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ACETYLCHOLINE MODULATION OF THE CONDUCTANCE OF INTERCELLULAR JUNCTIONS BETWEEN RAT LACRIMAL CELLS

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SUMMARY

1. The conductance of intercellular junctions between rat lacrimal cells was studied with the double whole-cell tight-seal recording technique.

2. This conductance decreases spontaneously with time as a result of the double-cell dialysis. The rate of this 'spontaneous' uncoupling is unaffected by changing the internal Ca concentration, $[Ca]_i$, between 10^{-8} M and 10^{-6} M.

3. This rate of uncoupling is greatly increased when $[Ca]_i$ is $\sim 10^{-5}$ M, and this effect does not involve changes in the internal proton concentration.

4. When $[Ca]_i$ is weakly buffered in one of the two cells, 1–2 μM-acetylcholine (ACh) both activates Ca-dependent channels in that cell (Marty, Tan & Trautmann, 1984) and uncouples the two cells. The uncoupling is not synchronous with the increase in $[Ca]_i$ as reflected by the Ca-dependent currents.

5. When $[Ca]_i$ is strongly buffered in both cells, ACh fails to activate Ca-dependent currents, but it can still uncouple the cells. This ACh-induced uncoupling is often preceded by a transient enhancing of the coupling.

6. In conclusion, ACh has several distinct effects on lacrimal cells: (1) activation of Ca-dependent channels in the plasma membrane, (2) closure of junctional channels involving a Ca-independent mechanism, and (3) sometimes, an increase in the junctional coupling by a Ca-independent mechanism.

INTRODUCTION

In many tissues, cells can communicate without involving extracellular messengers through arrays of intercellular channels organized in gap junctions (for a recent review see Peracchia & Girsch, 1985). This direct communication can, however, be modulated by extracellular molecules such as hormones and neurotransmitters. Some hormones have been found to participate in long-term (few hours to days) changes in gap junction permeability (Brown, Wiley & Dumont, 1979; Sims, Daniel & Garfield, 1982; Kessler, Spray, Saez & Bennett, 1984). In some of these cases, the channel-formation process was modified (Radu, Dahl & Loewenstein, 1982). On the other hand, two neurotransmitters have been shown to induce short-term (minutes) junctional-permeability modulation: acetylcholine (ACh) decreases the permeability of intercellular junctions between rat exocrine acinar cells (Iwatsuki & Petersen, 1978*a*; Findlay & Petersen,

1982) and dopamine acts similarly on gap junctions between retinal horizontal cells of lower vertebrates (Teranishi, Negishi & Kato, 1983; Piccolino, Neyton & Gerschenfeld, 1984; Lasater & Dowling, 1985). The intracellular mechanisms involved in such junction modulations are still not completely elucidated.

In different preparations, the gap-junction conductance can also be reduced by increasing intracellular concentrations of Ca (Rose & Loewenstein, 1976; Rose & Rick, 1978; Spray, Stern, Harris & Bennett, 1982), protons (Turin & Warner, 1977; Rose & Rick, 1978; Iwatsuki & Petersen, 1979; Giaume, Spira & Korn, 1980; Turin & Warner, 1980; Spray, Harris & Bennett, 1981; Spray *et al.* 1982) or, in retinal cells, cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (Teranishi *et al.* 1983; Piccolino *et al.* 1984; Lasater & Dowling, 1985).

A change in the intracellular Ca concentration, $[Ca]_i$, may be responsible for the modulation by ACh of gap junctions between the cells of rat lacrimal glands, since in this gland ACh increases $[Ca]_i$ (Trautmann & Marty, 1984; see also the reviews by Ginsborg & House, 1980; Petersen, 1980) and also decreases the electrical coupling between the cells (Iwatsuki & Petersen, 1978*a*). However, these results do not prove that an increase in $[Ca]_i$ actually mediates the ACh-induced uncoupling of these cells.

This question has been examined using the double patch-clamp technique that we recently used to characterize the properties of an intercellular junction at the molecular level (Neyton & Trautmann, 1985). With this technique, the two communicating cells are dialysed with solutions of controlled composition. We show here that even though a high internal level of $[Ca]_i$ can block the junction, the uncoupling effect of ACh can take place in the absence of any change in either $[Ca]_i$ or pH_i, the internal pH. Moreover, this ACh-induced decrease of gap-junction conductance appears to result from a decrease in the number of open channels rather than from a decrease in single-channel conductance.

METHODS

Experiments were performed at room temperature (20–25 °C) on freshly-isolated cell pairs obtained by enzymatic dissociation of exorbital rat lacrimal glands (Kanagasuntheram & Randle, 1976).

