to be given of the permeability ratio for cations *versus* anions, P_C/P_A, for the anionic channels

contributing most to I_i. Application of the Goldman–Hodgkin–Katz equation to the points in K-free saline at $E_{Cl} = +35$ mV gives $P_C/P_A \leqslant 0{\cdot}03$. In 5 mM-K solutions, roughly 25 % of the total conductance appeared to be due to the additional cationic conductance.

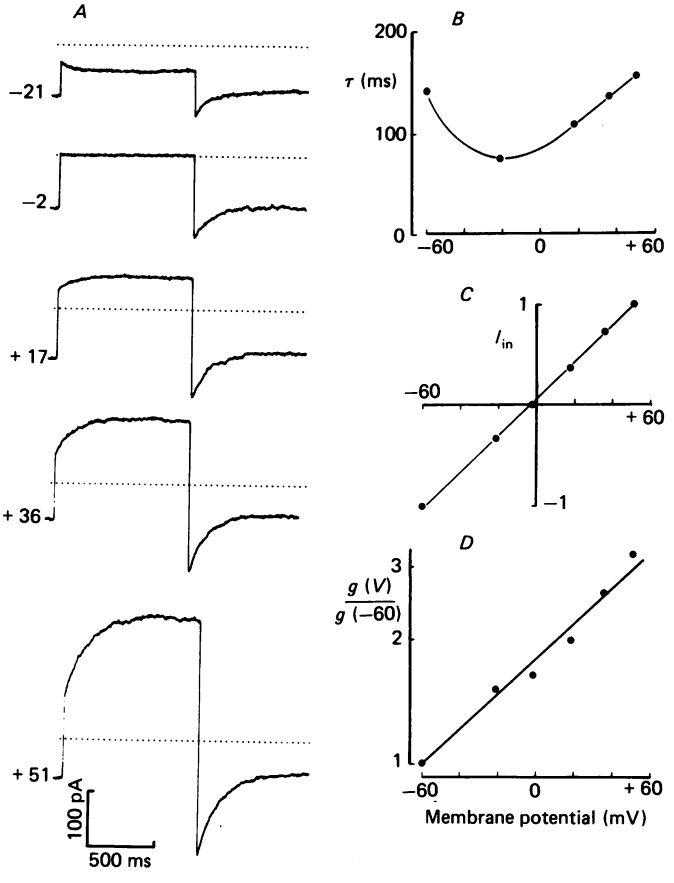

Fig. 7. Relaxation of I_i current. *A*, responses to voltage jumps applied from −60 mV following the application of 0·2 μM-A23187 and 10 mM-Ca. Na-dialysed cell; symmetrical Cl concentrations. Control currents were identical before ionophore application and after complete wash-out. They were subtracted from the traces shown. Test potentials are indicated for each sweep. Dotted lines indicate zero-current levels. *B*, relaxation time constant as a function of membrane potential. *C*, instantaneous current (I_{in}, arbitrary units) as a function of membrane potential. *D*, ratio of steady-state conductance at the test potential, V, to that at −60 mV, as a function of V. Logarithmic vertical scale.

(4) Kinetic properties of the Ca-induced inward current

Fig. 7*A* presents the results obtained when giving positive voltage pulses during an I_i response elicited by the application of 0·2 μM-ionophore in a Na-dialysed cell. I_i reversed near 0 mV. The voltage jumps elicited slow relaxations which could be fitted to single exponentials. In the traces shown, the corresponding time constant, τ, increased from 75 to 160 ms as the test potential was increased from −21 to

+51 mV. The off relaxations seen upon returning to the holding potential were also fitted to single exponentials (see example of a fit in Fig. 8). The time constants of the off relaxations were found not to depend strongly on the test voltage. They were close to 140 ms in the experiment of Fig. 7. The variation of τ as a function of potential is shown in Fig. 7*B*.

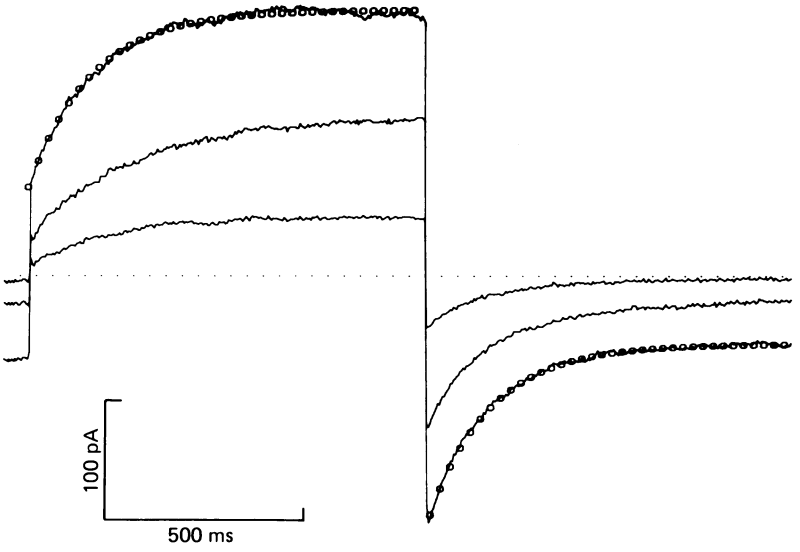

Fig. 8. I_i current relaxations for different mean currents. Current responses to identical voltage jumps (test potential = +51 mV) during recovery after A23187 application. Same experiment as in Fig. 7. Mean currents at −60 mV were 73 pA (single sweep), 24 pA (average of two traces), and 7 pA (average of four traces). Control currents have been subtracted. Zero-current level indicated by dots. Exponential fits are shown for the largest current. τ (+51) and g (+51)/g (−60) increase as the mean current at −60 mV decreases.

At each potential jump, an 'instantaneous' current value may be calculated by back-extrapolation of the exponential relaxation to the start of the jump. Instantaneous current ratios compared at the beginning and end of positive jumps were found to be identical. The instantaneous current curve constructed as a function of test potential was linear, as shown in Fig. 7*C*. Because of the linearity of the instantaneous *I–V* curve, the ratio of steady-state current to instantaneous current measured at a test potential V is equal to the ratio of the steady-state conductance at potential V, $g(V)$, over that obtained at the hold potential. In the experiment of Fig. 7, the $g(V)$ curve constructed with this method could be fitted with an exponential varying by an e-fold factor in 106 mV.

It was found that the exact value of τ, as well as the steepness of the $g(V)$ curve, were dependent on the level of the current. This is illustrated in Fig. 8, which shows results of potential jumps during recovery of the current for the experiment of Fig. 7. As the mean current decreased, values of $\tau(+51)$ increased and the $g(V)$ curve got steeper. The ratio between $g(+51)$ and $g(-60)$ for the lowest current shown corresponds to an e-fold variation in 50 mV. At +51 mV, τ values were 155 ms for the largest current (73 pA at −60 mV) and 215 ms for the smallest (7 pA at −60 mV).

At −60 mV, on the other hand, τ seemed almost independent of the current amplitude in this particular experiment.

In another experiment, a clear *acceleration* of the off relaxation was observed at the resting potential as the current decreased; at the same time, the kinetics of the off relaxation departed from a single exponential. Non-exponential relaxations were also found in other experiments, indicating that the channel opening obeys simple two-state kinetics only as a first approximation.

The results of Fig. 8 suggest that the level of $[Ca]_i$ exerts an influence on the kinetics of the I_i current. This may be one of the reasons why values of τ presented a rather large scatter. At potentials between −65 and −50 mV, τ values ranged between 100 and 250 ms. The corresponding mean value was 195±70 ms (mean ±S.D., five experiments).

