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PHYSIOLOGICAL MODULATION OF GAP JUNCTION PERMEABILITY

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SUMMARY

In many tissues cells communicate directly through arrays of intercellular channels which are organized to form gap junctions. These channels are permeant to inorganic ions as well as to small hydrophilic molecules up to M_r 2000. The electrical and chemical coupling provided by such junctions is under the control of intracellular and, in many cases, extracellular substances. The latter (hormones or neurotransmitters) function *via* the activation of intracellular second messengers. These can rapidly affect the state of opening of the junctions, or induce long-term modulation of the coupling. What are the second messengers and how do they control the functional state of the junctions? These questions remain largely unanswered, although several internal molecules are thought to be involved in these modulations (e.g. Ca^{2+}, H^+ or cyclic AMP). The double patch-clamp technique which enables control of both the intracellular *milieu* and high resolution measurement of transjunctional currents, has recently been applied to study these problems. In particular, it is now possible to examine at the single channel level how junctional conductance is modulated in terms, for example, of the number of open channels or channel elementary properties.

INTRODUCTION

In most animal tissues, direct intercellular communication occurs through gap junctions (see Bennett & Goodenough, 1978; Larsen, 1983). This form of communication can be temporarily increased, reduced or even interrupted. This review will consider where, how and why such modulations take place. Gap junctions were first defined morphologically as specialized intercellular junctions with a characteristic extracellular space (the gap) of 2–3 nm between the membranes of the two adjoining cells. In freeze fracture preparations, these junctions appear as typical aggregates of particles on the *p* face, and as complementary pits on the *e* face. There is a good correlation between the presence of morphologically identified gap junctions and the existence of intercellular communication (demonstrated by electrical coupling or by the passage of tracer molecules) between coupled cells, and it is now generally agreed that the gap junction is the structure which mediates this communication and that the intercellular channel is most probably contained in the gap junction particles (see below). Only rarely can electrical coupling not be correlated with the observation of a

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classical gap junction structure (Azarnia & Loewenstein, 1977; Williams & De Haan, 1981). Nevertheless, in these exceptional cases, sparse particles, similar to those found as clusters in gap junctions, have been observed to cross the extracellular space between the cells, and may constitute junctional channels (Larsen, Azarnia & Loewenstein, 1977). Thus, to avoid systematic circumlocutions, we will use the term 'gap junction' without limitation to specific morphological data, and even in cases where morphological identification of gap junctions has not been performed.

The first part of this paper will describe the heterogeneity of gap junction structure and function. Succeeding sections will deal with the long-term regulation (e.g. action of hormones) and the short-term modifications (e.g. those induced by neurotransmitters).

Both morphological and functional methods have been used to study the modulations of gap junctions. The morphological data have been reviewed and discussed elsewhere (e.g. Larsen, 1983; Peracchia & Bernardini, 1984; but see also the recent results of Page, Karrison & Upshaw-Earley, 1983; Hanna, Pappas & Bennett, 1984; Green & Severs, 1984; Miller & Goodenough, 1985). Functional studies deal with the transfer of molecules through gap junctions. The transfer of tracer molecules from one cell to another can be followed by optical techniques, for fluorescent compounds, or by autoradiography, for radiolabelled markers. These methods give interesting, but mainly qualitative information on the permeability state of a gap junction. They must therefore be used with caution when studying the mechanisms of gap junction modulation. As shown by Zimmerman & Rose (1985), the size of an open gap junction channel cannot be derived with certainty from measurements of cell-to-cell transfer of fluorescent dyes of various sizes, because the measures of this transfer also depend on the detection threshold and on the possibility of loss and sequestration of the tracers. Another approach is to study electrical coupling (i.e. the transfer of small ions between coupled cells). The simplest method used is to measure the coupling coefficient, the ratio $V2/V1$, between a voltage $V1$ applied in one cell and the resulting change $V2$ measured in a coupled cell. This technique does not allow a quantitative characterization of the properties of the gap junction channel because the coupling coefficient depends on the resistances of both the junctional and the nonjunctional membranes. Indeed, as pointed out by Socolar (1977), with this technique a marked change in the junctional conductance may leave the coupling coefficient almost unchanged and, conversely, a change in the nonjunctional membrane resistance can modify the coupling coefficient, even in the absence of gap junction modification. Examples of this latter case have been reported (Spira & Bennett, 1972; Carew & Kandel, 1976; Spira, Spray & Bennett, 1980).

A more satisfactory measure of junctional conductance can be obtained by voltage clamping the cells on both sides of a gap junction. This has been recently done, using classical intracellular electrodes, on isolated pairs of coupled cells (Spray, Harris & Bennett, 1981*a*; Obaid, Socolar & Rose, 1983). However, with this method, one does not know if alterations in the global junctional conductance are due to changes in the number of open channels or to modifications in their elementary properties.

This limitation can now be overcome in some cases, with the whole cell tight-seal recording method (Marty & Neher, 1983), which can be applied simultaneously to both coupled cells (Neyton & Trautmann, 1985). With this method, as in the case of a double voltage-clamp system (using four electrodes), the transjunctional current can be measured by stepping the voltage in one cell and measuring the induced current, at constant voltage, in the other. There are, however, several differences between the two techniques. For the double patch-clamp, only two electrodes are used instead of four. As a result, the voltages are selected, but not measured, and there may be important series resistance artifacts which have to be corrected for. It is possible to use this method with pairs of small cells (10–20 μm diameter) and to obtain such a low background noise from the whole cell membrane that, in many cases, single channel currents through the gap junctions are measurable, when only a few channels are simultaneously open (Fig. 1). The double patch-clamp method was used on a preparation of isolated pairs of lacrimal gland cells to measure the ionic selectivity of the junctional channel, its elementary conductance and to study its