Recording methods

Recordings were made with a double patch-clamp system (two List L/M EPC 7 amplifiers) allowing both cells of a pair to be simultaneously voltage clamped in the whole-cell tight-seal configuration (Marty & Neher, 1983). Patch electrodes were filled with solutions whose ionic compositions (and that of the bath) are listed in Table 1. Their resistances were 2–5 MΩ when measured in the bath. At the beginning of the double whole-cell recording, series resistances in each cell were estimated from the parameters used to cancel the capacitive surge of current at the beginning of a voltage jump simultaneously applied to both cells. Series-resistance values ranged from 5 to 20 MΩ and were kept constant (within a 20 % variation range) throughout the experiment by re-applying slight suction to the electrodes if necessary. This precaution was especially important when we used high $[Ca]_i$-containing solutions which often cause a clogging of the pipettes.

Measurement of the gap-junction conductance, G, was done as described in Neyton & Trautman (1985). Briefly, the potential of electrode 1 was stepped by V mV, whilst that of electrode 2 was maintained constant at V_2. This voltage step induced current changes ΔI_1 and ΔI_2 in cell 1 and 2 respectively. If r_1 and r_2 are the series resistances in cell 1 and 2, and if R_2 is the non-junctional membrane resistance of cell 2 at potential V_2, the junctional conductance G is given by:

$$G = \Delta I_2 (1 + r_2/R_2)/[V - (r_1 \Delta I_1 - r_2 \Delta I_2)].$$

Note that the correction factors $r_1\Delta I_1$ and $r_2\Delta I_2$ introduced by the series resistances are additive because ΔI_1 and ΔI_2 have opposite polarities.

Coupling stability

A serious drawback of the double whole-cell recording approach is the lack of coupling stability observed in the so-called 'control conditions' (i.e. low $[Ca]_i$, solutions A or C in Table 1). As previously reported (Neyton & Trautmann, 1985), we always observe under these conditions a time-dependent fade in the coupling. The origin of this fade is still obscure. However, its time constant appears to depend very much on the quality of the preparation. As in the present study effects of high $[Ca]_i$ were analysed by measuring the rate of uncoupling of paired cells, for each preparation control experiments were performed.

TABLE 1. Ionic composition in mM of the bath solution and of the solutions used to fill the micropipettes, all adjusted at pH 7·2

Solution	KCl	NaCl	EGTA -KOH	$CaCl_2$	pCa	$MgCl_2$	HEPES -KOH
Bath	5	140	0	1	3	1	5
A	140	0	5·5	0·5	8	2	5
B	140	0	0·5	0		2	5
C	84	0	20	2	8	2	30
	NaCl	NaMS	HEDTA -NaOH	CaMS	pCa	HEPES -NaOH	
D	4	36	40	11	6	20	
E	4	16	40	32	5	20	

MS, methanesulphonate.

Ca buffering

Two different Ca chelators have been used in these experiments: EGTA (ethyleneglycol-bis-(β-aminoethylether)*N,N'*-tetraacetic acid), and HEDTA ((2 hydroxyethyl) ethylenediamine-*N,N,N'*-triacetic acid).

The apparent dissociation constants, K_{app}, for Ca of these two buffers were calculated from the values of the stability constants given in Martell & Smith (1974). EGTA is a good Ca buffer at pH 7·2 and for low Ca concentrations, since $K_{app} = 0{\cdot}1\ \mu M$. In order to buffer $[Ca]_i$ at higher concentrations, HEDTA was preferred ($K_{app} = 2{\cdot}6\ \mu M$ at pH 7·2). Note that Mg could be included in solutions buffered by EGTA because this Ca chelator has a low affinity for Mg. However, the binding of Mg to HEDTA has a K_{app} of 40 μM at pH 7·2, so that Mg had to be omitted from the HEDTA-buffered solutions.

Determination of single-channel amplitude

As already described (Neyton & Trautmann, 1985), only a fraction of the conductance changes at a weakly-coupled junction are quantal. Quantal conductance changes can be demonstrated in two ways: as occasional step-like current changes during a transjunctional voltage jump, or as evenly distributed peaks in the amplitude histogram. To build an amplitude histogram, a continuous recording is selected and every digitized point during the successive voltage jumps is counted as one occurrence. The height of one peak is thus proportional to the time spent at this current level. Intermediate levels of conductance appear sometimes randomly distributed between clearly separated peaks, but they can also be found at some preferential levels, giving unevenly distributed peaks (see Fig. 4). In the latter situation, the single-channel conductance cannot be determined with certainty. To present the amplitude histograms, we have chosen to fit Gaussians only to the major peaks, without looking necessarily for multiples. The smallest major peak was chosen as the most probable (though not certain) level of single-channel conductance, and used as such to draw dotted lines in the continuous current traces, corresponding to the presumptive levels of 0, 1, 2, 3 or 4 channels open. Step-like current changes during one voltage jump between two such levels, when they exist, reduce the uncertainty in the determination of the single-channel conductance.

