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Cell Communication: A Cyclic-AMP Mediated Phenomenon

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Summary. Incubation of the salivary glands of the larvae of *Drosophila hydei* in a control medium containing 2×10^{-3} M cyclic adenosine monophosphate (cAMP) induces a considerable increase in passive electrical cell communication. This is caused by a decrease in permeability of the nonjunctional membrane part, together with an increase of the permeability of the low-resistance junctions. Similar changes in intercellular communication in the salivary gland of *Drosophila hydei* were seen in a majority of experiments in which 10^{-3} M dibutyryl cyclic adenosine monophosphate (dBcAMP), 5×10^{-3} M theophylline or ecdysterone (100 μg/ml) were added to the control medium. Hyperpolarization of the gland cells can be observed concomitant with the increase in communication. A hypothesis is discussed for a possible molecular regulation of passive electrical cell communication in which the intracellular cAMP level plays a significant part.

The development of special electrophysiological techniques made it possible to determine electrotonic spread within many cell systems, both *in vivo* and in cultures. It often appears that electrolytes can freely move from one cell to another as they can be transported through special junctional membrane pathways that seem to be much more permeable to electrolytes than the nonjunctional membrane part. This phenomenon is called passive electrical cell communication (Loewenstein, 1966, 1968; Furshpan & Potter, 1968).

Recent evidence given by Gilula, Reeves and Steinbach (1972) and Azarnia, Michalke and Loewenstein (1972) also suggest that specialized low-resistance junctions are a prerequisite for metabolic cooperation (Bürk, Pitts & Subak-Sharpe, 1968; Cox, Kraus, Balis & Dancis, 1972) as they allow the transfer of physiologically relevant compounds of different molecular size. These experiments support the hypothesis postulated by Loewenstein, that communication via junctional membranes directly from cell to cell is important to cell differentiation and regulation of tissue and cellular growth (Loewenstein, 1968).

3′,5′-Cyclic adenosine monophosphate (cAMP), an intermediate in the action of many hormones (Robison, Butcher & Sutherland, 1971), has been reported to be involved in the regulation of cellular growth by inducing respectively restoring contact-inhibition of cell proliferation (Bürk, 1968; Hsie & Puck, 1971; Otten, Johnson & Pastan, 1971; Peery, Johnson & Pastan, 1971; Sheppard, 1971, 1972; Burger, Bombik, Breckenridge & Sheppard, 1972; Frank, 1972; Froehlich & Rachmeler, 1972; Seifert & Paul, 1972; Smets, 1972; Teel & Hall, 1973). Furthermore, the intracellular cAMP level seems to be very important for the regulation of cell metabolism. High intracellular levels of cAMP inhibit cell proliferation and stimulate cell metabolism, while low intracellular cAMP concentrations are found in growing cell cultures (Sutherland, 1970).

In view of the above-mentioned arguments, the influence of an enhancement of the cAMP level upon the phenomenon of passive electrical cell communication has been studied. Salivary glands of the larvae of *Drosophila hydei* are incubated in a control medium containing cAMP, dibutyryl-cAMP (dBcAMP), theophylline or ecdysterone.

For the quantitative evaluation of the passive electrical cell communication in the salivary gland models have been developed. With the help of these models, junctional (R_j) and nonjunctional (R_u) membrane resistance estimates may be derived with an accuracy of about 10% from electrotonic spread measurements (van Venrooij, Hax, van Dantzig, Prijs & Denier van der Gon, 1974). Also the capacity can be computed for the nonjunctional membrane part.

Evidence is presented in this paper that passive electrical cell communication may be a cAMP-mediated phenomenon. If a salivary gland is treated with cAMP, or one of the other agents that, in other tissues, are known to raise intracellular cAMP concentrations, reversible decreases occur in the resistance of the junctional membrane part, whereas the resistance of the nonjunctional membrane part increases, thus causing a high communicative state of the cell system. This is always accompanied by hyperpolarization of the nonjunctional membrane part, the capacity of which proves to be a stable parameter which does not change significantly.