Small voltage-dependent Ca currents were often observed under the ionic conditions of Fig. 7 in the control runs (before drug application). These currents will be described in a future publication. Since CCh increases the rate of labelled Ca uptake in lacrimal glands (Keryer & Rossignol, 1976), it seemed possible that Ca currents would be affected by the ionophore. The subtraction procedure used to obtain I_i would then clearly not be justified. However, no kinetic component corresponding to the rate of deactivation of Ca currents (which occurred within 10–20 ms) was apparent in the control-subtracted I_i relaxations measured after returning to the holding potential. Thus, voltage-dependent Ca currents did not seem to be markedly changed following stimulation by the ionophore. Ca currents may also have affected the time course of I_i currents by transiently raising $[Ca]_i$. This second effect, if present, could not account for *all* of the voltage sensitivity of I_i currents since (1) relaxations were observed in potential ranges where Ca currents were negligible (when stepping the potential between −100 and −30 mV, or raising the test voltage above +20 mV), and (2) one experiment performed in a Ca-free bath solution containing 0·5 mM-EGTA gave normal-looking relaxations. It is nevertheless possible that, in the usual 1 mM-Ca solution, the values of τ measured for test potentials ranging between −20 and +20 mV, as well as the value obtained for the off relaxation, were to some extent affected by Ca entry through voltage-dependent channels.

As the relaxations described in Figs. 7–8 were obtained in standard saline, the possibility that the measurements may have been affected by the K-sensitive conductance described in section (*3*) was considered. The fact that the slow relaxations reversed near E_{Cl} in asymmetrical Cl concentrations, as illustrated in Fig. 6, demonstrated that the underlying conductance was indeed Cl selective. On the other hand, no time-dependent current appeared to reverse near 0 mV in the cases where E_{Cl} deviated from E_i. This indicates that, if a K-sensitive conductance contributes to I_i, it does not have a marked voltage sensitivity, and thus is not likely to have introduced large errors in the measurement of Cl-current relaxations, except perhaps on steady-state current values.

(*5*) *Noise analysis*

I_i current induced either by A23187 or by micromolar concentrations of CCh was accompanied by a small noise increase. The relation between noise variance and mean current provides an estimate of the elementary current (i_{el}) under the hypothesis

that the response results from the opening of a homogeneous population of channels independent of each other (Katz & Miledi, 1972; Neher & Stevens, 1977). The major difficulty encountered in measuring fluctuations associated with the I_i current was to eliminate effectively other noise sources. Contamination by K currents could be avoided by dialysing the cells with Na, but in this situation, few cells were responsive

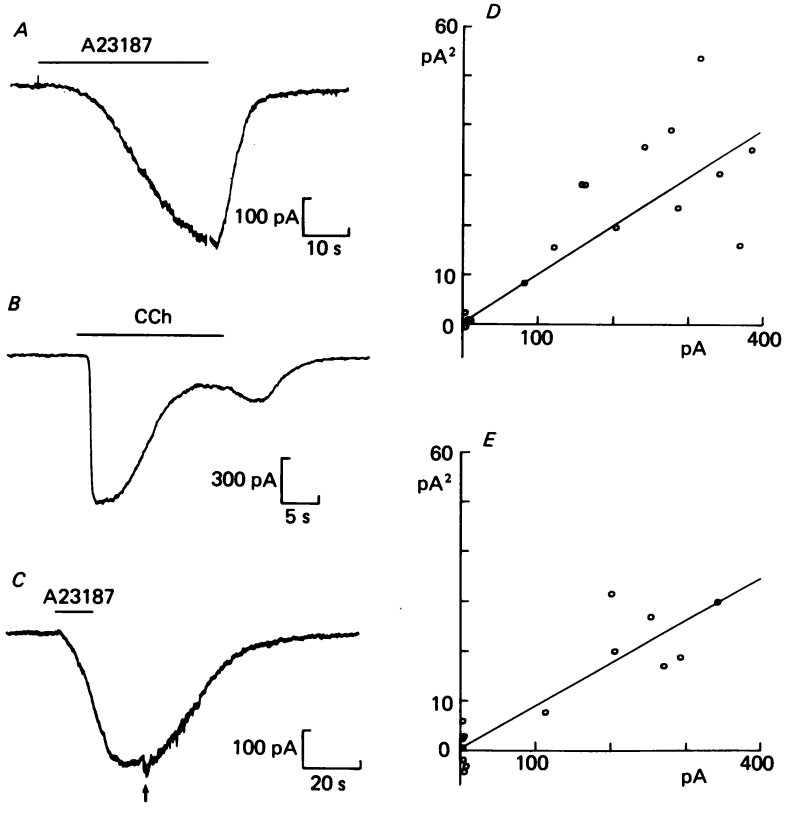

Fig. 9. Variance analysis of I_i current. *A*, current induced by the application of 0·4 μM-A23187 (plus 10 mM-Ca) in a cell held at −80 mV with a K-filled pipette (the short gap near the end of the record corresponds to an artifact due to the perfusion system). *B*, current induced by the application of 2 μM-CCh in another cell held at −83 mV with a K-filled pipette. *C*, current induced by 0·2 μM-A23187 (plus 10 mM-Ca) in a cell held at −60 mV with a Na-filled pipette. Note (arrow) the sudden increase of noise accompanied by a small increase of current. *D* and *E*, the variance of the records shown in *A* and *B* have been plotted in *D* and *E* respectively for a current range of 0–400 pA. The variance was measured as indicated in Methods on 2 s long segments sampled at 500 Hz. The average variance from control runs was subtracted. The slopes of the regression lines give elementary currents of 0·09 (*D*) and 0·08 pA (*E*).

to CCh (see above). Alternatively, K-filled pipettes were employed, and the potential was held close to the K equilibrium potential (−84 mV in the standard ionic conditions). Two other noise sources were occasionally encountered, which precluded the use of the records for I_i noise analysis. In some experiments, a sudden noise increase appeared in the course of an I_i response, presumably because of the activation of the 25 pS cationic channels to be described below. An example of such

a recording is shown in Fig. 9 *C*. In other cases, specially in the presence of CCh, I_i presented slow fluctuations ('waves') presumably due to changes of $[Ca]_i$ (see Fig. 3 above). Finally, the K-sensitive conductance may have affected the noise results to an unknown extent. As a control, occasional voltage jumps were given during noise experiments to test for the presence of slow relaxations, in order to ensure that a large part of the I_i current recorded was actually flowing through Cl-selective channels.

Fig. 9 illustrates various examples of I_i-associated fluctuations. The top record (Fig. 9 *A*) shows a response to the application of 0·4 μM-ionophore. I_i developed slowly and recovered rapidly upon washing. As shown in Fig. 9 *D* the relation between variance and mean current may be fitted with a straight line in the range of current studied (0–400 pA). In the experiment shown the i_{el} value derived from the slope of the line is 0·09 pA. When corrected for the limited band width used in the variance analysis (see Methods), this value becomes 0·14 pA. Assuming a reversal potential of 0 mV under the ionic conditions of the recording, as justified by the results of Fig. 6, the elementary conductance of the channel turns out to be 1·8 pS. From six such experiments, the amplitude of the conductance of the elementary I_i current activated by the ionophore was estimated at $1{\cdot}2 \pm 0{\cdot}4$ pS (mean $\pm$ S.D.). The rather large dispersion is probably due to the fact that a large part of the I_i noise variance is carried by very low frequencies, as will be shown below.

Fig. 9 *B* illustrates a response to the application of 2 μM-CCh in another K-dialysed cell held at -83 mV. As in the previous examples shown, it may be seen by comparing Fig. 9 *A* and *B* that the CCh-induced current develops more quickly than the ionophore-induced current. The record of Fig. 9 *B* presents an overshoot after the end of the CCh application. The variance analysis of the record of Fig. 9 *B* is shown in Fig. 9 *E*. The analysis has been restricted to a current range of 0–400 pA in order to fulfil the conditions of low channel-opening probability. The elementary current calculated from the straight line in Fig. 9 *E* is 0·08 pA (0·125 pA once corrected as above). The corresponding elementary conductance is 1·6 pS. Another recording gave a value of 0·6 pS. Thus, CCh-induced I_i currents had a unitary conductance in the same range as ionophore-induced I_i currents. This result supports the view that CCh and A23187 activate the same channels.