RESULTS

Uncoupling induced by increasing $[Ca]_i$

In order to examine whether a change in $[Ca]_i$ is able to modulate the junction conductance between two lacrimal cells, the rate of progressive uncoupling was measured in a series of pairs of cells dialysed with various Ca concentrations. Several precautions had to be taken in these experiments. When one attempts to buffer $[Ca]_i$ at a value higher than normal, the cell may influence $[Ca]_i$ by its own Ca-buffering capacity, and in some cases by some Ca-induced Ca-release mechanism (Fabiato & Fabiato, 1975). For these reasons when pCa_i was < 7 we used high concentrations of a Ca chelator (40 mM-HEDTA, see Methods). Another precaution dealt with the control of pH_i. It has been shown that in a cell, the controls of $[Ca]_i$ and pH_i might not be independent (Meech & Thomas, 1977; Rose & Rick, 1978) and the point has often been raised of whether the effects of Ca or protons on gap junctions are direct or via the other cation (see e.g. Peracchia & Girsch, 1985). To ascertain whether variations of pH_i would bias our experiments at various $[Ca]_i$ levels, pH_i was also strongly buffered (30 mM-HEPES at pH 7·2).

Finally, in order to reduce the problem of voltage drop through series resistances, it was important to work using the smallest possible currents. The amplitude of the Ca-activated currents was reduced by appropriate ionic substitutions: both K and Cl (Ca-activated) currents were practically blocked by substituting Na for K inside the pipettes, and methanesulphonate for Cl in both internal and external solutions. As a result, Ca-induced currents were very moderate at a pCa_i of 6 (typically less than 0·5 nA at -50 mV) but at a pCa_i of 5, a large Ca-activated cationic current was present (2–3 nA at -50 mV).

Fig. 1 shows the time course of uncoupling observed in a series of experiments performed at three different values of pCa_i (8, 6 and 5). At a pCa_i of 8 (solution A in both cells) and large initial coupling conductance ($\geqslant 25$ nS), the junctional conductance fell to half its initial value in approximately 9 min. When $[Ca]_i$ was increased 100 times (pCa_i 6), and with our recording conditions improved by the ionic substitutions (solutions D in both cells), the stability of the coupling of initially strongly coupled cells was not markedly different from that of control conditions, since G fell to half its initial value in 7·5 min on average. The slight difference between the results obtained with solutions A and D could result either from the differences in pCa_i, or from the other ionic substitutions (Na for K and methanesulphonate for Cl). A large number of experiments would have been necessary to clarify this point, because of the smallness of the effect and of the important day-to-day variability in the rate of uncoupling. We can nevertheless conclude that $pCa_i \geqslant 6$ has little effect on the coupling conductance. In contrast, when pCa_i was lowered to 5, the cells were completely uncoupled after 3 min of recording (solution E in both cells, five experiments). However, the Ca-activated currents were too large to allow a reasonably accurate correction for the series-resistance artifact discussed above, and in these conditions the initial junctional conductance could not be measured. To reduce this difficulty, we did not load the first cell with Ca, using instead a light Ca buffer (solution B). The high $[Ca]_i$ and large Ca-activated currents were then only present in the second cell. In these conditions, the gap-junction conductance could be measured.

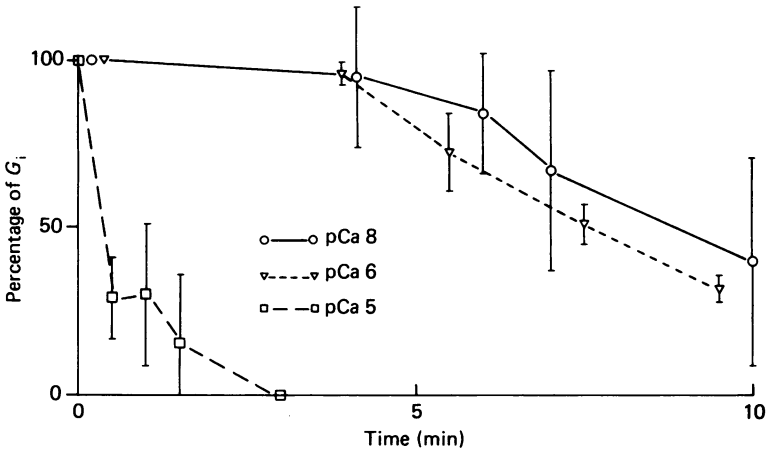

Fig. 1. Effect of $[Ca]_i$ on the rate of uncoupling of lacrimal cell pairs. The value of the junctional conductance G, is expressed as a percentage of its initial value, G_i, and plotted as a function of time following the establishment of the double whole-cell recording (time 0). The symbols give the average and the bars the S.D. of the measurements made in three or four experiments. Three series of experiments were done with pCa_i 8 (control, solution A), pCa_i 6 (solution D) and pCa_i 5 (solution E), shown by different symbols as indicated in the Figure.