Materials and Methods

Culture Methods and Media

All measurements were performed with a wild type stock of *Drosophila hydei*. The standard nutritive medium used for culturing larvae is described by Poels (1970). The culture temperature was 23 °C. The age of the larvae used in each experiment was 155 to

165 hr after oviposition. After dissection in a drop of control medium the two salivary glands were transferred to a perspex chamber filled with the incubation medium. The transfer of the salivary glands was carried out with a forceps placed upon the duct to avoid damage of gland cells. The bottom of the perspex chamber was covered with a thin translucent gel layer. The salivary glands were fixed by means of a staple placed over the duct. The control medium used was a modified Shields and Sang medium described by Poels (1972). The incubation media used during the experiments were: (*a*) Control medium; (*b*) Control medium $+ 2 \times 10^{-3}$ M adenosine 3′:5′-cyclic-monophosphoric acid (Sigma Chemical Company); (*c*) Control medium $+ 10^{-3}$ M N^6, $O^{2'}$-dibutyryl adenosine 3′:5′-cyclic-monophosphoric acid (monosodium salt) (Sigma Chemical Company); (*d*) Control medium + 100 μg ecdysterone/ml; (*e*) Control medium $+ 5 \times 10^{-3}$ M 1,3-dimethylxanthine (theophylline) (Sigma Chemical Company). The final osmolality of these solutions was 260 mosm.

Experimental Procedure

Electrophysiological procedures and data processing as described in the companion paper (van Venrooij *et al.*, 1974) were applied to determine junctional and nonjunctional membrane resistances. All measuring series were assayed for usefulness by calculating the confidence interval. Those series referring to glands which meet the requirements of the reliability criterion (van Venrooij *et al.*, 1974) during the whole experiment, were used for Figs. 2–5.

Results

Incubation of the salivary gland in control medium containing 2×10^{-3} M cAMP drastically changes the potentials in it as shown in Fig. 1. The observed changes may be reduced to a decrease in permeability of the outer nonjunctional membrane part, whereas the permeability of the low-resistance junctions is increased (Fig. 2*A*, *B*). Those permeability changes are significant as can be deduced from error estimates (van Venrooij *et al.*, 1974) as well as from the fact that the reactions of all the glands are similar. It follows from these findings that passive electrical cell communication is increased.

It is notable, furthermore, that an increase in membrane potential takes place during the incubation of the gland in cAMP-containing medium (*see* Fig. 2*C*). This phenomenon was also observed in rat liver cells (Friedman & Dambach, 1973) and in salivary gland cells of *Drosophila melanogaster* (Rensing & Hardeland, 1972).

To check whether incubation in the medium containing cAMP gave rise to any damage of the gland cells, this medium was replaced by the control medium. As can be seen in Fig. 2 the resistances of the junctional as well as the nonjunctional membrane parts tend to regain their original values, suggesting that no damage took place. The membrane potential also decreases to normal values.

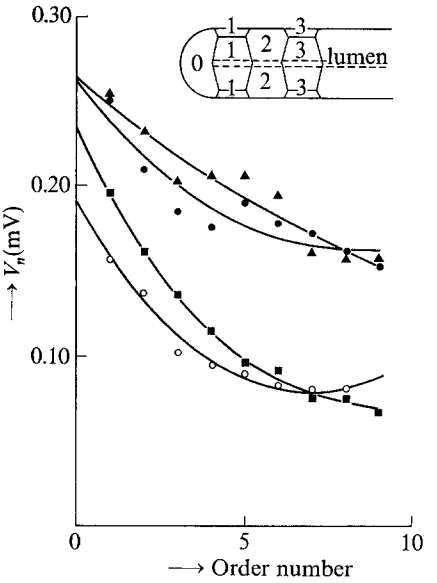

Fig. 1. The relation between the potential V_n, caused by the injection of pulse current $I=10^{-9}$ A in the most distal cell (order number: 0) and the order number n is given. The potentials indicated are measured values. The drawn lines are computed values obtained by applying the minimization procedure of Fletcher and Powell (1963) on the function: $\Sigma_n(V_n^{\text{theor}}-V_n^{\text{exp}})^2$ in which $V_n^{\text{theor}}=c_1t^n+c_2t^{-n}$, the general solution of the difference equation: $V_{n-1}+V_{n+1}=\alpha V_n$, describing the potential path within the gland. V_n^{exp} = experimental measured potentials. ○: Salivary gland in control medium; ●: salivary gland incubated 30 min in medium containing 2×10^{-3} M cAMP; ▲: salivary gland incubated 60 min in medium containing 2×10^{-3} M cAMP; ■: 60 min after restitution of the control medium. *Inset*: Model of the configuration of the cells in the salivary glands. Each cell has an order number $n(n=0\rightarrow9)$. The most distal cell has the order number 0; the cells belonging to the ring adjacent to the most distal cell have the order number 1, etc.

The capacity of the nonjunctional membrane part does not change significantly when the salivary gland is incubated in the cAMP-containing medium.