It should be noted that the value of the elementary conductance (1–2 pS) estimated for both the ionophore- and the CCh-induced currents may be affected by several errors sources. First, non-linear variations of the channel-opening probability during the sampling periods would result in an over-estimate of the variance. Secondly, I_i may have been contaminated by cation-selective channels with large (25 pS) or small unit conductance.

Given an elementary conductance value of 1–2 pS, a minimum value may be calculated for the number of channels contributing to the I_i current. Since peak currents of 500 pA to 1 nA were obtained at -60 mV, the cells must have contained at least 5000 to 20000 channels. This corresponds to a minimum average density of 4–16 channels/μm^2 (calculated for a standard 20 μm diameter cell). Thus, the I_i channels are far more numerous than BK channels, which have only an average density of 1 channel/5–15 μm^2 (Trautmann & Marty, 1984).

Kinetic information can also be obtained from I_i current noise, provided that the

length of available recordings is large compared to the duration of the elementary current. Such long recordings could be obtained only with ionophore-induced I_i. In the example shown in Fig. 10, 30 s of control noise recorded before the ionophore application and 70 s of noise accompanying a I_i current of 100–130 pA have been analysed in a frequency range of 0·1–50 Hz. The recording was obtained at −60 mV

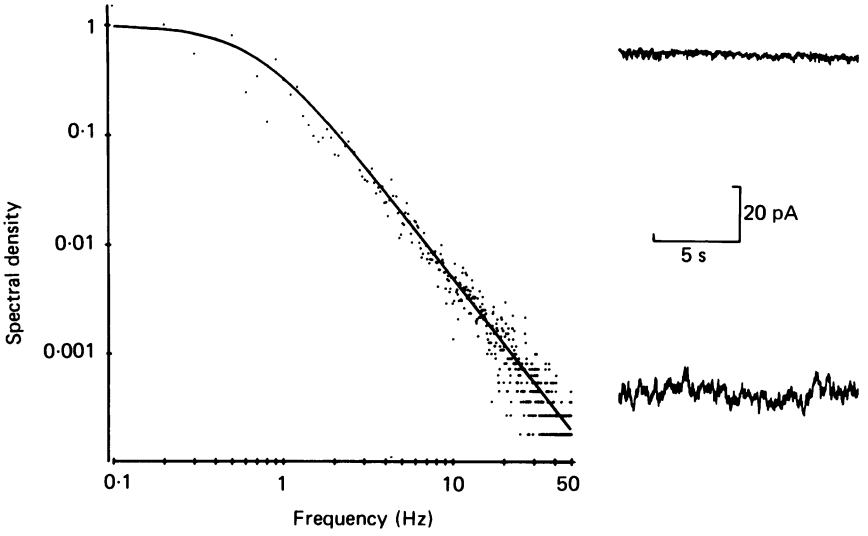

Fig. 10. Power spectrum of I_i current noise. $V_H = -60$ mV. Na-filled pipette. Two sections of current trace taken before (top) and during (bottom) development of I_i current are shown in the right part of the Figure. In this experiment I_i developed very slowly; the spectrum was obtained by averaging seven spectra calculated on 10 s long recordings sampled at 100 Hz, and subtracting the average of three control spectra taken in the same conditions. The mean current was 120 pA. The power spectrum was fitted by eye with a single Lorentzian, with a cut-off frequency of 0·69 Hz, corresponding to a mean channel open time of 231 ms.

with a Na-filled pipette. The resulting spectrum was fitted by eye with a single Lorentzian. The cut-off frequency (0·69 Hz) gives a mean channel open time of 231 ms (results from another experiment could be fitted with the same time constant.) This is within the range of τ values obtained at the same voltage in relaxation experiments. Noise and relaxation experiments therefore appear to reveal the same kinetic step of channel activation.

(6) Responses in cell-attached patches

Cell-attached recordings showed voltage-dependent single-channel openings which were attributed to BK channels. An indication of the value of the cell resting potential was obtained by comparing elementary currents measured in cell-attached and isolated patches. The values ranged between −40 and −70 mV. BK channels were activated only by strong positive pulses (50 mV or more). By comparing again results from cell-attached and isolated patches (see Trautmann & Marty, 1984), it was estimated that the value of $[Ca]_i$ was no more than 10 nM in resting cells. (This assumes that, apart from the value of $[Ca]_i$, additional factors influencing BK channel

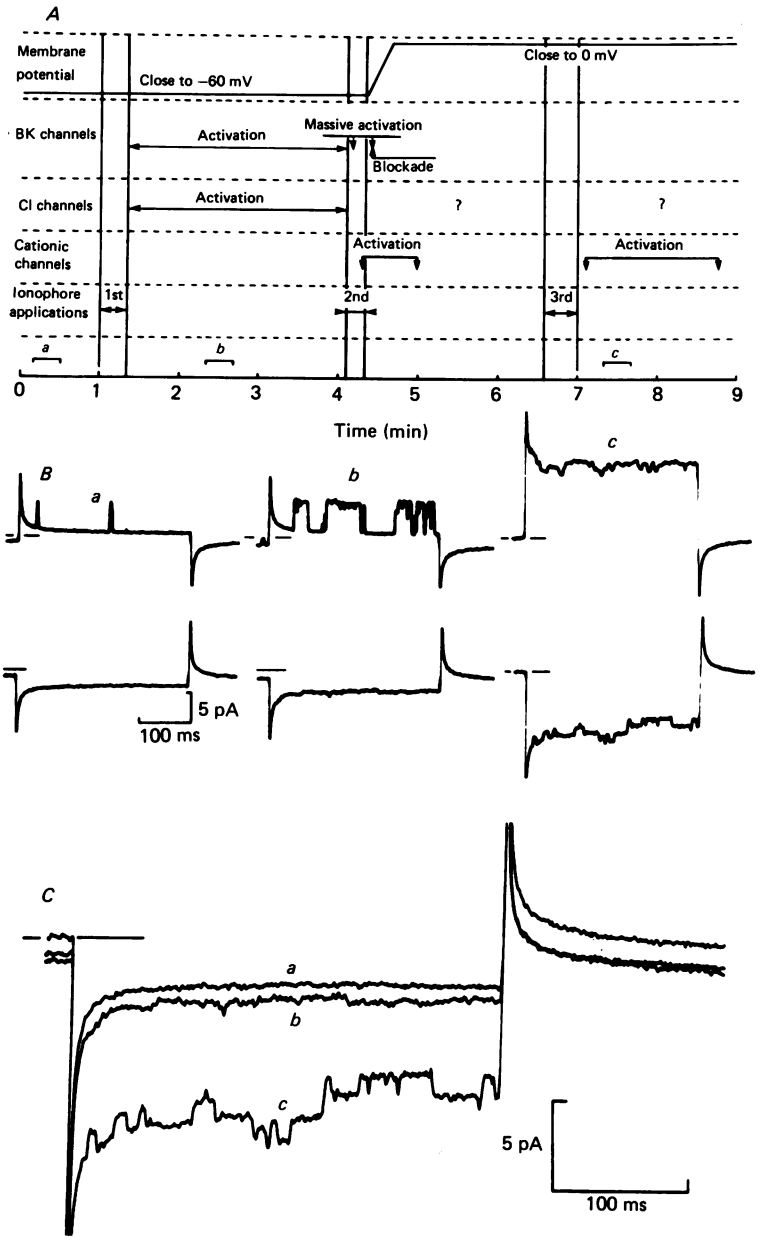

Fig. 11. Responses to the Ca ionophore in a cell-attached patch. Pipette solution, Ca-free saline (with 140 mM-Na). Bath solution, normal saline. *A*, summary of the experiment. The ionopore (0·2 μM) was applied three times. The pipette was held at the bath potential, so that the patch holding potential was equal to the cell resting potential E_r. Voltage jumps of various amplitudes were given at 1 Hz on either side of E_r. The value of E_r was roughly estimated by comparing the single-channel amplitudes of BK channels and of 25 pS cationic channels to the values obtained in isolated patches under similar ionic conditions. E_r was found to depolarize irreversibly within about 20 s following the second ionophore

activation were similar in isolated patches and in intact cells.) This value compares with a concentration of 10–100 nM found with the same method in pig pancreas cells (Maruyama *et al.* 1983).