It fell to half its initial value in less than 30 s and, as with the symmetrical solutions, it became negligibly small 3 min after the beginning of the double-cell dialysis.

In conclusion, a high $[Ca]_i$ level ($> 10^{-6}$ M) induces a closure of gap junctions between lacrimal cells, independently of any simultaneous pH_i change.

Is the ACh-induced uncoupling of lacrimal gland cells dependent on internal Ca and pH?

To elucidate the mechanism by which ACh induces an uncoupling of acinar cells, we have first compared the time course of $[Ca]_i$ changes (deduced from the changes of the Ca-activated currents) and that of the ACh-induced uncoupling. One of these experiments is illustrated in Fig. 2*A*. ACh-induced currents were allowed to develop in only one cell (C_1) in order to reduce the magnitude of series-resistance artifacts associated with large currents. In C_1, where $[Ca]_i$ was weakly buffered by 0·5 mM-EGTA, the membrane potential was repetitively stepped from -50 to -20 mV, whilst the potential of the other cell (C_2) was kept constant at -50 mV. Before the ACh application, the current necessary to step V_1 from -50 to -20 mV was essentially flowing through the gap junction (the contribution of the non-junctional channels of the plasma membrane to this current is given by the difference between I_1 and I_2). Upon application of 1 μM-ACh with a fast-perfusion system (Marty *et al.* 1984), an outward K current appeared in C_1 at -20 mV, after a delay of about 2 s. This current was maintained throughout the ACh application, and returned to the small initial amplitude several seconds after ACh wash-out. It is likely that the K current instantaneously follows the $[Ca]_i$ changes. The lag between the ACh application and the K current therefore corresponds to the time needed to induce a high enough increase in $[Ca]_i$ at the onset, and a sufficient decrease in $[Ca]_i$ at the

offset of the ACh application (Marty *et al.* 1984). At −50 mV, an inward Cl current was also transiently activated in C_1; it started slightly later than the K current and disappeared before this K current. These kinetic differences are presumably due to the fact that a higher $[Ca]_i$ level is required to activate the Cl current (Marty *et al.* 1984). In C_2, where $[Ca]_i$ was kept near 10 nM by 5·5 mM-EGTA, no ACh-induced Ca-activated current was observed (the small inward current in C_2 synchronous with the inward current in C_1, can entirely be accounted for by the imperfect voltage clamping of C_1 due to the large current appearing in this cell, and flowing through the series resistance r_1, see Methods). The main effect of ACh measurable in C_2 was a decrease of the transjunctional current induced by the voltage jumps in C_1. This indicates a decrease in the junctional conductance G, which is plotted as a function of time in Fig. 2*B*. Note that the decrease in junctional conductance can also be observed in C_1: the amplitude of the current induced by the voltage jumps in that cell became smaller after ACh wash-out than before ACh application. In this experiment, at the peak of the Ca-activated currents (and probably of $[Ca]_i$), the junctional conductance was transiently increased. This effect was not systematically observed. But in every experiment, the onset of the uncoupling presented a delay with respect to the Ca-activated currents. Moreover, the uncoupling always progressed after $[Ca]_i$ had returned to low levels, as judged by the disappearance of the Ca-activated currents. The ACh-induced uncoupling was sometimes reversible (Fig. 2*C*) but not always (Fig. 2*A*, *B*). When present, the recovery was generally slow and started 1–5 min after ACh removal.

The absence of a strict correlation between the level of $[Ca]_i$ and the coupling conductance suggests that an increase in $[Ca]_i$ may not mediate the effect of ACh on lacrimal cells junctional conductance. However, this point had to be examined more carefully, since we have also shown above that a high intracellular concentration of Ca is indeed able to decrease the junctional conductance. Internal acidification, also known to induce uncoupling of lacrimal cells (Iwatsuki & Petersen, 1979), could also be involved in the ACh modulation of the junctional conductance. In another series of experiments, illustrated in Fig. 3, ACh was applied on pairs of cells in which pCa_i and pH_i were strongly buffered at 8 and 7·2 respectively by 20 mM-EGTA and 30 mM-HEPES (solution C). Under these conditions, as expected, no Ca-dependent current was activated by ACh (see Fig. 3*A*). Despite the absence of any detectable $[Ca]_i$ increase, ACh could evoke the uncoupling of the cells, which was most often reversible, as in the two different experiments illustrated in Fig. 3.