A similar increase in passive electrical cell communication and hyperpolarization of the outer membrane could be observed very rapidly (within 5 min) after incubation of the salivary glands in the medium containing 10^{-3} M dBcAMP (*see* Fig. 3*A–C*). Replacement of this medium by the control medium did not induce a return to the original communicative state. On the contrary, the high communicative state reached during the dBcAMP treatment was maintained. Also the membrane potential remained high.

A phosphodiesterase which converts cAMP to 5′-AMP is the only enzyme presently known to inactivate it. A competitive inhibitor for the

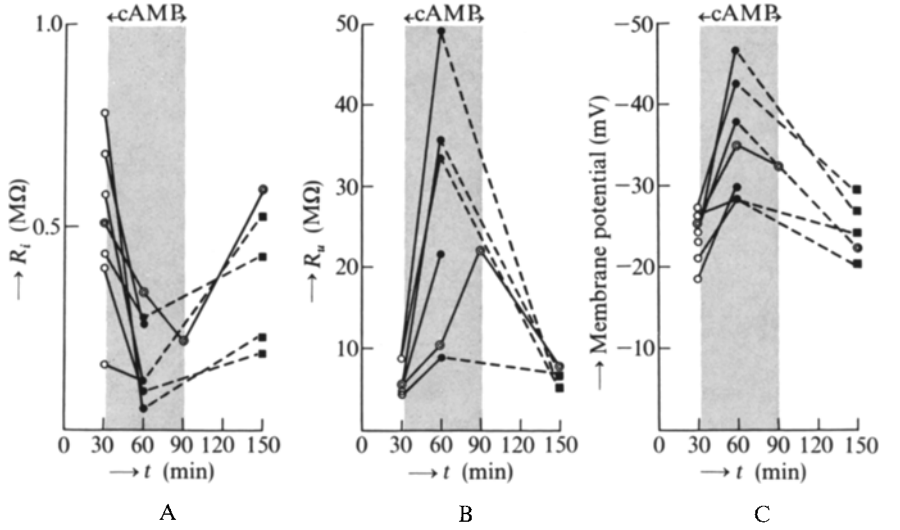

Fig. 2. Fluctuations in the resistance of the junctional (R_i) and the nonjunctional (R_u) membrane part due to the incubation of the salivary gland in medium containing 2×10^{-3} M cAMP. Furthermore, the time course of the membrane potential is given. The marks have the same meanings as in Fig. 1. ◎: These points are from the experiment of Fig. 1

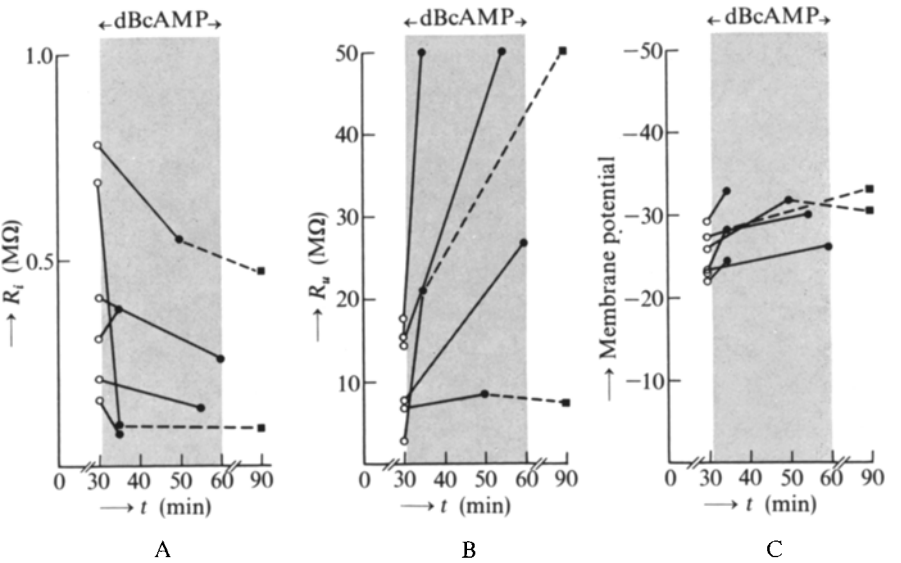

Fig. 3. Fluctuations in the resistance of the junctional (R_i) and the nonjunctional (R_u) membrane part due to the incubation of the salivary gland in medium containing 10^{-3} M dBcAMP. Furthermore, the time course of the membrane potential is given. ○: Control; ●: 10^{-3} M dBcAMP; ■: control