With low CCh or ionophore concentrations, the cell hyperpolarized (as determined by measuring the amplitude of BK single-channel currents) and the frequency of opening of BK channels increased. If larger doses were applied, the same effects were obtained at first, but the cell was found to depolarize later on. Thus, cell-attached experiments gave dual responses matching the sequential activation of BK current and of I_i current in whole-cell recordings.

Fig. 11 illustrates one of the cell-attached experiments. Fig. 11 *A* gives a summary of the channel events observed after three successive ionophore stimulations. The pipette holding potential was equal to the bath potential, so that the *membrane* holding potential was equal to the cell resting potential, E_r. Voltage jumps were given at a frequency of 1 Hz. Before ionophore application, BK channel openings were obtained with low frequency, and E_r was estimated at -60 mV. The first ionophore application resulted in a slight hyperpolarization to -65 mV and in a 10-fold increase of the opening probability of BK channels near -10 mV (Fig. 11 *B*). At the same time, a small, noisy inward current was observed both at E_r and at $E_r - 50$ mV. The noisy inward current was interpreted as being carried by the 1–2 pS Ca-dependent Cl channels contributing to I_i in whole-cell recordings. Although the exact value of E_{Cl} is not known in lacrimal gland cells, it is very likely to be less negative than E_r, so that the opening of Cl-selective channels would tend to depolarize the cells. In the experiment of Fig. 11 it appears that simultaneous activation of BK channels and of Cl channels roughly cancelled each other's effect and resulted only in a slight hyperpolarization.

Records labelled *b* in Fig. 11 *B* were taken as the cell was slowly recovering from the first ionophore application. Before recovery was complete, a second ionophore application was performed and a new electrical response was observed during the wash-out. BK channels were further activated at first; they then started to present numerous fast interruptions, and were eventually entirely blocked. Since it is known that large Ca concentrations induce a voltage-dependent channel-blocking effect with fast (Marty, 1981) and slow kinetics (Vergara & Latorre, 1983), it seems likely that $[Ca]_i$ rose to values where it blocked BK channels entirely. At the same time, single-channel currents of a new type were observed. These events were attributed to

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application (upper part of the graph). Single-channel openings followed each ionophore application as indicated. Vertical arrows point to rather precisely timed events, whereas horizontal arrows indicate events whose boundaries were not known exactly. *B*, responses to 50 mV jumps positive (upper records) and negative (lower records) from E_r. The records were taken at the three periods (*a*, *b*, *c*) indicated in *A*. Zero-current levels are indicated near each record. In the control (*a*) only rare BK channel openings were seen for 50 mV positive jumps. After the first ionophore application (*b*), BK channel openings were much more frequent during positive jumps. A small, noisy inward current was observed during negative jumps. After the third ionophore application (*c*), unitary steps due to Ca-dependent cationic channels may be seen during positive and negative jumps. At this stage, E_r was close to 0 mV, so that the holding current was also close to 0. *C*, superimposed responses to hyperpolarizing jumps in *B*. Trace *b* presents small fluctuations attributed to Ca-dependent Cl channels.

Ca-dependent cationic channels, to be described below (Fig. 13). From the size of these single-channel currents, the membrane potential was estimated to be close to −70 mV for the first openings. The single-channel current amplitude decreased gradually in the following 20 s, bringing the estimated cell potential to near 0 mV. This depolarization is probably the consequence of a massive activation of the cationic channels over the entire cell membrane. As expected (Fenwick *et al.* 1982), the holding current measured at E_r subsided simultaneously (Fig. 11*C*). The cell did not recover a normal resting potential later on, but remained depolarized. Cationic channels could still be transiently activated by renewed application of the ionophore. This is shown in the right part of Fig. 11*B*, which illustrates responses to voltage jumps following a third ionophore application. The single-channel currents apparent at this stage of the experiment had a slope conductance of 23 pS, close to that of the Ca-dependent channels observed in isolated patches (Fig. 13, below). No reopening of BK channels was observed after their blockage during the cell depolarization. It seemed plausible that the small currents attributed to Cl channels remained activated throughout the experiment after the first ionophore application, since the patch current remained noisy after closure of cationic channels. However, this could not be ascertained, hence the two question marks in Fig. 11*A*. To sum up, the results shown in Fig. 11 were consistent with the notion that cell membranes contained three classes of Ca-dependent channel: BK channels with high sensitivity to Ca; small-conductance channels, which were activated at intermediate $[Ca]_i$ levels; and 25 pS channels which were the least sensitive to $[Ca]_i$. At the high $[Ca]_i$ levels where the 25 pS channels were activated, BK channels were blocked.

Results from nine other experiments were consistent with the findings of Fig. 11. A clear increase in BK channel activity was found in four patches with moderate stimulations, and subsequent blockage was observed in three cases. The 25 pS channels were activated in four patches, and six patches displayed the noisy inward current response to the ionophore. In some of these patches, steps of *ca.* 0·2–0·4 pA could occasionally be distinguished, as shown in Fig. 12, but in most cases single-channel events were not resolved. It seems unlikely that the noisy inward current was due to unspecific membrane damage caused by the ionophore, since (1) the size of the underlying elementary events, as judged from the noise variance, was consistent from one experiment to the next, and (2) in several patches the membrane resistance and the noise level were found to recover fully after prolonged washing. In the experiment of Fig. 12, single-channel openings of about 0·4 pA were observed at the onset and at the end of the response, when overlaps were rare. These openings were obtained during 50 mV hyperpolarizing voltage jumps from the cell resting potential E_r. Assuming that $E_r = -65$ mV, as in Fig. 11, and that $E_{Cl} = -30$ mV, one obtains a unit conductance of 5 pS. Ensemble noise analysis (see Methods) performed on the traces shown in Fig. 12 gave i_{el} estimates of 0·09 pA at E_r, and of 0·16 pA at $E_r - 50$ mV. (The total number of channels in the patch, N, was estimated as 10.) The corresponding unit conductance is 1·9 pS. The discrepancy between unit conductances derived from single-channel measurements and from noise analysis may be due to a faulty estimate of N or to an error in the subtraction of the background current with the second method. Such errors are likely to be large when performing noise analysis on such small currents as those shown in Fig. 12. Alternatively, the

channel may assume three-state kinetics similar to those displayed by electroplaque Cl channels (Miller, 1982). These channels fluctuate between one closed and two open states with single and double unit conductances. The fluctuations occur on a millisecond time scale and are grouped in bursts of long duration. Such an effect would provide an explanation for the discrepancy between the two i_{el} estimates if the 0–1–2 fluctuations are fast compared to the filtering frequency (500 Hz in Fig. 12).

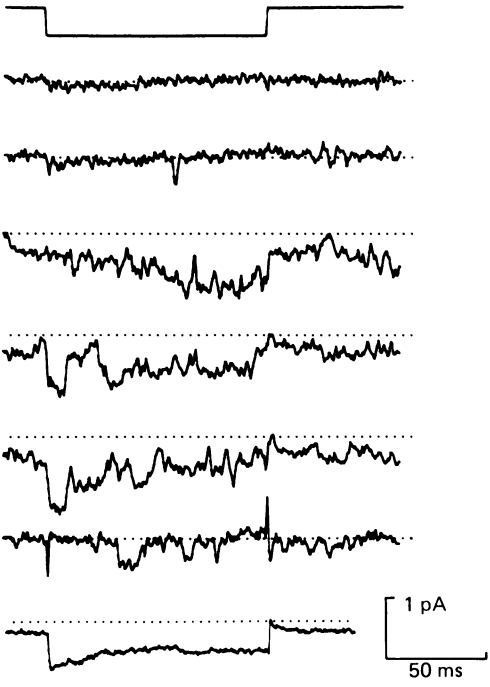

Fig. 12. Activation of Cl channels in a cell-attached patch. Responses to 50 mV hyperpolarizing voltage jumps (uppermost trace) during an ionophore application. Pipette holding potential = bath potential (membrane holding potential = cell resting potential). Six single-sweep records are shown after subtraction of leakage and capacitive currents. Top record, before ionophore application. Second record, short single-channel current, during ionophore application. Third record, development of a noisy inward current. Fourth and fifth records, noisy currents during maximal response. Sixth record, single opening during wash-out. Bottom record, average of thirty-two sweeps during ionophore response (control current was subtracted). 500 Hz low-pass filter.