These experiments strongly suggest that the effects of ACh on lacrimal cells junctional conductance do not require any change in intracellular concentrations of Ca or protons. Besides this main result, it is worth noting that, with strongly buffered internal solutions, the initial effect of ACh on G was often a transient increase (Fig. 3) similar to that described in Fig. 2*B*, but which was more marked and prolonged when $[Ca]_i$ was not allowed to change. Clearly, the activation of cholinergic receptors has multiple indirect effects on junctional conductance.

ACh effects at the single-channel level

The dual effect of ACh on lacrimal cell junctions (transient increase in junctional conductance followed by a closure of the junction) could *a priori* result either from

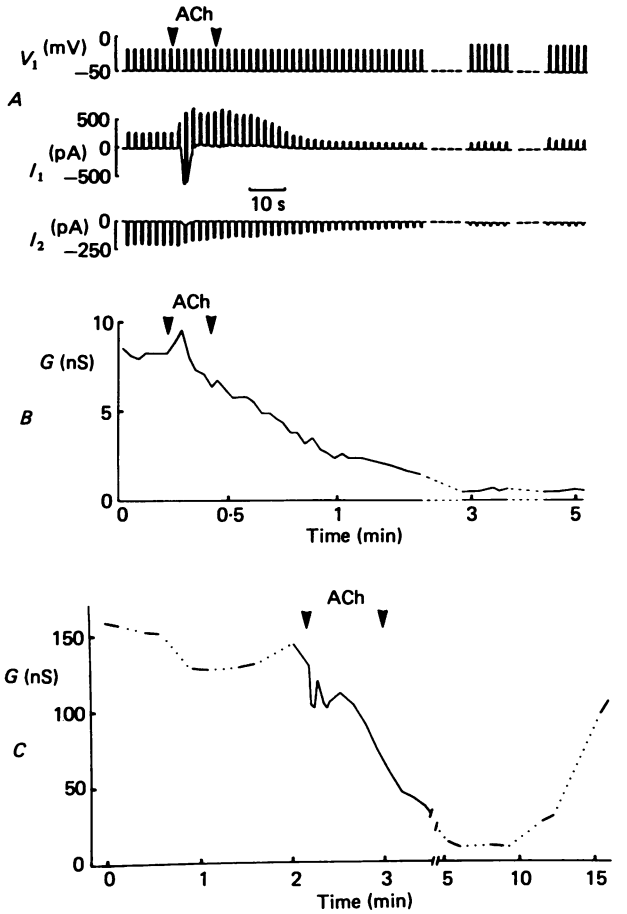

Fig. 2. Effect of ACh on the junctional conductance of cell pairs where $[Ca]_i$ was allowed to move in cell one (C_1) (solution B) but not in cell two (C_2) (solution A). The duration of the ACh applications is marked by arrows. *A*, experimental protocol: V_1 is the voltage applied to C_1, I_1 and I_2 the currents measured in C_1 and C_2 respectively. Holding potential of C_2 is −50 mV. The dotted lines are interruptions of 90 and 100 s respectively. ACh concentration 1 μM. Note that the voltage-pulse amplitude has been increased after the first interruption. *B*, the junctional conductance measured in the experiment shown in *A* is plotted as a function of time. The uncoupling provoked by ACh was irreversible in this particular experiment. *C*, junctional conductance as a function of time in another experiment in which the ACh-induced uncoupling was reversible. During the periods shown by dotted lines, the tape recorder was stopped. Note the change in time scale during the recovery. ACh concentration 2 μM.

variations in the number of open junctional channels, or from changes in their elementary conductance.

To examine this question, it was necessary to follow the effect of ACh on a pair of cells almost completely uncoupled, where single channel fluctuations could be individually resolved. These experimental conditions are difficult to obtain, either because the convenient level of coupling is not maintained long enough or because, as often observed, cell pairs which have been dialysed for a long time in order to obtain