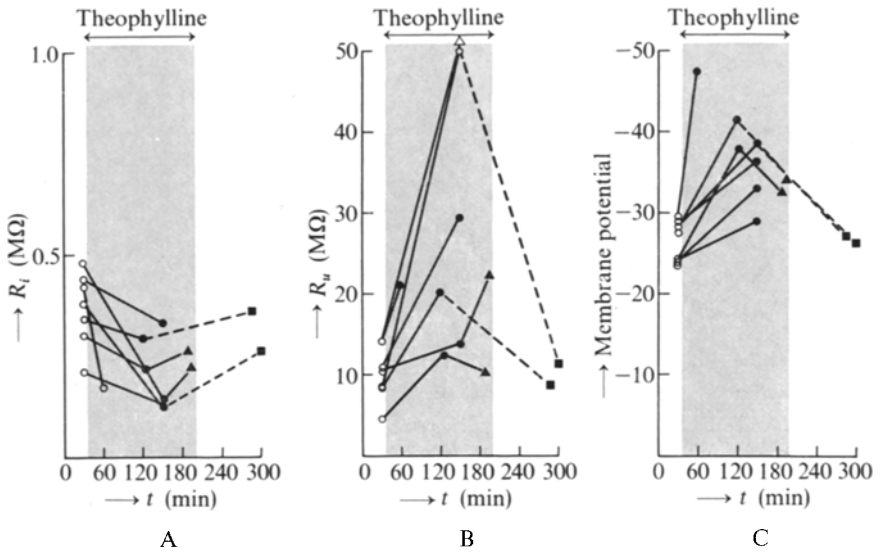

Fig. 4. Fluctuations in the resistance of the junctional (R_i) and the nonjunctional (R_u) membrane part due to the incubation of the salivary gland in medium containing 5×10^{-3} M theophylline. Furthermore, the time course of the membrane potential is given. ○: Control; ●▲: 5×10^{-3} M theophylline; ■: control

phosphodiesterase is theophylline (Honda & Imamura, 1968). If an active adenyl-cyclase system should be present in the salivary glands, incubation with theophylline would increase the intracellular cAMP level, resulting in an increase of intercellular communication. As shown in Fig. 4*A–C* incubation of the glands in the medium containing 5×10^{-3} M theophylline does indeed increase the intercellular communication, whereas the membrane potential is increased concomitant with the increased communication.

Incubation of the salivary gland of *Drosophila hydei* with ecdysterone (100 μg/ml), a hydroxylated derivative of the insect molting hormone ecdysone, has been reported to increase the intracellular cAMP level (Leenders, Wullems & Berendes, 1970). Fig. 5*A–C* shows the effects induced by the steroid hormone ecdysterone. A straight increase as well as an initial decrease in junctional permeability was observed here. After 3 hr, however, the gland cells are strongly coupled. The initial decrease in junctional permeability may be caused by the strong stimulation by ecdysterone of the adenyl cyclase leading to a rapid increase of the intracellular cAMP concentration (Leenders *et al.*, 1970). The high cAMP level may induce mobilization of Ca^{++} ions (Friedman & Park, 1968) causing the initial decrease in intercellular communication (*see also below*: Mechanism of regulation of

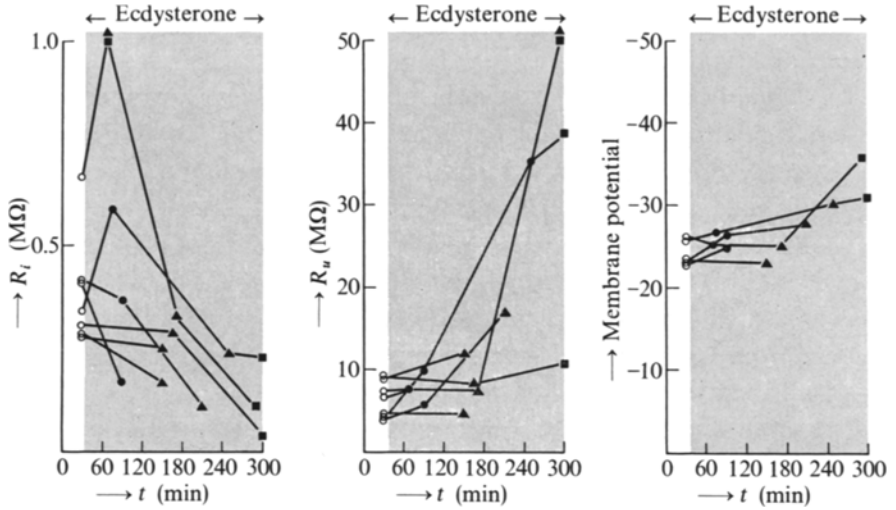

Fig. 5. Fluctuations in the resistance of the junctional (R_i) and the nonjunctional (R_u) membrane part due to the incubation of the salivary gland in medium containing ecdysterone (100 μg/ml). Furthermore, the time course of the membrane potential is given. ○: Control; ●▲■: ecdysterone (100 μg/ml)