Whatever the exact explanation, the fact that noise analysis performed on cell-attached patches gives a unit conductance close to that performed on whole-cell recordings suggests that the two types of current are carried by the same channels, which we assume to be Cl selective and Ca sensitive. The finding of a slow conductance decrease upon hyperpolarization in the average current of Fig. 12 further supports this proposal.

No systematic spatial segregation between K-, Cl- and cation-selective channels appeared from cell-attached results. Thus, the patch illustrated in Fig. 11 possessed all three types of channels, whereas that of Fig. 12 had only the small-conductance Cl channels.

(7) Ca-activated channels in isolated patches

Cell-attached experiments revealed a population of Ca-dependent channels with a slope conductance of about 25 pS (Fig. 11). Ca-dependent unitary currents apparently due to the same channels were found in inside-out patches, as illustrated in Fig. 13*A*. The bottom trace was taken as the cytoplasmic side of the patch membrane was

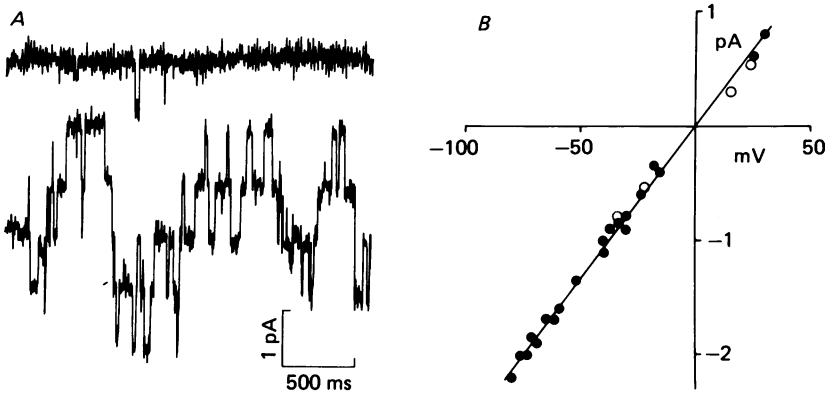

Fig. 13. Ca-dependent cationic channels in inside-out patch. *A*, recordings from an inside-out patch held at −40 mV. Pipette and bath solutions contained mainly NaCl. Upper record, 1 mM-EGTA bath solution; lower record, 1 mM-Ca bath solution. The pipette solution was Ca free. In 1 mM-Ca between one and five channels were open in the trace shown. *B*, *I–V* curve of the single-channel currents shown in *A* (filled circles). The results have been fitted with a straight line having a slope of 27 pS. Open circles, results from another inside-out patch where 4/5 of the Cl in the pipette solution was replaced with glutamate (approximate single-channel conductance, 24 pS).

bathed with a NaCl solution including 1 mM-Ca. Between one and five channels were open during the recording. (Since the fifth level was reached many times in high-Ca solutions, whereas a sixth level was never observed, the patch most probably contained just five channels.) The top trace was taken shortly after replacing Ca with 1 mM-EGTA. All channels closed, except for the short reopening shown in the trace. The single-channel currents depended linearly on potential (filled circles in Fig. 13*B*), with a unit conductance of 27 pS in the experiment shown. The average unit conductance was 25·7 ± 2·4 pS (mean ± S.D.) in a total of five patches. A noise power spectrum obtained from the recording illustrated in Fig. 13 gave a time constant t_0 of 90 ms. The mean fraction of open channels, p_0, was 0·6 in this experiment. Assuming that, to a first approximation, the channels obey simple two-state kinetics, the mean channel-open time may be calculated as $\tau = t_0/(1-p_o)$, that is 225 ms. Thus, the 25 pS channels have slow kinetics, in the same time scale as that of the Cl currents of sections (4) and (5) above. To test the ionic selectivity of the 25 pS channels, inside-out patches were formed, still with symmetrical Na concentrations, with a pipette solution containing 4/5 glutamate and 1/5 Cl. *I–V* curves obtained under these conditions (open circles in Fig. 13 *B*), with $E_{Cl} = -41$ mV, were indistinguishable from the curves in symmetrical Cl concentrations. These experiments show that the 25 pS channels are not the main carriers of the I_i current of sections

(*3*)–(*5*). They further indicate that the channels are impermeable to Cl (unless they transport glutamate as readily as Cl), and that they are selective for cations. Since cell-attached experiments, with mostly K ions on one side of the membrane and Na ions on the other, also gave a reversal potential of 0 mV (Fig. 11), the channels do not discriminate between these two cations. Channels with similar unit conductance, mean duration, ion selectivity and Ca sensitivity have been previously described by Colquhoun *et al.* (1981) in cardiac cells, by Yellen (1982) in neuroblastoma, and by Maruyama & Petersen (1982*a*) in pancreatic acinar cells. From the limited number (three) of inside-out patches where different $[Ca]_i$ values were tested, it appears that the 25 pS channels are activated at 10 μM-internal Ca but not at 0·1 μM.

Ca-dependent activation of small-conductance Cl channels was also investigated in inside-out patches. In three experiments, a clear, reversible inward current response was obtained when increasing $[Ca]_i$ from 10^{-7} M to either 10^{-5} or 10^{-4} M in the bath solution. (Two out of the three patches also displayed Ca-dependent unitary steps due to the 25 pS cationic channels.) Quantitative noise analysis was not possible on these patches, but occasional steps of *ca.* 0·2 A were obtained at −40 mV during the inward current, corresponding to a unitary conductance of 5 pS. This is the same unit conductance as that estimated from Fig. 12. These results confirm that the noisy inward current obtained in cell-attached experiments (Figs. 11 and 12) is due to Ca-dependent channels and not to some artifact elicited by the Ca ionophore. They further suggest that the channels involved have an activation threshold in the micromolar concentration range.

BK channels were not studied in inside-out patches, but outside-out patch experiments were performed with $[Ca]_i$ values of 10 nM, 200 nM, 10 μM and 100 μM. The opening probability of the channel, p_0, presented at a given voltage a fairly large cell-to-cell variability, but the shape of the p_0–V curve was very similar in all cases. As previously indicated (Trautmann & Marty, 1984), the channels were half-activated near +15 mV in 10 nM-internal Ca. This $[Ca]_i$ value is markedly lower than those needed to activate the Cl channels or the 25 pS channels. Fig. 14 shows the variation of the channel opening probability as a function of potential for three values of $[Ca]_i$. At 10 nM-internal Ca, p_0 increases with the membrane potential. At 200 nM-internal Ca, p_0 is high at all potentials. At 100 μM-internal Ca, p_0 varies with potential in the direction opposite to that obtained at low $[Ca]_i$ values. The latter effect, also evident at 10 μM-internal Ca, is probably due to a slow channel-blocking action of internal Ca (Vergara & Latorre, 1983). In Fig. 14*D*, records obtained from an outside-out patch at 100 μM-internal Ca are shown. Openings at +30 mV are much shorter than at −10 or −30 mV. (Much of the background noise in these records is probably due to the small-conductance Ca-dependent channels of sections (*3*)–(*5*).)