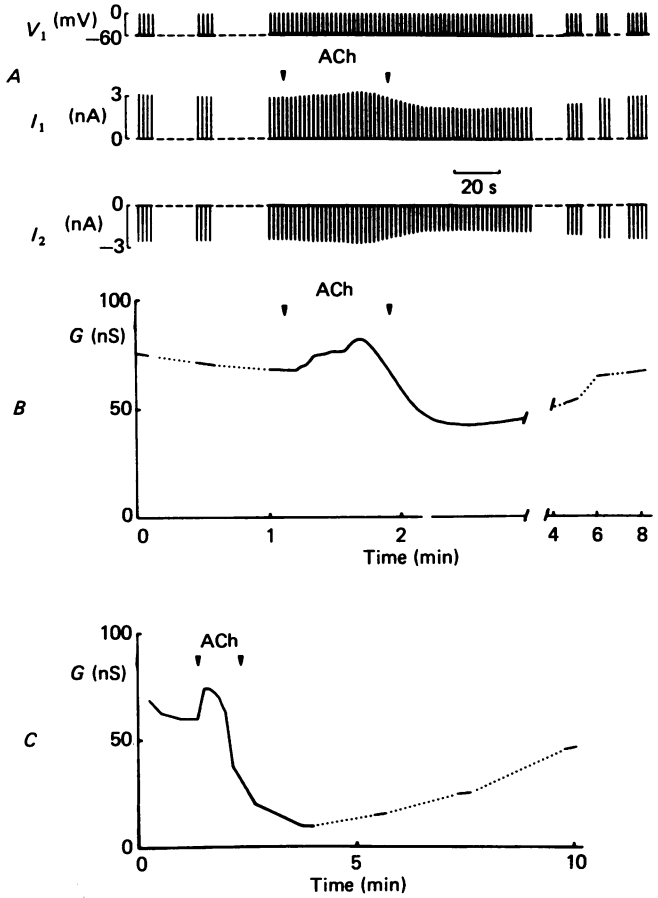

Fig. 3. Effect of ACh (2 μM) on the junctional conductance of pairs of cells where $[Ca]_i$ and $[H]_i$ were strongly buffered (solution C). *A*, the experimental protocol is similar to that described in Fig. 2. *B*, junctional conductance, *G*, measured in the experiment shown above, and plotted as a function of time (the time scales in *A* and *B* are identical; note the record interruption of 1 min, and the change in time scale during the recovery). *C*, junctional conductance, as a function of time in another experiment. In *A*, *B* and *C* the tape recorder was stopped during the dotted lines.

a large spontaneous reduction of the coupling, lose their sensitivity to ACh (note that, as shown below, the Ca-dependent currents and the coupling do not always lose their sensitivity to ACh at the same time). However, in one experiment, illustrated in Fig. 4, these conditions were met. The beginning of the experiment on this cell pair has been described in Fig. 2*A* and *B*: an initial ACh application first triggered two Ca-activated currents in C_1 (dialysed with solution B) and a slight increase in coupling, followed by a marked uncoupling, observed in both C_2 (dialysed with solution A), and C_1. The low level of coupling reached 3 min after the ACh wash-out remained unchanged for the next 6 min.

At this time, a second application of ACh was performed (see arrowheads on the continuous current recording shown in Fig. 4*A*). At this low junctional conductance