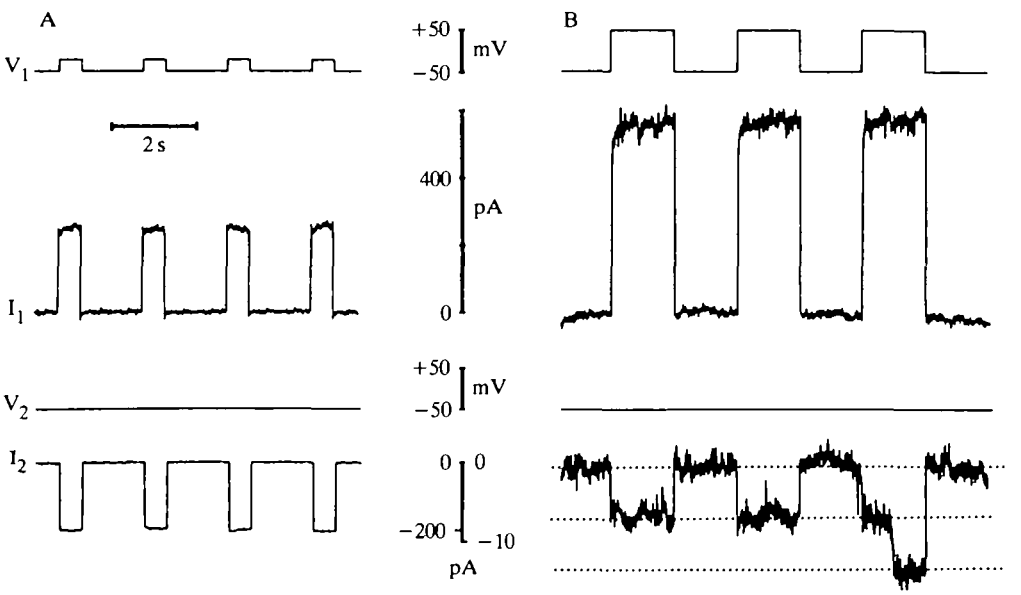

Fig. 1. Measurement of a junctional conductance with the double patch-clamp method at initial (A) and late (B) phases of recording in an isolated pair of rat lacrimal gland cells. V_1 and V_2 are the command voltages of cells 1 and 2, respectively, and I_1 and I_2 are the currents measured in these cells. At a common voltage of −50 mV all currents (leak and junctional) are null. The current changes in cell 2 in response to voltage jumps in cell 1 (V_2 being constant) correspond mainly to the currents which flow through the junction. The junctional conductance, G, is given by the ratio between the changes in I_2 and V_1, after the corrections for the series resistances have been made (see Neyton & Trautmann, 1985). In Figs 3 and 4, only V_1 and I_2 will be given. Note that one can obtain the current flowing through nonjunctional membrane in cell 1 by adding I_1 and I_2. The junction, initially widely open (A, G = 8 nS) was almost closed after 11 min (B). The quantal variation of junctional conductance of 70 pS, observed in the third transjunctional voltage jump in B, was presumably due to the opening of a single junctional channel.

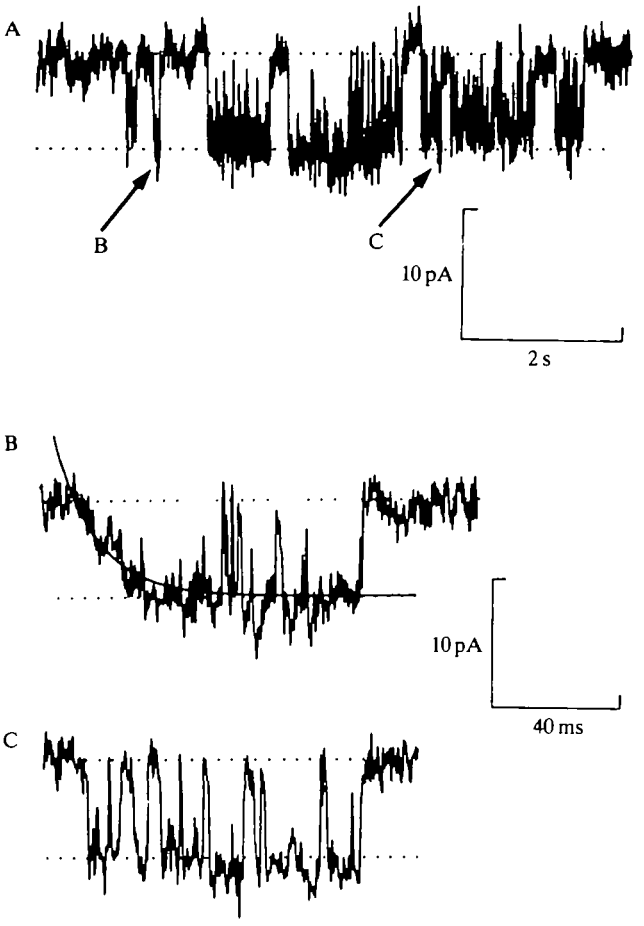

Fig. 2. Kinetics of a junctional channel in a pair of rat lacrimocytes. With a steady transjunctional voltage ($V_2 = -40$ mV, $V_1 = +50$ mV), all the junctional channels were closed before one opened. It then oscillated for 5·6 s between the open and closed states (A). At two instants marked by the arrows, the current trace I_2 was expanded, showing that transitions may be either much slower (exponential time constant = 14 ms in B) or faster (C) than the time detection threshold (0·5 ms in this experiment).

voltage sensitivity (Neyton & Trautmann, 1985). It was found that transitions between the open and closed states are often unusually slow, extending over tens of milliseconds. Besides these slow transitions, fast transitions (in the submillisecond range) can occur (Fig. 2).

THE INTERCELLULAR CHANNEL

We will here briefly review the gap junction channel properties that may be of interest in understanding the various types of junctional modulation. More extensive reviews on the biochemistry, structure and functioning of the junctional channel

can be found elsewhere (Loewenstein, 1981; Peracchia, 1985; Revel, Nicholson & Yancey, 1985; Spray & Bennett, 1985).

Structure of the junctional channel

It is generally admitted that the gap junction particles observed in freeze fracture are the morphological counterpart of the intercellular channel. X-ray diffraction (Caspar, Goodenough, Makowski & Phillips, 1977; Makowski, Caspar, Phillips & Goodenough, 1977) and Fourier analysis of electron micrographs (Unwin & Zampighi, 1980) have shown that a junctional channel consists of two hexameric proteins, one contributed by each membrane. These paired hexamers form along their central axis an aqueous pore which connects the cytoplasms of the two cells.

This general scheme raises different questions. What are the subunits of this dodecameric protein complex? Are they identical in different gap junctions? What is the three-dimensional structure of the complex? Partial answers have been given to these questions. The isolation and characterization of the subunit of the gap junction channel complex is still a subject of controversy. Most authors, nevertheless, agree that in several tissues the subunit is a protein of $M_r = 26\,000$–$28\,000$ (e.g. review by Revel *et al.* 1985; but for other values see also Finbow, Shuttleworth, Hamilton & Pitts, 1983; Manjunath, Goings & Page, 1984; Warner, Guthrie & Gilula, 1984). Peptide maps (Gros, Nicholson & Revel, 1983) and partial sequencing (Nicholson *et al.* 1983; Revel, Nicholson & Yancey, 1984) indicate that the primary sequence of the subunits appears to vary widely accordingly to the tissues, even in the same species. Such a broad variation was *not* observed between different species for the *same* tissue (Nicholson *et al.* 1981; Zigler & Horwitz, 1981). Nevertheless, the subunits isolated from different tissues often share common antigenic properties (for review see Hertzberg, 1985). Thus, parts of these proteins have probably been conserved, while other parts have somehow become tissue specific. Consequently, heterogeneity is expected in the three-dimensional structure of the junctional channels of different origins. Such heterogeneity has, in fact, been observed in isolated gap junctions from heart and liver (see Page & Manjunath, 1985). The cytoplasmic surfaces of cardiac gap junctions (isolated in the presence of proteolysis inhibitors) are covered with a layer of fuzzy material which was never seen in liver junctions. The presence of this material is associated with the isolation of a major protein (presumably the subunit) of $M_r = 44\,000$–$47\,000$. This protein is cleaved to $M_r = 29\,000$ when proteolysis is not inhibited. Thus, a large part of the cardiac junction subunit appears to protrude on the cytoplasmic face of the junction. An equivalent of this protruding part is absent in liver junctions. In this tissue, however, the cytoplasmic part of the subunits has a complex organization. In particular, the hexamers together form a small structure which can plug the mouth of the channel (Makowski *et al.* 1984). This result supports the idea that gating mechanisms involve changes in the structure of the cytoplasmic part of the channel (Makowski *et al.* 1984) and may not be identical in all gap junctions.