Intercellular Communication). Again the parallelism, although less pronounced, between the hyperpolarization of the outer membrane and the increase in communication seems to be present.

Discussion

The points in Figs. 2–5 are derived by multistep analyses of primary measurements such as those in Fig. 1: a set of points (each curve) like those given in Fig. 1 results in, e.g., one point in Fig. 2*A* and one point in 2*B*. The companion paper (van Venrooij *et al.*, 1974) describes the analytical machinery for generating the estimates of junctional and nonjunctional membrane resistances. Furthermore, it is indicated in that paper that the morphological as well as the "arithmetic" errors are not relevant, if measurements on the same gland are compared. As, following van Venrooij *et al.* (1974), the reliability criterion was applied and taking into account that the average value of G appears to be 0.04 $(mV)^2$, the average inaccuracy of the derived parameters in Figs. 2–5 is not likely to exceed 10%. This means that the changes in membrane permeability, namely the increased junctional and decreased nonjunctional permeability, caused by adding cAMP to

the bathing fluid are significant. Consequently, intercellular communication is facilitated significantly.

We would like to emphasize that incubation of the salivary glands in the medium containing cAMP did not lead to damage of the gland cells. A return to the original communicative state occurred after replacement of the incubation medium by the control medium.

The reactions of some salivary glands to cAMP did not conform to the normal pattern of facilitation of intercellular communication. However, those measurements also did not fulfill the reliability criterion and were excluded from the Figures. Besides, changes in the luminal volume, suggesting an enhancement of secretion were observed then.

The set of experiments described in this paper suggest strongly that the observed changes in permeability resulting from a change in cAMP concentration in the bathing fluid, are not induced directly by action of cAMP on the outer membrane. Our experiments as well as those by Leenders *et al.* (1970) on the specific action of the steroid ecdysterone on the activity of certain genes, indicate the existence of an active adenyl-cyclase system in the salivary glands of *Drosophila hydei*. Increased cAMP levels may be reached by stimulating this adenyl-cyclase system through the hormone ecdysterone or by inhibiting the catabolic activity of a phosphodiesterase through theophylline. The presence of cAMP or its derivative dBcAMP in the incubation medium will also increase their intracellular levels. A high intracellular cAMP level thus seems to facilitate intercellular communication. Figs. 2–5 obviously show that the increased communication is caused by a decrease of the permeability of the nonjunctional membrane part, while simultaneously the junctional membrane is increased in permeability. Although the reported changes of R_i and R_u are significant in a majority of cases, sometimes no significance for the apparent changes can be claimed. However, even in those cases the induced changes are in the same direction as the significant ones and add in that way to the overall significance of the findings. It is notable, furthermore, that always opposite changes in permeability for the different membrane parts occur, suggesting that a change in a particular part of the cell membrane has repercussions for other membrane parts, too.

The induced changes in membrane potential agree with experiments described earlier (Loewenstein, 1966; Loewenstein, Nakas & Socolar, 1967; Politoff, Socolar & Loewenstein, 1969; Rose & Loewenstein, 1969, 1971). The permeability of the low-resistance junction seems to depend on the potential across the nonjunctional membrane. A direct evidence for a parallelism between the membrane potential and the extent of intercellular

communication was given by Rose (1970) and Socolar and Politoff (1971). Under conditions in which the cell communication was depressed and depolarization occurred, the junctional permeability could be restored by repolarizing currents. Theophylline occasionally induced a decrease in membrane potential. This was indeed attended by a decrease in intercellular communication. These findings are excluded from Fig. 4 as the reliability criterion was exceeded.

In addition, we should mention that there was no clear correlation between the size of decrease of R_i, or increase of R_u, and the size of hyperpolarization. Also, there was no indication of the possibility that a significant decrease in R_i may be occurring only in those preparations that hyperpolarize beyond a certain threshold of negativity. The changes in membrane potential may result directly from the increased resistance of the nonjunctional membrane part. Other explanations, however, are also possible; e.g., an activation of a membrane-bound $Na^+/K^+/Ca^{++}$ pump or an accumulation of divalent ions in intracellular pools.