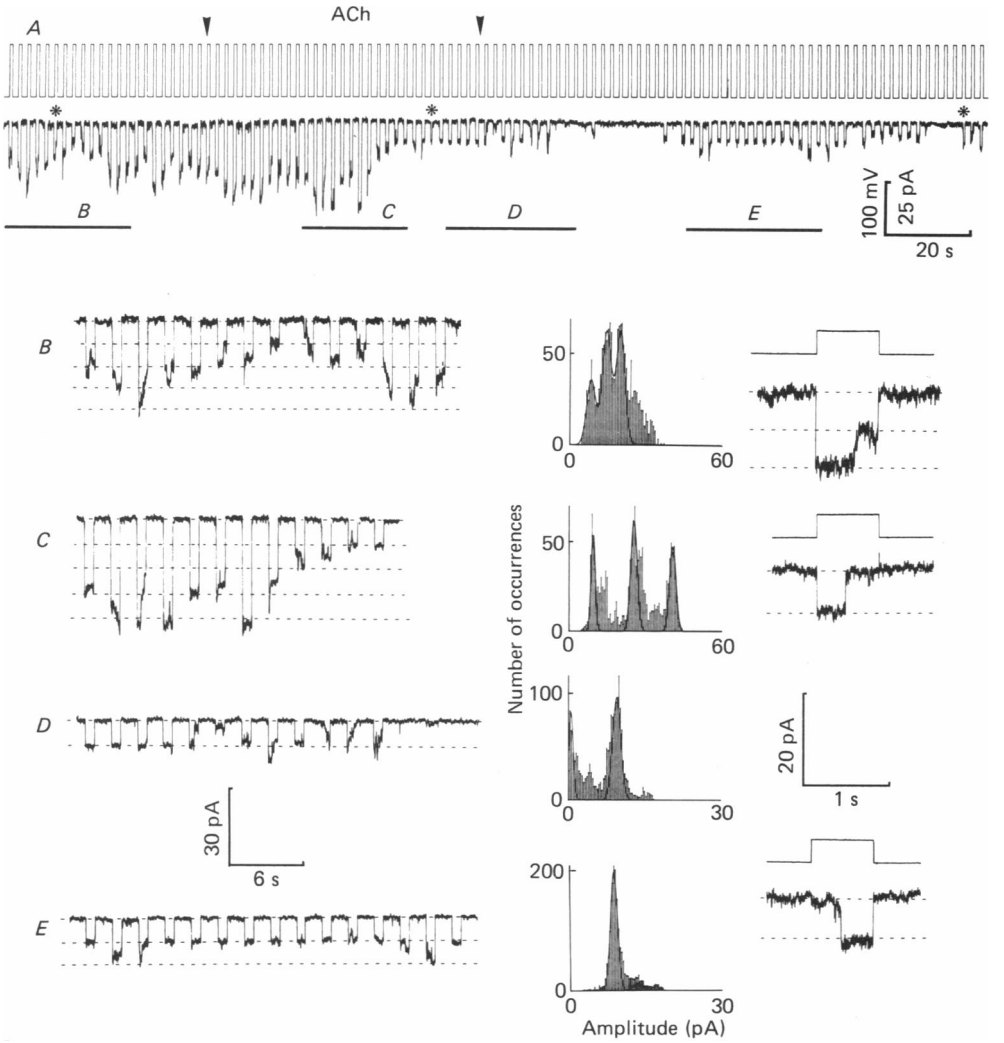

Fig. 4. ACh modulation of the junctional conductance of a weakly coupled pair of cells. The same pair of cells, at two different levels of coupling, has been used in Fig. 2 *A* and *B* and this Figure. Note that the voltage jumps were applied in cell one (C_1) in Fig. 2, and in cell two (C_2) in this Figure. *A*, continuous recording of V_2 (top) and I_1 (bottom), i.e. of the voltage applied to C_2 (100 mV jumps) and the current measured in C_1 respectively. The voltage drop through r_2 was 2·2 mV (1100 pA through 4 MΩ with 50 % series resistance compensation). The voltage drop through r_1 was negligible. C_2 was dialysed with solution A and C_1 with solution B. The duration of the 2 μM-ACh application is marked by the arrows. The rest of the Figure gives more information on the currents measured during selected periods, indicated by the line segments under the current trace in (*A*) before (*B*), during (*C* and *D*) and after (*E*) the application of ACh. For each period, the amplitude histogram corresponds to the data shown on its left (the points are taken only during the voltage jumps). The Gaussians fitted by eye to the major peaks are centred at the following values: *B*: 8·4, 14·5 and 19·9 pA; *C*: 9·4, 25·4 and 40·7 pA; *D*: 0 and 9·5 pA; *E*: 8·7 pA. The dotted lines are drawn on the left and right traces as multiples of the amplitudes italicized above. The far right-hand traces show step-like transitions of I_1 during the different voltage jumps in V_2 marked by * on the top trace in *A*, before (*B*), during (*C*) and after (*E*) ACh application.

level, ACh still affected the coupling in two phases, as in the previous application: first a transient increase and then a decrease which occasionally completely blocked the junction (zero transjunctional current during the voltage pulses). A partial recovery apparently took place 1 min after ACh wash-out. Surprisingly, ACh failed to evoke any inward current in C_1. Thus, even if ACh did not at this time induce any significant increase in $[Ca]_i$, the cells became uncoupled. This argues once more against a direct link between ACh-induced uncoupling and $[Ca]_i$ variations.

To investigate the effects of ACh on the elementary properties of the junctional channels, we analysed more carefully the transjunctional currents recorded during different periods before (Fig. 4*B*), during (Fig. 4*C* and *D*) and after (Fig. 4*E*) ACh application.

The amplitude histograms obtained from these recordings show either one peak (Fig. 4*E*), or several unevenly-distributed peaks (Fig. 4*B*, *C* and *D*). The determination of the single-channel conductance from these recordings (see Methods) cannot thus be devoid of some uncertainty. The estimates that are obtained in this experiment are 86 pS before ACh was applied, 96 and 97 pS during the initial and late phase of ACh application, and 89 pS during the recovery. These figures, confirming mere inspection of the I_2 current trace, show that ACh does not cause a blockade of the junction by reducing the single-channel conductance but by changing the number of open channels. It is even possible that the single-channel conductance is increased during the ACh application (such an effect could play a role in the initial, transitory action of ACh) but this point should be presently taken with caution.

DISCUSSION

The present study was aimed to test the possible involvement of Ca in the intracellular mechanism of gap-junction modulation by ACh in rat lacrimal cells. We found that high $[Ca]_i$ levels ($> 10^{-6}$ M) uncouple lacrimal cells. However, the mechanism of the gap-junction closure by ACh does not require an increase of $[Ca]_i$.