The junctional channel, as with other membrane ionic channels, can assume different functional states. Three physiologically relevant factors are known to affect

their permeability directly: transjunctional or transmembrane voltage, intracellular calcium concentration and internal pH. Yet the sensitivities to these parameters vary among gap junctions of different tissues.

Voltage dependence

Some junctions are highly sensitive to voltage applied across the junction. This is particularly true for certain electrical 'rectifying' synapses (Furshpan & Potter, 1959; Auerbach & Bennett, 1969; Nicholls & Purves, 1972), where a strong rectification can occur (i.e. currents pass through the junction more easily in one direction than in the other). At these junctions, dye transfer also depends on the polarization of the junction (Margiotta & Walcott, 1983; Giaume & Korn, 1984). These channels thus possess a dipole-like, voltage-sensitive structure which may move in the electric field. Other junctions present a symmetrical, high sensitivity to voltage applied either across the junction (Spray, Harris & Bennett, 1979; White, Spray, Campos de Carvalho & Bennett, 1982; Spitzer, 1982) or between the interior and the exterior of each of the coupled cells (Obaid *et al.* 1983; Spray *et al.* 1984). Such junctions probably possess two voltage-sensitive structures, instead of one as in the rectifying electrical synapse. More commonly, other junctions are apparently devoid of such structures since they are totally voltage insensitive (see review by Spray, White, Verselis & Bennett, 1985).

An intermediate situation is found in lacrimal glands. Fully open junctions are always voltage insensitive. This is also the case for some partly closed junctions. In Fig. 3A, the junction exhibited a similar voltage insensitivity at the beginning of the recording (G = 40 nS) as it did 9 min later, when it was almost closed (G = 600 pS). However, a voltage sensitivity was observed in some junctions that were almost completely closed. In these cases, the number of open channels depended upon the transjunctional voltage (but not on the membrane potential). In the experiment illustrated in Fig. 3B, a transjunctional voltage of one polarity caused more channels to open, whereas voltage jumps of the other polarity induced channel closure. When one or two channels at a time were open, their conductance could be measured, and apparently it was unaffected by voltage (see dotted lines). Thus, in gap junctions between lacrimocytes, when a voltage dependence is present, the potential appears to affect the number of open channels and not their elementary conductance. A similar situation seems to exist in the *Chironomus* salivary gland (Zimmerman & Rose, 1985). At least three kinetic processes can be revealed by the voltage dependence in the lacrimocyte preparation. They can occur in the submillisecond range (apparently instantaneous rectification), and in the range of tens of milliseconds and of tens of seconds (Fig. 4, see the legend for more details). The variability in the voltage sensitivity of lacrimal gland gap junctions suggests that this is not an intrinsic property of the lacrimocyte junctional channels.

pH and calcium dependence

A great deal of evidence shows that an increase in intracellular Ca^{2+}, Ca_i^{2+}, or a decrease in internal pH, pH_i, can reduce gap junctional permeability (see reviews by

Lowenstein, 1981; Spray & Bennett, 1985). However, the range of Ca^{2+} and H^+ intracellular concentration at which these modulations do occur, and the independency of their mechanisms, are still a matter of debate. For example, the minimum Ca_i^{2+} concentration inducing a decrease in gap junction permeability has been claimed to be either in the micromolar range (Dahl & Isenberg, 1980) or in the millimolar range (Spray, Stern, Harris & Bennett, 1982). Junction gating, by a decrease in pH_i, seems to occur in a more restricted range with a pKa for the gating varying between 6·4 for mammalian liver junctions (Spray & Hertzberg, 1985) and 7·3 for coupled fish blastomeres (White *et al.* 1982). An exception is nevertheless found in the lens, where coupling between the fibres appears to be poorly sensitive to CO_2 exposure (Scheutze & Goodenough, 1982).

Some of these discrepancies may result from differences in the experimental approach used. Cell regulations of internal Ca^{2+} and pH are coupled (e.g. *via* Ca^{2+}/H^+ exchange, or Ca^{2+}/Na^+ and Na^+/H^+ exchanges) so that increasing the