From the results of isolated patch experiments, a rough concentration scale may be suggested for the cell-attached experiments illustrated in Figs. 11 and 12. Activation of BK channels requires very low $[Ca]_i$ levels, in the range 10–100 nM. Cl channels are then activated, probably in the range 100 nM–1 μM. Opening of the 25 pS channels, which shortly precedes the closure of BK channels, occurs above 1 μM. In the experiment of Fig. 11, BK channels were entirely blocked after the second ionophore application. $[Ca]_i$ must then have risen well above 100 μM, since the slow Ca-induced channel block is only partial at this concentration (Fig. 14). Furthermore,

the last single-channel currents seen before total blockade of BK channels presented numerous short interruptions due to another blocking action of Ca with fast kinetics. This fast Ca-induced channel block occurs only with concentrations above 1 mM in chromaffin cells (Marty, 1981; and unpublished observations), and was not present at 100 μM in the experiments of Fig. 14. This gives another indication that, after prolonged ionophore applications, $[Ca]_i$ rose steeply to large values, probably in the millimolar concentration range.

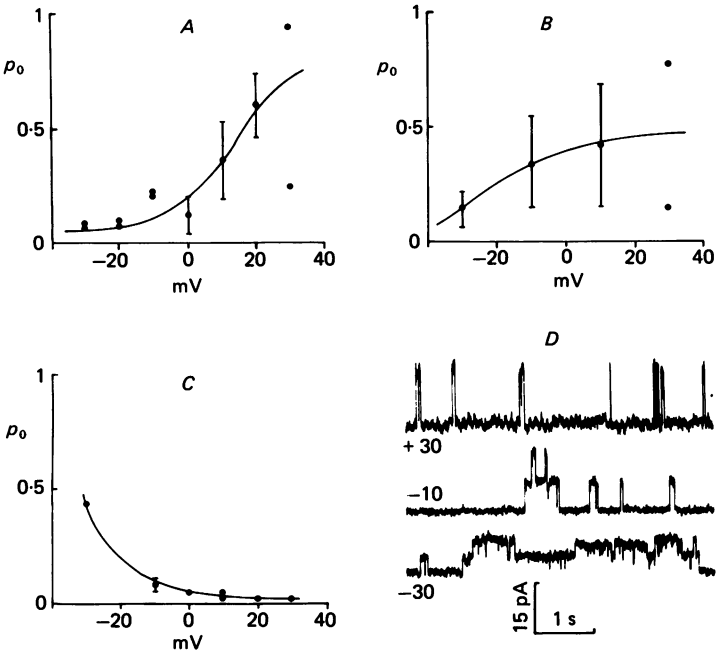

Fig. 14. Effects of internal Ca concentration and membrane potential on BK channels. Probability of channel opening, p_0, as a function of membrane potential with $[Ca]_i$ values of 10 nM (*A*), 200 nM (*B*) and 100 μM (*C*). Outside-out patches. External solution, nominally Ca-free, unbuffered saline. When more than two values were available, mean ±S.D. is shown. *D*, recordings from a patch with 100 μM-internal Ca, at three membrane potentials (−30, −10 and +30 mV).

DISCUSSION

The present work shows that different classes of Ca-dependent channel are activated by cholinergic agonists in lacrimal gland cells. Four different classes of Ca-dependent channels are present in the cells, of which three types could be studied in some detail: K channels with large unit conductance (BK channels), Cl channels with low unit conductance, and 25 pS cation-selective channels. In the following, two main questions are considered: (1) how is the finding of various Ca-dependent channels related to previous electrophysiological work? and (2) what may be the role of Ca-dependent channels in fluid secretion?

(1) Polyphasic electrical responses in lacrimal and salivary glands

A number of previous studies have described electrical responses to secretory agents using conventional micro-electrode recordings on intact glands or on cell clusters. This type of measurement is prone to numerous types of error in exocrine tissue notably because of the strong electrical coupling linking adjacent cells. Nevertheless, this early work clearly showed that the responses to secretory agents involve several conductance pathways. Thus, membrane potential changes are usually polyphasic. In all lacrimal or salivary glands investigated (insect salviary glands: reviewed in Ginsborg & House, 1980; and in House, 1980; mammalian salivary glands: Roberts, Iwatsuki & Petersen, 1978; Gallacher & Petersen, 1980; Wakui & Nishiyama, 1980; and mammalian lacrimal glands: Iwatsuki & Petersen, 1978*b*), one of the potential changes observed was a hyperpolarization due to an increase of K conductance. This probably corresponds to the opening of BK channels in the present work. As in our cell-attached recordings, it was found in previous work on lacrimal glands that low doses of ACh only evoke a hyperpolarization, and that for higher doses the hyperpolarization is followed by a depolarization (Iwatsuki & Petersen, 1978*b*).

A second part of the complex responses observed in previous micro-electrode studies was a depolarization involving a conductance increase. In several preparations (lacrimal glands: Iwatsuki & Petersen, 1978*b*; and parotid glands: Roberts *et al.* 1978) the depolarization was abolished by Cl removal. As removing external Cl ions shifted E_{Cl} to positive potentials, a larger depolarization was expected in Cl-free solution than in the control. The authors argued then that the depolarization was not due to a Cl conductance. But the possibility that Cl removal could affect the opening of Cl channels was not considered. Yet, it has been shown recently that certain Cl channels have an opening probability which depends on internal Cl concentration, and possibly also on external Cl concentration (Chesnoy-Marchais, 1983). If such a phenomenon also occurs in the channels of salivary and lacrimal glands, then the results of the studies quoted above are in fact compatible with an involvement of Cl channels in the depolarization induced by secretory agents. The presence of such a Cl conductance in blowfly salivary glands was proposed by Berridge, Lindley & Prince (1975).

Finally, previous studies concluded on the basis of Na-substitution experiments that an increase in Na conductance was contributing to the depolarization (Iwatsuki & Petersen, 1978*b*; Roberts *et al.* 1978; Wakui & Nishiyama, 1980). This conclusion is corroborated by our finding that Ca-dependent cationic channels are activated by strong stimuli.

In conclusion, the polyphasic responses found in previous studies on lacrimal and salivary glands may be explained on the basis of the three types of Ca-dependent channel described in the present work. In rodent exocrine pancreas, acetylcholine (ACh) also evokes complex conductance increases to K, Na and Cl, but the part of Cl is more prominent than in salivary and lacrimal glands (Iwatsuki & Petersen, 1977*b*). It is quite possible that the three types of channel are present in these preparations as well, but that the proportion of Cl channels is larger than in the cells investigated in the present work while that of K channels would be smaller.

(2) Properties of Ca-dependent Cl channels

The Ca-dependent Cl channels found in the present investigation have distinct properties. (1) The presence of Ca is an absolute prerequisite for their activation. They differ in this respect from the BK channels. (2) They present a low unitary conductance of about 1–2 pS. As a consequence, they are difficult to resolve in isolated patches. (3) They are selective for Cl ions. Because of probable contamination with Ca-dependent cation-selective channels, it is difficult to assess exactly the selectivity of the channels for Cl over Na in normal saline. Fortunately, it appears that the contaminating current is much reduced in the absence of external K ions. In K-free solutions, the permeability ratio of the channels for Na and Cl ions, P_{Na}/P_{Cl}, is less than 0·03. (4) They are activated by membrane depolarization. The steepness of the conductance–voltage curve is, however, much less marked than for voltage-dependent Na, Ca or K channels. (5) They present slow kinetics, with a mean channel open time in the range 100–250 ms. In addition, some results indicate the presence of a faster kinetic process, with a time constant smaller than 1 ms. (6) They are activated by micromolar concentrations of internal Ca.

The observation of waves of Cl current (Fig. 3) suggests that the intracellular Ca concentration oscillates during a stimulation. Similar effects are observed in skinned muscle fibres where oscillations in fibre tension were attributed to cyclic variations of $[Ca]_i$ due to Ca-induced Ca release from the sarcoplasmic reticulum (Weber, 1971*a*, *b*; Fabiato & Fabiato, 1975). It is therefore tempting to attribute the oscillations observed in lacrimal gland cells to Ca-dependent release and uptake systems of the endoplasmic reticulum. Such Ca-regulated systems may also explain why, in ionophore experiments, $[Ca]_i$ apparently rose abruptly to comparatively large values once a certain threshold was reached. The inhibitory effects of internal Na and Cs on CCh responses may also be explained if these ions affect the Ca exchange between cytoplasm and reticulum.