Ca-induced uncoupling of lacrimal cells

In our recording conditions, all the cell pairs tend to spontaneously uncouple, even when $[Ca]_i$ and internal proton concentration were strongly buffered at low values, which suggests that the spontaneous uncoupling is not due to changes in concentration of one of these two ions. This 'spontaneous' uncoupling is a consequence of the cell dialysis following the whole-cell-recording establishment. It could be due, for example, to the slow diffusion of a large molecule, e.g. a protein, involved in the control of the opening of the intercellular channels. Such a functional perturbation could interfere with the junction gating by Ca or H ions. One could also wonder if the $[Ca]_i$ concentration seen by the junction is identical to that present in the pipette. Despite our precautions (using strongly buffered solutions), the two Ca concentrations might differ if the junctions were very close to an efficient cellular system able to modify $[Ca]_i$, e.g. the endoplasmic reticulum. Nevertheless, the apparent threshold value for the $[Ca]_i$ uncoupling found in this study is rather close to that obtained in *Chironomus* salivary glands, where $[Ca]_i$ was monitored with aequorin (Rose & Loewenstein, 1976; Rose & Rick, 1978).

There is one other study of the modulation of a junctional conductance by $[Ca]_i$ where the junction was bathed, but on one side only, with a strongly buffered solution (Spray *et al.* 1982). In that preparation (*Fundulus* blastomeres), the sensitivity of the junction to internal Ca was much lower (50% uncoupling with pCa_i 3·3) than in lacrimal cells (fast and complete uncoupling with pCa_i 5). Aside of species and tissue differences, one source of the difference in Ca sensitivity might come from the more drastic change in the environment of the *Fundulus* junctions (which involved the destruction of one of the two cells of a pair).

ACh modulation of the junctional conductance between lacrimal cells

The experiments with solutions strongly buffered at pH_i 7·2 and pCa_i 8 clearly demonstrate the existence of a Ca-and pH-independent mechanism for the ACh-induced uncoupling. The existence of transient uncontrolled variations of $[Ca]_i$ following ACh application is very unlikely: if present, they would have been revealed by the activation of Ca-dependent currents, detectable when $[Ca]_i$ is higher than 10^{-7} M (Marty *et al.* 1984). Similarly, the existence of transient variations in pH_i appears unlikely because of the high HEPES concentration of our internal solutions. Moreover, a similar ACh-induced uncoupling could be observed in an experiment where both cells were dialysed with a solution strongly buffered at pCa_i 8 and pH_i 7·8. Nevertheless, when $[Ca]_i$ is allowed to move, (in the *in vivo* situation, for example) it is quite possible that $[Ca]_i$ plays a role in the ACh-induced uncoupling (see Iwatsuki & Petersen, 1978*b*) in addition to the Ca-independent mechanism. This could, in particular, explain why the ACh uncoupling tends to develop faster when $[Ca]_i$ is weakly buffered than when it is clamped at a low value.

The fact that ACh can induce both Ca-dependent non-junctional currents and a Ca-independent uncoupling suggests that, in lacrimal cells, more than one second messenger system might be under the control of the activation of cholinergic receptors. Recent results of Evans & Marty (1986) suggest that in lacrimal cells, as in other systems (for review, see Berridge & Irvine, 1984), the trisphosphoinositol may link the muscarinic activation to the intracellular Ca release. At which level the pathways controlling the level of $[Ca]_i$ and the gap-junction conductance become divergent remains to be determined.

This paper also shows that ACh has more than one action on the junctional conductance. The most obvious effect is the junction closure. Although partial conductance-states of the single junctional channels can be observed under control conditions, the blocking action of ACh is not due to an increase in the relative frequency of these states of lower conductance, but instead it results from a decrease in the number of fully open channels. Paradoxically, ACh can also cause a transient increase in junctional conductance. This effect is observed more clearly when $[Ca]_i$ is clamped at a low value, probably because the uncoupling in this case develops more slowly than when $[Ca]_i$ increases. Is the coupling increase due to a change in the number of open channels or in their elementary conductance? We have no clear answer to this question but our results are compatible with both possibilities. Part of the difficulty in examining this point was the existence in the related experiments of preferential levels of intermediate conductance (i.e. not quantal). This suggests that there may be more than one stable level of single junctional channel conductance.

This paper raises several questions: it was previously thought that Ca was the most likely internal messenger linking the cholinergic activation to the cell uncoupling. We have shown that ACh is able not only to uncouple but also to increase the coupling of two cells, and that Ca is not the internal messenger involved in these two actions. The nature of these messengers is obviously the next question to address.

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