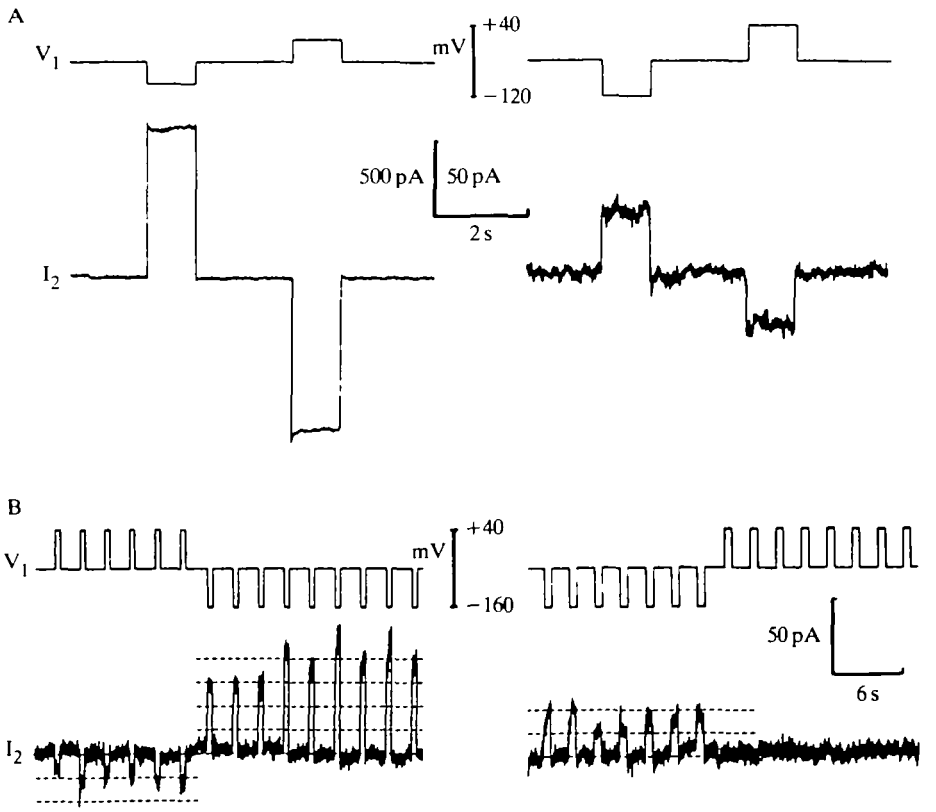

Fig. 3. Voltage dependence of junctional channels in pairs of lacrimocytes. (A) An example of a junction which is insensitive to voltage both in the initial high-conductance state (left) and in a later almost uncoupled state (right). (B) In another cell pair the junctional conductance was voltage-dependent. At a moment of the recording (left), one or two channels were open if the current was flowing in one direction and three to five if the current flowed in the other direction. 30 s later, the current could flow in only one direction (right). At a later stage (not shown), all channels were definitely closed.

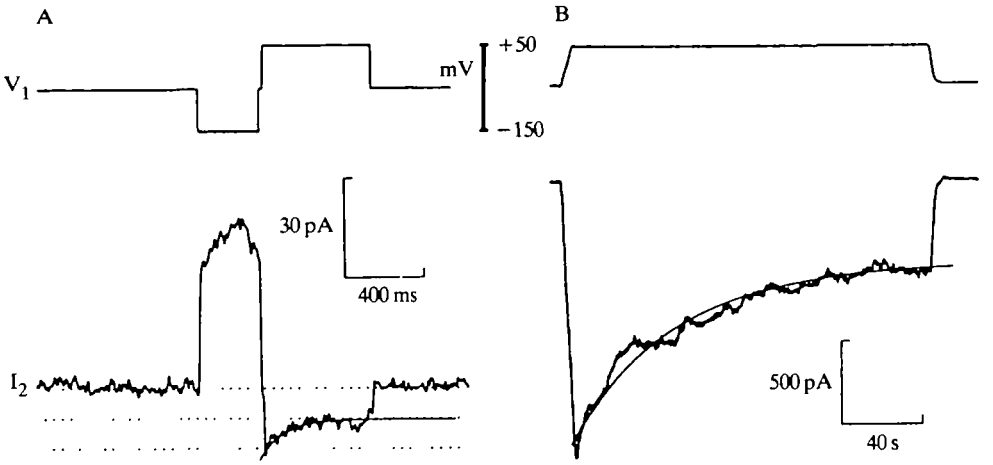

Fig. 4. Voltage dependence of junctional channels in pairs of lacrimocytes. (A) An example of a junction where a transjunctional voltage of one polarity increases the number of open channels, whereas the other polarity causes a channel closure, by two different kinetic processes. The first one, apparently completed in a few milliseconds, reduced the number of open channels from five (at the end of the negative voltage jump) to two (at the beginning of the positive voltage jump). During that later jump, another channel closed, with a time constant of 118 ms. Processes on the same time scale have been described in Fig. 2. (B) The level of coupling was too high in this other junction to allow a resolution of the transjunctional current at the single channel level. A transjunctional voltage of +100 mV slowly reduced the junctional current, with a more-or-less exponential time course (time constant of the exponential fit = 51 s). The meaning of the current changes observed on one channel and on a population of channels are, of course, completely different.

intracellular concentration of one ion may lead to an undesired increase of the other (Meech & Thomas, 1977; Rose & Rick, 1978). This problem has been avoided using experimental protocols in which the concentration of one ion was increased, whilst the concentration of the other was constantly monitored. These experiments show that an increase in pH_i, at constant Ca_i^{2+} (Rink, Tsien & Warner, 1980), as well as an increase in Ca_i^{2+} without pH_i variation (Rose & Rick, 1978), actually decrease junctional permeability. Another approach consists of perfusing the cell interior with media of controlled composition. This method produces a quantitative change in the intracellular concentration of only one ionic species at a time. Surprisingly, variable results have been obtained with this strategy: (a) in pairs of *Fundulus* blastomeres (in which only one cell was internally perfused) a high sensitivity to pH (pKa = 7·3) was observed, whereas a low sensitivity to calcium (pK = 3·5) was recorded (Spray, Stern, Harris & Bennett, 1982); (b) in pairs of rat lacrimal gland cells (perfused on both sides of the junction using patch-clamp electrodes) a higher sensitivity to calcium was found (Neyton & Trautmann, 1986); (c) in crayfish axon septate junctions, perfusion of both sides of the junction resulted in a loss of sensitivity to both calcium and pH (Johnston & Ramon, 1982). The latter authors have suggested that internal cell perfusion may cause the disappearance of a molecule, or of a part of the channel structure, which is directly involved in the channel gating. Calmodulin,

a protein which shows conformational changes upon binding with calcium or protons (see e.g. Pundak & Roche, 1984), could be this intermediate. This protein indeed binds to lens and liver gap junction proteins (Hertzberg & Gilula, 1981; Welsh *et al.* 1982). Moreover, calmodulin inhibitors inhibit the CO_2-induced uncoupling of amphibian embryonic cells (Peracchia, Bernardini & Peracchia, 1983; Peracchia, 1984) and can also produce uncoupling in the epidermis of an insect (Lees-Miller & Caveney, 1982). Lastly, calmodulin is required to confer a calcium-sensitive gating to channels obtained by reincorporation into liposomes of lens isolated gap junction proteins (Girsch & Peracchia, 1985). Calmodulin may thus be directly involved, at least in the lens, in the gating mechanism of junctional channels by calcium. However, an indirect involvement of calmodulin (e.g. through the control of a protein kinase), or even the possibility of a direct action of H^+ or Ca^{2+} ions on the channel structure itself cannot be excluded.

LONG-TERM MODULATION OF GAP JUNCTIONS

Long-term modulation of direct cell-to-cell communication has been described in several tissues, during development or in response to extracellular messengers. These modulations could result from changes in the properties of the elementary channels or in their number (by acting in the latter case on their turnover mechanisms). Gap junctions form in the so-called formation plaques (Johnson, Hammer, Sheridan & Revel, 1974) where channel assembly and functional maturation is thought to occur (see Larsen, 1985). Gap junctions may disappear, and their removal from the surface of contact between the coupled cells takes place through an endocytosis by one of the two cells (see Larsen, 1985). We will review some examples of long-term modulations, illustrating and discussing these different mechanisms.