The Cl current of lacrimal gland cells presents several similarities to the ACh-activated Cl current described by Kusano, Miledi & Stinnakre (1982) in *Xenopus* oocytes. As in lacrimal glands, the ACh response found in oocytes is muscarinic, and the ACh-induced potential presents a marked delay (0·5–20 s). Furthermore, ACh responses in oocytes are oscillatory, and it was suggested that these oscillations were due to fluctuations of the concentration of some intracellular component controlling the opening of a Cl channel. Subsequent studies (Miledi, 1982; Barish, 1983) strongly suggest that Ca-dependent Cl channels are present in this preparation, so that the 'second messenger' involved in the muscarinic response may well be internal Ca. Recently, a Ca-dependent Cl conductance was also described in rod inner segments from salamander retinas (Bader, Bertrand & Schwartz, 1982).

(3) Role of Ca-dependent channels in fluid secretion

It is likely that the electrical response to muscarinic agonists are somehow linked to the secretion process, even though the mechanism involved remains a matter of debate (see Petersen & Maruyama, 1984). In this section, we show that various modes of secretion may be explained on the basis of Ca-induced activation of the three types of channel described in the present work. Since quantitative information on fluid

secretion is more complete in salivary glands than in lacrimal glands, we will often refer to results obtained in salivary glands. This is justified in view of the striking similarities between electrical responses obtained in the two types of preparation (Petersen, 1980).

Initial phase of secretion: KCl release. It was shown in a previous work (Trautmann & Marty, 1984) that very low doses of agonist exclusively open Ca-dependent K channels (BK channels). This by itself should lead to a hyperpolarization of the acinar cell without significant ion movements, since the leakage conductance is very low. In accord with this expectation, Petersen (1970*a*) found in submandibular glands that very low concentrations of ACh hyperpolarize the cells without stimulating secretion.

Following somewhat larger cholinergic stimulation, both BK channels and Ca-dependent Cl channels are activated. This should lead to extrusion of KCl, insofar as E_K is more negative than E_{Cl}. If the acinar cell membranes have a sufficient water permeability, extrusion of KCl will be followed by extrusion of water and cell shrinkage. Cell shrinkage was indeed observed during fluid secretion (Burgen, 1967). If K- and Cl-selective channels are located both on the basolateral and on the luminal sides of the acinar cells, then KCl will be released both towards the exterior and towards the blood. KCl-rich secretions are found both in salivary (Burgen, 1956; Young & Schögel, 1956) and in lacrimal (Alexander, van Lennep & Young, 1972) glands at low levels of stimulation. Concerning the blood–gland exchange, it has been shown in dog salivary glands that at the start of a nerve stimulation, K is transferred from the gland both to the saliva and to the blood (Burgen, 1956).

The above proposal of a KCl secretion at a low stimulation level conflicts with one series of findings, however. Micropuncture samples collected directly at the output of acini in various glands invariably gave a NaCl-rich solution (Martinez, Holzgreve & Frick, 1966; Young & Schögel, 1966; for review, see Schneyer, Young & Schneyer, 1972). From these results it has been widely accepted that the 'primary secretion' of acinar cells is a plasma-like solution. To account for the fact that the final secretion has in all cases a much higher K concentration than plasma, an *ad hoc* Na–K exchange system was assumed to function in secretory ducts. But this theory is incompatible with quantitative results on K, Na and Cl exchange during secretion. Burgen (1956) noted that a 3 min long stimulation depleted 40 % of the initial K content of dog salivary glands. The corresponding K ions must have come mostly from acinar cells, because these cells occupy the major part (about 90 %) of the gland volume. Although some K release to the blood occurred at the beginning of the stimulation, most of the K was released into the saliva. Thus, acinar cells must secrete K, not Na. Duct cells, on the other hand, do not contain enough K ions initially to account for the large amount of K ions secreted; nor can they take the K from the blood, because in fact blood takes up K from the gland (at least during the early part of secretion: see below). Such considerations indicate that the micropuncture results should be taken with great caution. It should be kept in mind that the samples may have originated from interstitial fluid rather than from secreted fluid. Finally, it is interesting to note that in the blowfly, where the primary saliva can be collected without ambiguity because of a favourable anatomical arrangement, this solution is essentially an isotonic KCl solution (Oschman & Berridge, 1970).

The possible involvement of Ca-dependent K and Cl channels in the early phase

of secretion is depicted in the left part of Fig. 15. Likely figures of the various ion concentrations encountered are given in the Appendix. Quantitatively, the currents observed in the present work are adequate to explain the large loss of K observed during secretion (Burgen, 1956; Poulsen & Oakley, 1979). Specifically, a 200 pA K current would lead to the loss of about 20 % of the intracellular K within 1 min, and would thus be consistent with the 40 % loss observed over 3 min in Burgen's experiments.

Early and sustained secretion. The above model of secretion can clearly not last since the cells have a finite pool of KCl. In his detailed study of Na, K and water movements during saliva secretion in dog submaxillary glands, Burgen (1956) demonstrated two phases in the secretion process. The early phase consists of a KCl release both to the blood and to the mouth, as discussed above. In the sustained phase, the saliva is less concentrated (but the main cation is still K). A transfer of KCl and water is then established from the blood to the saliva through the gland. During the sustained phase, the secreting cells have lost a large part of their internal K and they have gained Na. Secretion in other mammalian salivary and lacrimal glands is not as well characterized as in dog submaxillary glands, but the available evidence also indicates differences between early and sustained phases of secretion. Rat exorbital lacrimal glands, for instance, secrete an isosmotic NaCl-rich solution during sustained secretion and an isosmotic KCl-rich solution during the early phase (Alexander *et al.* 1972).

It was found in the present work that Ca-dependent cationic channels are activated after prolonged stimulation. Following the opening of cationic channels, accumulation of internal Na and external K is likely to take place in the gland, so that the Na–K pump should be activated. (Activation of the pump is also likely to follow the external K accumulation brought about by the opening of BK channels.) The presence of an ouabain-sensitive Na–K pump has been well documented in exocrine glands (for review, see Petersen, 1980). Finally, it has been shown that BK channels are blocked at intracellular Ca levels similar to those needed to activate the non-specific cationic channels. These various events may be combined in several ways to model either a sustained KCl secretion or a sustained NaCl secretion.

Sustained KCl secretion. In salivary glands, KCl is transferred through acinar cells during steady-state secretion. Thus, K and Cl enter a cell at the basolateral face and leave it at the luminal face. A scheme suggesting how these salt transfers may occur is shown in Fig. 15. On the basolateral membrane, activation of non-specific cationic channels and of Cl channels leads to the entry of NaCl. (In comparison to the initial phase of secretion, the Cl flux at the basolateral membrane is now inverted. A quantitative justification of the various directions of ion fluxes shown in Fig. 15 is given in the Appendix.) Simultaneous activation of the Na–K pump leads to the extrusion of Na and entry of K, so that the net movement is an entry of KCl. On the luminal face, the scheme of Fig. 15 assumes that non-specific cationic channels and the Na–K pump are both lacking. On this membrane, only BK channels and Cl channels are activated, and KCl is extruded. BK channels are assumed to be blocked on the basolateral membrane only, for reasons discussed in the Appendix. Finally, active salt uptake is assumed to occur in the ducts, so that the fluid which is secreted is hypotonic. Such an uptake system has been demonstrated in the distal segment of blowfly salivary glands (Oschman & Berridge, 1970).

In accord with the model of Fig. 15, Cl removal was found to inhibit secretion in salivary glands (Petersen, 1970*b*). Similarly, Singh (1984) recently reported that labelled Rb release from pancreatic segments could be inhibited by substituting SO_4 for Cl, or by including furosemide (which abolishes transmembrane Cl movement) in the incubation medium.