Uterine muscle

In the uterine muscle of pregnant mammals, a change in the concentration of circulating hormones appears to occur just prior to parturition and may be responsible for it (see Thorburn & Challis, 1979). It consists of an increase in oestrogen and prostaglandins F2, E1 and E2, and a decrease in progesterone and prostaglandin I2. These hormonal changes are paralleled by the appearance of large and numerous gap junctions between myometrial cells (Garfield, Sims & Daniel, 1977), an increase in electrical coupling (Sims, Daniel & Garfield, 1982) and metabolic cooperation (Cole, Garfield & Kirkaldy, 1985). This correlation strongly suggested that gap junction formation was hormonally controlled. This hypothesis is supported by the *in vivo* and *in vitro* application of exogenous analogues of the hormones (see the review of Cole & Garfield, 1985).

The precise role of each hormone and the mechanisms involved in their action are still unclear. Oestrogen alone can induce the gap junction formation in the uterine muscle, and this effect requires RNA and protein synthesis (Garfield, Merrett & Grover, 1980). This may suggest a direct control by oestrogen of junctional channel subunit synthesis, through the activation of nuclear steroid receptors. Alternatively,

oestrogen might activate the neosynthesis of macromolecules involved in the control of *other* stages of the gap junction formation process (e.g. affecting cell surface properties or channel subunit assembly). Such an alternative has indeed been found in a very different preparation: the C1-1D cell line (a malignant subline of mouse L-cells), in which the formation of gap junctions is induced by an increase in intracellular cyclic AMP (Azarnia, Dahl & Loewenstein, 1981). Even if, in this case, the junction formation requires protein synthesis, the amount of 27-kD junctional channel subunit, measured with specific antibodies, does not change when the junction formation is induced (see Hertzberg, 1985).

Ovarian follicle

In the mammalian ovarian follicle, the oocyte is enclosed by a population of granulosa cells whose multiplication and differentiation is controlled by gonadotropins and steroid hormones. At different stages of follicular growth, gap junctions are found between granulosa cells and between granulosa cells and the oocyte (see e.g. Burghardt & Matheson, 1982). Interestingly, the level of intercellular communication in the follicle appears to be hormonally controlled through a modulation of the processes of gap junction formation and removal. During the early growth of ovarian follicles in hypophysectomized animals, the appearance of gap junctions between granulosa cells does not require any hormonal stimulation, but the number and size of the junctions are markedly enhanced by follicle-stimulating hormone (FSH) (Burghardt & Matheson, 1982). In the later stages of follicular growth, FSH also stimulates the internalization of gap junctions in granulosa cells.

In contrast, a few hours after the application to hypophysectomized animals of ovulatory doses of hCG (human chorionic gonadotropin, a hormone which binds to granulosa cell receptors for LH, the luteinizing hormone), the coupling between granulosa cells and oocyte decreases, and a dissociation of granulosa cells is observed in preovulatory follicles (Gilula, Epstein & Beers, 1978; Moor, Smith & Dawson, 1980; Eppig, 1982). These hCG effects are paralleled by a decrease in the number of gap junctions between oocyte and granulosa cells (Gilula *et al.* 1978) and by a stimulation of gap junction removal in granulosa cells (see Larsen, 1985). The hCG effects were mimicked in some *in vitro* preparations by applications of LH (Dekel, Lawrence, Gilula & Beers, 1981) and in other preparations by FSH (Moor *et al.* 1980). It should be emphasized that ovulation *in vivo* is preceded by a peak in the plasma oestrogen concentration, followed rapidly by a surge in LH and FSH concentrations.

In summary, a hormonal control of the formation and removal of gap junctions can be observed in the ovarian follicle of mammals, and involves at least gonadotropins, LH and FSH. It is worth noting that FSH has two opposite actions: on one hand, it induces an increase in the number of gap junctions during follicle growth, and on the other it decreases this number just prior to ovulation. The mechanisms involved in the hormonal modulation of ovarian follicle gap junctions are poorly understood. Cyclic AMP might be a second messenger, since both FSH and LH induce an increase of its intracellular concentration in granulosa cells (see Dufau & Catt, 1978)

and, in some preparations, pharmacological treatments that increase internal cyclic AMP mimic the hormones' actions on granulosa cell gap junctions (Dekel *et al.* 1981). An increase in intracellular cyclic AMP stimulates gap junction formation in other preparations including various mammalian cell lines (Flagg-Newton, Dahl & Loewenstein, 1981; Azarnia *et al.* 1981; Radu, Dahl & Loewenstein, 1982) and cultured rat sympathetic neurones (Kessler, Spray, Saez & Bennett, 1984). In these preparations, the cyclic-AMP-induced gap junction formation requires RNA and protein synthesis (Azarnia *et al.* 1981; Kessler *et al.* 1984) and may result from protein phosphorylation by a cyclic-AMP-dependent protein kinase (Wiener & Loewenstein, 1983). The target(s) of this kinase and the consequences of this protein phosphorylation are unknown.

Insect larval tissues

In the salivary glands of *Drosophila hydrei* larvae (Hax, van Venroogi & Vossenberg, 1974) and in the larval epidermis of the beetle *Tenebrio molitor* (Caveney & Blennerhasset, 1980), the moulting hormone 20-hydroxyecdysone induces, within a few hours after application, an increase in electrical coupling. This results from modifications of both the junctional and nonjunctional membranes. In contrast with the two examples given above (uterine muscle and ovarian follicle), the increase in coupling conductance observed in these cases is due to a modification of the properties of pre-existing junctional channels (presumably a change in their gating kinetics, see Caveney & Safranyos, 1985) rather than to an addition of new ones to the junctions (Caveney, Berdan & McLean, 1980). The hormonal action does not involve protein synthesis, but, surprisingly, can be prevented by actinomycin D, an inhibitor of RNA synthesis (Caveney *et al.* 1980). An increase in internal cyclic AMP has been claimed both to reverse the effect of 20-hydroxyecdysone, in the beetle epidermis (Caveney, 1978), and to have the contrary action, by mimicking the action of the hormone in the *Drosophila* salivary gland (Hax *et al.* 1974). In the latter case, however, a rise in internal cyclic AMP induces a hyperpolarization of the cells which, *per se*, elicits an increase in the voltage-sensitive junctional conductance (see above).

SHORT-TERM MODULATION OF GAP JUNCTION CONDUCTANCE

In a few cases, physiological stimuli have been shown to cause changes in gap junction conductance, in the second to minutes range. These modulations are too fast to be due to a change in the junctional channel turnover. They most probably result from alterations in the elementary properties of the channels. The fastest modulation is that exerted by voltage on some gap junctions. This point has been discussed above. Although most junctions are sensitive to changes in internal calcium and pH, a direct role of these ions in a physiological modulation has not yet been demonstrated (see e.g. the discussion in Ramon, Zampighi & Rivera, 1985). In two cases, it was clearly shown that a neurotransmitter could reversibly decrease gap junction permeability: acetylcholine (ACh) can uncouple cells of exocrine glands,

either pancreas or lacrimal glands (Iwatsuki & Petersen, 1978*a*; Neyton & Trautmann, 1986) and dopamine (DA) can uncouple horizontal cells of turtle or fish retina (Gerschenfeld, Neyton, Piccolino & Witkovsky, 1982; Teranishi, Negishi & Kato, 1983; Piccolino, Neyton & Gerschenfeld, 1984).