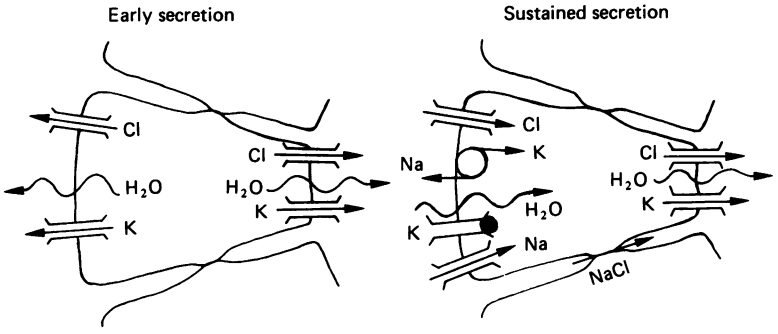

Fig. 15. Model of fluid secretion in mammalian salivary and lacrimal glands. Each diagram shows an acinar cell with its basolateral membrane on the left and its luminal membrane on the right. Three types of Ca-dependent channel are depicted, which are selective for K, for Cl and for monovalent cations (in which case mainly Na ions are transported in the ionic conditions encountered). During early secretion (left diagram), K and Cl leave the cell on both sides through K- and Cl-selective channels. During sustained secretion (right diagram), transport occurs on the luminal side as during the initial phase. At the basolateral membrane, Cl channels and unselective cationic channels are assumed to be activated, and BK channels are blocked by internal Na and Ca ions. This leads to entry of Na and Cl. The Na–K pump is activated, so that the final balance on the basolateral membrane is an entry of KCl (and water) into the cell. (Some passive extrusion of K is also likely to occur on the basolateral membrane via BK channels and cation-selective channels, as discussed in the Appendix.) In certain glands (such as lacrimal glands), where the sustained secretion is rich in Na ions, NaCl may enter directly the lumen through leaky intercellular junctions.

Another implication of the model is that sustained secretion should be inhibited by blocking the Na–K pump, whereas the initial secretion should not be affected. This is in agreement with the finding that K removal, as well as application of dinitrophenol or ouabain, selectively blocks the sustained phase of secretion (Petersen, 1970*a*, *b*; Poulsen, 1974). These treatments do not affect the early phase of secretion or the hyperpolarizing response to low ACh applications.

Sustained NaCl secretion. Following sustained stimulation, rat exorbital lacrimal glands secrete an isosmotic NaCl-rich solution containing 40 mM-KCl (Alexander *et al.* 1972).

One way to account for this result is to assume that the scheme shown on the right part of Fig. 15 is also valid in lacrimal glands, and that in addition, the cell-to-cell contacts are comparatively leaky so that NaCl may be transferred directly from the interstitial fluid to the lumen (see discussion in Petersen, 1980, chapter 6).

Another way to account for a NaCl-rich secretion is to assume that the blood pressure within the gland is increased during secretion, and that NaCl transfer occurs via activation of Ca-dependent cationic and Cl channels on both basolateral and luminal membranes.

Conclusion. To sum up, plausible models of the early and sustained phases of secretion have been developed for KCl- and NaCl-secreting glands. The models assign specific roles to the three types of Ca-dependent channel described in the present work, as well as to the Na–K pump. In addition, they assume the presence of active salt uptake by duct cells in one case, and of leaky cell-to-cell junctions in the other. The models do not take into consideration other factors likely to play a role in secretion, such as bicarbonate transport. Also, it is clear that exocytosis may contribute to the secretion of water and salt. From what is known about the ionic content of granules (Sasaki, Nakagaki, Mori & Imai, 1983), exocytosis should provoke a secretion about equally rich in Na and in K. The proportion of fluid secretion which may be ascribed to Ca-dependent conductance changes and to exocytosis is at present unknown.

Petersen & Maruyama (1984) recently proposed another model of fluid secretion which shares some of the features of that of Fig. 15. These authors do not however make any proposal about the events occurring at the luminal membrane, where they assume that the 'primary secretion' has a plasma-like composition. On the basolateral membrane, they place a Na–K–Cl_2 carrier similar to that supposed to be involved in transport phenomena in kidney tubules and in other tissues. This carrier is replaced in Fig. 15 by the three classes of Ca-dependent channel described in the present work.

APPENDIX

It may not be apparent at first sight that the schemes of Fig. 15 actually allow the passive transfer of Na, K and Cl in the directions indicated. In the following, we give specific figures for the concentrations in the basolateral extracellular space ($[Na]_1$, $[K]_1$, $[Cl]_1$), in the intracellular compartment ($[Na]_i$, $[K]_i$, $[Cl]_i$), and in the lumen ($[Na]_2$, $[K]_2$, $[Cl]_2$), and for their respective potentials, V_1, V_i and V_2 which are consistent with available information (e.g. Poulsen & Oakley, 1979; Mori, Murakami, Nakahari & Imai, 1983; Sasaki *et al.* 1983) and which yield fluxes in the directions shown in the Figure. For simplicity, only Na, K and Cl are considered.

During the first phase of secretion. In this phase we take (in mM) $[Na]_i = 20$, $[K]_i = 140$, $[Cl]_i = 40$. On the basolateral face, the external fluid has a composition close to that of plasma, but with an increased [K]: $[Na]_1 = 130$, $[K]_1 = 30$, $[Cl]_1 = 160$. On the luminal side, the secretion is a KCl solution which is diluted by salt uptake in the ducts: $[K]_2 = 60$, $[Cl]_2 = 60$. These assumptions lead to the following approximate values (in mV, at 37 °C): at the basolateral membrane $E_K = -41$, $E_{Cl} = -37$, $V_i - V_1 = -39$; at the luminal membrane $E_K = -23$, $E_{Cl} = -11$, $V_i - V_2 = -17$, assuming that the conductances of K and Cl channels are equal on both the basolateral and the luminal membrane. The fact that E_K is more negative than E_{Cl} at both membranes implies that K and Cl leave the cell on both sides. Note that we expect a transepithelial potential $V_2 - V_1 = -22$ mV.

During sustained secretion. During this phase the cell has gained Na and lost K, while $[Cl]_i$ remained steady. Likely values are $[Na]_i = 100$, $[K]_i = 60$, $[Cl]_i = 40$. The external fluid on the basolateral side is again similar to plasma. In dog submaxillary glands, blood flow is greatly increased during sustained secretion, and K is lost by the blood. This implies that $[K]_1$ must be somewhat *less* than in the blood. We thus take $[Na]_1 = 155$, $[K]_1 = 4$, $[Cl]_1 = 160$.

With these figures, $E_K = -72$, $E_{Cl} = -37$, $E_{Na} = +12$ at the basal membrane,

$V_i - V_l$ is likely to be close to -20 mV; Na and Cl tend to enter the cell, while K leaves it both through unselective cationic channels and through BK channels. The passive extrusion of K (which we assume to be more than compensated by the Na–K pump) is limited by two factors. First, only the basal membrane is in direct contact with the blood, and in the lateral clefts the external [K] may be much larger than 4 mM, resulting in a diminished K outflux. Secondly, BK channels at the basal membrane may be blocked at the elevated $[Na]_i$ and $[Ca]_i$ levels occurring during sustained secretion. BK channels located in other parts of the cell (as on the luminal face) are not likely to be affected by the same mechanism since Na and Ca block are very dependent on external [K] (Marty, 1983; Vergara & Latorre, 1983).

The fluid found in the lumen during sustained KCl secretion is a rather dilute KCl solution: $[K]_2 = 30$, $[Cl]_2 = 30$. On the luminal membrane $E_K = -19$ and $E_{Cl} = -8$. Since E_{Cl} is less negative than E_K, both K and Cl are extruded.

During sustained NaCl secretion, likely lumen ionic concentrations are $[K]_2 = 40$ and $[Cl]_2 = 120$. Passive extrusion of KCl requires that $[K]_i$ remains high, e.g. $[K]_i = 140$, in order for E_K to be more negative than E_{Cl} at the luminal membrane.

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