Modulation of the permeability of horizontal cell gap junctions by dopamine

The axon terminals of the H1 horizontal cells (H1ATs) in the turtle retina, like the H1 cell bodies in the fish retina, are electrically coupled by extensive gap junctions (see e.g. Witkovsky, Owen & Woodworth, 1983) and constitute large functional networks. The hyperpolarizing responses of these cells to light spot stimuli still increases when the diameter of the spot enlarges far beyond their anatomical arborization under control conditions. Thus these cells possess a very large receptive field. Preliminary experiments by Negishi & Drujan (1979) showed that DA causes a narrowing of the receptive field profile of fish horizontal cells. A similar effect of DA was also found in the turtle retina (Gerschenfeld *et al.* 1982; Piccolino *et al.* 1984). The hyperpolarization provoked in an H1AT by a light spot of small diameter (i.e. which covers only the central part of its receptive field) is increased in the presence of DA at micromolar concentrations (maximal effect at 2–10 μmol l^{-1}). When the light stimulates the periphery of the receptive field of an H1AT (annulus of light) the light response of the peripheral axon terminals is less well transmitted to the central one in the presence of DA. The onset of the DA action (2–5 min) is related to the DA diffusion in the tissue and, possibly, to the production of second messengers (see below). Such a reduction of the size of the H1AT receptive field indicates that DA reduces the coupling in the H1AT network. This decrease of coupling could result either from an increase in the nonjunctional membrane conductance of the axon terminals, or from a decrease in their junctional conductance. The latter case has been demonstrated by two in-dependent methods. First, the spread of intracellularly injected current in the H1AT network was examined. The potential change measured in an H1AT, in response to the injection of current in another H1AT (located at less than 0·1 mm from the first one), was *increased* by DA. This result may appear paradoxical. Yet reduction of gap junction conductance induces a shrinkage of the functional network. This will reduce the spread of current in the furthest H1ATs, but produce in the injected axon terminal and its closest neighbours larger voltage changes for a given current injection. In contrast, an increase of the nonjunctional conductance would also shrink the network, but the amplitude of the electrotonic potentials evoked by the current injection would decrease in *all* the cells of the network.

That DA affected the junctional conductance between H1ATs was confirmed in experiments with Lucifer Yellow, a fluorescent dye which can permeate junctional channels, but not nonjunctional membranes (Stewart, 1978; Bennett, Spira & Spray, 1978). This dye, when injected into one H1AT under control conditions, diffuses to the others through the junctions and thus stains a large network (see Fig. 5A). The mean size of the stained network is markedly reduced when the preparation is exposed to DA (see Fig. 5B). Three types of measure (size of the

receptive field to light stimuli, spread of current, spread of a dye) thus give convergent results, suggesting that DA closes the gap junctions between H1ATs in the turtle retina. The DA receptors involved in that modulation are certainly localized on the horizontal cell itself and not in another interneurone, because the effects of DA persist when synaptic transmission is blocked by 2 mmol l^{-1} cobalt. Moreover, it was shown that an endogenous release of DA may physiologically modulate the coupling in the turtle H1AT network. Indeed, the effects produced by DA can be mimicked by the application of drugs known to stimulate the release of DA by nerve terminals: both amphetamine and DOPA reduce the H1AT receptive field.

Cyclic AMP appears to be involved as a second messenger in the action of DA on the gap junctions of H1ATs. Its inhibitory action on gap junction conductance is quite different from that previously described on long-term modulation. The evidence for this is as follows.

(1) The effects of DA on H1AT gap junctions are blocked by DA antagonists specific for the D1 receptor type; in the retina, as in the brain, the activation of dopamine D1 receptors stimulates the adenylate cyclase (Kebabian, Petzold & Greengard, 1972; Brown & Makman, 1972; Watling & Dowling, 1981).

(2) Forskolin (a compound known to directly activate the cyclase) causes a closure of gap junctions between H1ATs as measured by the three methods described above (see Fig. 5C).

(3) Similar effects are obtained by inhibiting the degradation of cyclic AMP: if the action of the phosphodiesterase is inhibited by isobutylmethylxanthine (IBMX) or theophylline, the H1ATs gap junctions appear to close (see Fig. 5D).

Finally, the intercellular communication in the network of the axon terminals of turtle H1 horizontal cells may be physiologically modulated by dopaminergic terminals, which, by releasing DA, could evoke an increase in the concentration of cyclic AMP in the H1ATs. A similar conclusion has also been reached in experiments with an isolated fish retina preparation (Teranishi *et al.* 1983), or on pairs of cultured, isolated horizontal cells (Lasater & Dowling, 1985). However, it is still not known if cyclic AMP acts directly on the gap junction channels of horizontal cells, or if another intermediate, like a cyclic-AMP-dependent protein kinase, is needed in its action.

Cyclic AMP appears also to be involved in a short-term modulation of the gap junctions between mammalian cardiac cells (De Mello, 1984) and between rat hepatocytes (Saez *et al.* 1985). However, in these two preparations, increasing intracellular cyclic AMP induces a fast *increase* in junctional conductance. With hepatocytes, the cyclic-AMP-induced modulation may be physiologically relevant, since application of glucagon or stimulation of β-adrenergic receptors also produces a rapid increase of gap junction conductance, which can be blocked by the Walsh inhibitor (of cyclic-AMP-dependent protein kinase). Moreover, the catalytic subunit of this kinase can apparently phosphorylate the major intrinsic liver gap junction protein (presumably the junctional channel subunit). Thus glucagon, or β-adrenergic agonists may increase the conductance of gap junctions between hepatocytes

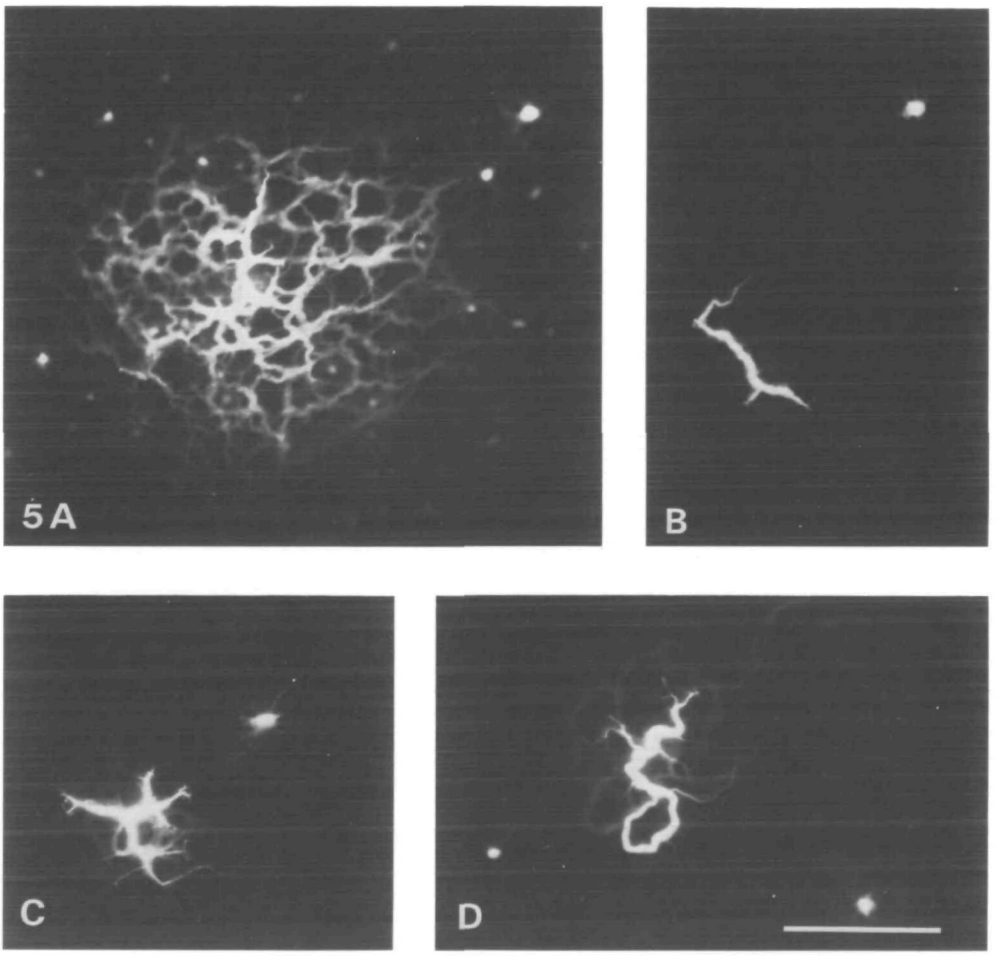

Fig. 5. Diffusion of Lucifer Yellow in the network of axon terminals of turtle H1 horizontal cells. (A) The dye injection was performed in a retina bathed in normal Ringer solution. The Lucifer Yellow diffused from the injected H1AT into a complex network of interconnected H1ATs. The dye also backfilled some H1 cell bodies through the fine axon fibres which connect the cell bodies to their axon terminal. (B) In this retina, after a bath application of 10 μmol l^{-1} dopamine, the diffusion of the dye was restricted to the injected H1AT and its cell body. (C) A similar restriction is observed with a bath application of 10 μmol l^{-1} forskolin. (D) Restriction of Lucifer Yellow diffusion induced by 50 μmol l^{-1} isobutylmethylxanthine. (Results from Piccolino, Neyton & Gerschenfeld, 1984). Scale bar, 100 μm.

via an increase in intracellular cyclic AMP and a subsequent phosphorylation of the junctional channels.

Modulation of a gap junction permeability by acetylcholine

In various exocrine glands (pancreas, salivary and lacrimal glands), ACh controls a series of properties of the glandular cells: it evokes exocytosis, the opening of different calcium-dependent channels, and a closure of gap junctions. The first

description of gap junction closure by ACh in these glands was based on the coupling coefficient method (Iwatsuki & Petersen, 1978*a*) with its unavoidable uncertainties. The apparent simultaneity of junction closure and increase in internal calcium provoked by ACh suggests a causal link between the two phenomena (Iwatsuki & Petersen, 1978*b*). We have re-examined this problem using the double patch-clamp technique on isolated pairs of lacrimal gland cells (see above). We first studied the effect of internal calcium on the junction by measuring, under various intracellular calcium concentrations, the stability of the coupling as a function of time after double dialysis. The possible interactions between Ca_i^{2+} and pH_i could be avoided in this system, because the concentrations of both ions were strongly buffered. The accuracy of the measurements was limited to some extent by a systematic and spontaneous rundown of the coupling following double-cell dialysis, even when Ca^{2+} and H^+ internal concentrations were kept low. At first sight this is a disturbing phenomenon, but it reveals more interesting processes. There is a high level of noise in the transjunctional current during this rundown, indicating that a number of channels are fluctuating between two or more levels of conductance. The functional elimination of channels during the rundown is thus not an instantaneous process: the junctional channels appear to be open most of the time at the beginning of an experiment, then oscillate for some time between the open and closed states, and finally shut.

The effect of increased Ca_i^{2+} on the coupling was estimated, statistically, by measuring the rate of junctional conductance rundown. As expected, increasing Ca_i^{2+} could speed up this rundown, but quite high concentrations were needed to get an effect: $1\,\mu mol\,l^{-1}$ was ineffective; $10\,\mu mol\,l^{-1}$ Ca^{2+} caused a closure of the junction within a few seconds. These values, which are similar to those reported in other preparations (Rose & Loewenstein, 1976; Rose & Rick, 1978; seem too large to be likely in a living cell.

The action of ACh was then examined with the same double patch-clamp method. ACh was applied with a fast perfusion system on paired cells. One cell had its internal calcium weakly buffered to follow the various calcium-dependent currents, namely: a potassium current, flowing through a few large channels which open when $Ca_i^{2+} = 10^{-7}\,mol\,l^{-1}$; a chloride current, flowing through many small channels, opening when $Ca_i^{2+} = 2\times10^{-7}\,mol\,l^{-1}$, and occasionally, a cationic current resulting from the opening of intermediate-sized channels which are activated at even higher calcium concentrations (Marty, Tan & Trautmann, 1984). The appearance of these currents was prevented in the adjacent cell by adequate calcium buffering, to follow more easily the current flowing through the gap junction when the two cells were clamped at different voltages. As expected, ACh produced both calcium-activated currents and uncoupling (Fig. 6A). Calcium-dependent currents (activated by ACh for 1–2 min) disappeared after application of the agonist. But uncoupling always occurred after the calcium-activated currents had disappeared and, thus, presumably after the return of Ca_i^{2+} to a low level. In addition, the junctional conductance frequently showed a transient increase at the peak of Ca_i^{2+} concentration. These observations clearly do not indicate a direct involvement of calcium in gap junction

closure by ACh. This suspicion became a certainty with a second series of experiments in which the internal calcium concentration was strongly buffered in both cells at a low value (pCa 8, see Fig. 6B). Under these conditions, ACh failed to increase Ca_i^{2+} (as judged by the absence of calcium-dependent currents), but uncoupling (preceded by a transient increase in coupling) still occurred. The uncoupling was obviously not calcium- or proton-dependent (since the internal pH of these cells was strongly buffered at pH 7·2).

Do the effects of ACh result from changes in the number of open junctional channels or alteration in their elementary properties? This is a difficult question to answer at the single channel level, because several levels of conductance occur which correspond to the closed state, one, or possibly more, fully open state in the range

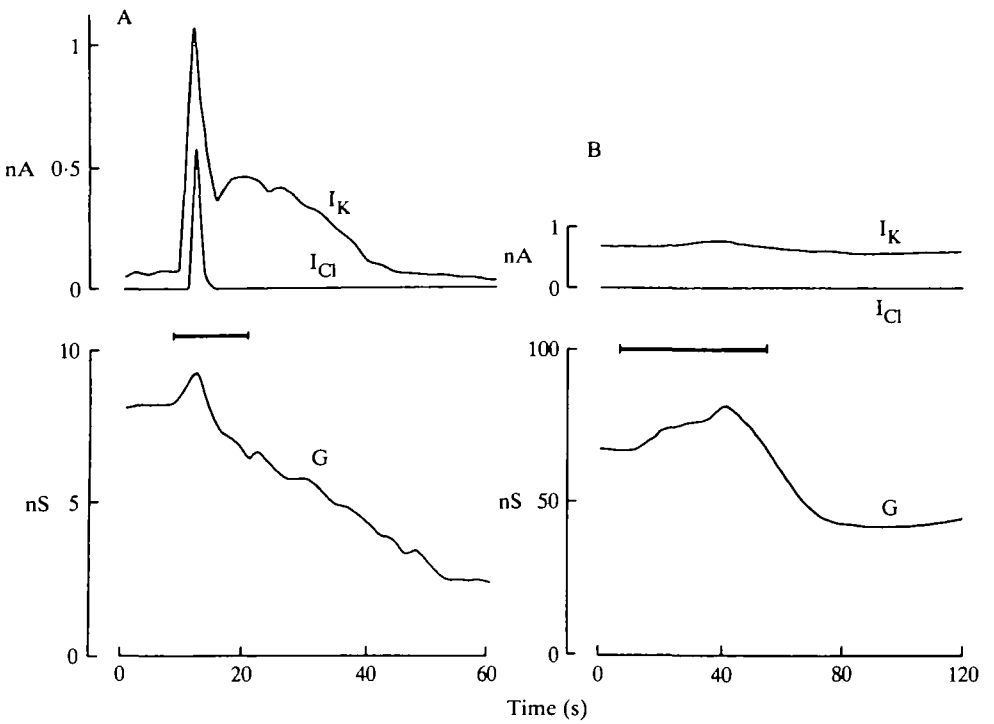

Fig. 6. Acetylcholine-induced calcium-dependent currents and decrease of gap junction conductance in pairs of lacrimal gland cells. Two experiments are illustrated here. In both cell pairs, Ca_i^{2+} in cell 2 was clamped at pCa = 8 with 5·5 mmol l^{-1} EGTA in A, and 20 mmol l^{-1} EGTA in B. Ca_i^{2+} of cell 1 was allowed to change in A (0·5 mmol l^{-1} EGTA) but not in B (20 mmol l^{-1} EGTA). pH_i in both cells was clamped at 7·2 by 30 mmol l^{-1} Hepes in B. In the top panels the nonjunctional currents in cell 1 are shown, measured as indicated in the legend of Fig. 1. The outward currents measured at −20 mV (A) or 0 mV (B) are mainly carried by potassium, whereas the inward currents measured at −50 mV (A) or −60 mV (B) are mainly chloride currents (all currents are shown upwards here). In both cases, the application of acetylcholine (1 μmol l^{-1} in A, 2 μmol l^{-1} in B), indicated by the bars, causes a biphasic change of the junctional conductance G; a transient increase followed by a long-lasting decrease. Changes in nonjunctional currents are seen only in A.

70–180 pS, and several intermediate states (partially opened or partially obstructed). Despite this complexity, it is clear that closure of the junction by ACh does not result simply from an increased probability of the channel being in an intermediate, low-conductance state. It seems that during ACh action some channels close completely (but reversibly) while others remain fully open. Thus, the mechanism which had been previously suggested for calcium-induced closure of gap junctions (i.e. decrease of the diameter of the junctional channels: Rose, Simpson & Loewenstein, 1977) does not apparently occur in the case of the ACh-induced modulation of the gap junctions between lacrimocytes. It should also be emphasized that the experimental evidence for the hypothesis of partial channel closure, which is based on measurement of the diffusion of various fluorescent molecules, does not appear to be reliable (Zimmerman & Rose, 1985). The mechanism of the transient increase in gap junction conductance at the beginning of the ACh action is still unclear. It might result from a slight *increase* in the elementary conductance of the open junction channels.

In summary, an application of ACh on acinar cells from lacrimal glands causes an increase in Ca_i^{2+}. This increase controls the secretion of both enzymes, by exocytosis, and of ions, through channels and pumps (Marty *et al.* 1984). On the other hand, ACh induces a delayed and long-lasting closure of gap junction channels which can be reversible. We ignore at the moment the nature of the second messenger involved in this latter phenomenon, but it clearly does not result from a rise in Ca_i^{2+}. Trisphosphoinositol is apparently involved in the internal calcium release induced by ACh in these cells (Evans & Marty, 1986). This raises the question of the role of phosphoinositide breakdown in the ACh-induced uncoupling. What the function of this junction closure could be is a puzzling question. We suggest that it could be used during prolonged gland stimulation, which probably requires a lot of energy and produces important concentration changes inside the cells. By disconnecting the lacrimocytes, ACh might allow a discontinuous secretion by each individual cell with resting periods. But to answer this question it is necessary to know the extent and the kinetics of junction closure *in vivo* (i.e. under the action of nerve-released ACh, in nondialysed cells).

CONCLUDING REMARKS

The biological functions of gap junctions are understood in only a few, very simple situations: in a non-vascularized tissue like the lens (where the gap junctions are probably involved in trophic functions) and in the heart (where they ensure a synchronous contraction of groups of cardiac muscle cells). These extreme cases have led to the idea that gap junctions allow cellular ensembles to behave like syncitia. This is probably one of their functions, but the existence of junctions between heterogeneous cell types, of different functions (e.g. the endocrine pancreas, see review by Meda, Perrelet & Orci, 1984), does not fit with this somehow simplistic idea. The existence, in many tissues, of physiological modulations of gap junctions also indicates that the presence of gap junctions does not necessarily imply

a syncitial behaviour. Indeed, functional differences between two coupled cells may be preserved by a reversible closure of the junctional channels. It is likely that a limited number of intracellular mechanisms are directly involved in the control of junctional channel gating, and that different physiological modulators are able to activate or inhibit such common mechanisms.

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