In the companion paper (van Venrooij *et al.*, 1974) it is mentioned that the impalement of the gland cells by microelectrodes was much easier after lysolecithin treatment of the salivary glands. In other words, in a situation in which the resistance of the outer membrane is decreased, the mechanical stability of this very membrane seems to be decreased too. Incubation of the glands in media containing cAMP, dBcAMP, theophylline or ecdysterone leads to an increased resistance of the nonjunctional membrane part. The membrane seems to become more rigid now: it proves difficult to impale the cell with a microelectrode while on the other hand the membrane is damaged easily if the microelectrodes are not handled carefully.

Mechanism of Regulation of Intercellular Communication

The normal intracellular cAMP level is provided on the one hand by the formation of cAMP from ATP, catalyzed by the adenyl cyclase and on the other hand by the catabolic activity of a phosphodiesterase which converts it into 5′-AMP. Knowing that the adenyl-cyclase system is a membrane-bound enzyme, a likely place to find it is the nonjunctional membrane part. The phosphodiesterase is a cytoplasmic enzyme. Hormonal activation of the adenyl-cyclase system results in an increase of the intracellular cAMP level. Such an increase now will induce changes in the permeability properties of the junctional and nonjunctional membrane parts according to the evidence presented in this paper. Consequently, the flow of "information" from cell to cell may be enhanced (*see* Fig. 6). A decreased intracellular concentration of cAMP due to either inhibition of the adenyl-cyclase system

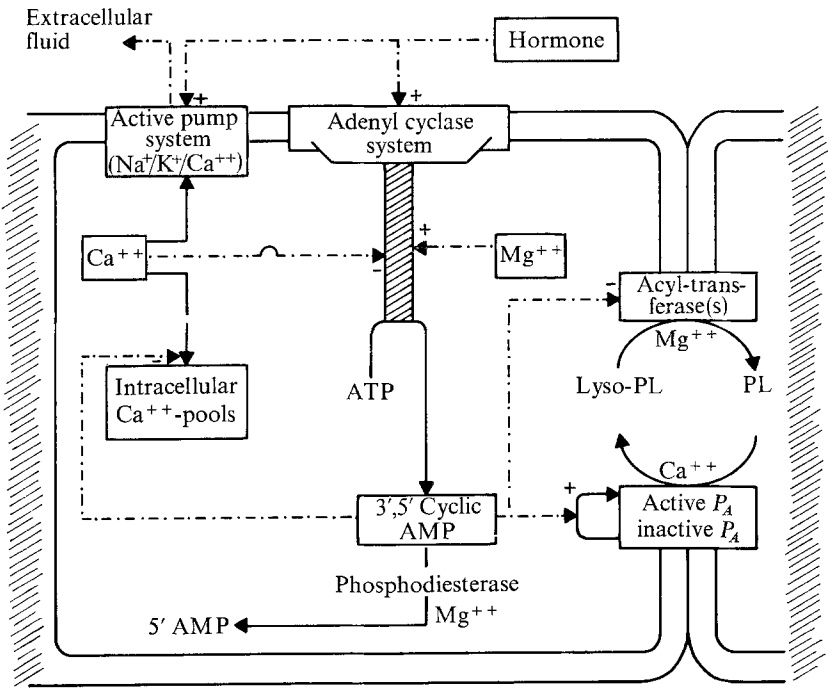

Fig. 6. The basic aspects of the proposed adenyl-cyclase control mechanism. Hormonal activation of the adenyl-cyclase system results in an increase of the intracellular cAMP level. The increased cAMP concentration may indirectly and/or directly activate the conversion of an inactive precursor of a membrane-bound phospholipase A into an active phospholiphase A. The resulting increase of the lysophospholipid content of the junctional membrane part induces an increase in intercellular communication. Low concentrations of divalent (Ca^{++} and Mg^{++}) ions constitute a basis for a concerted action of the involved enzymes, resulting in an optimal cell communication. Increased levels of divalent ions, due to mobilization of these ions from intracellular pools by high cAMP concentrations may act as a feed-back inhibitor of further adenyl-cyclase activation. Conversion of lysophospholipids into phospholipids by (an) acyltransferase(s) may induce a decrease in intercellular communication under these circumstances. During the highly communicative phase an active pump mechanism may cause hyperpolarization of the gland cells

and/or stimulation of a phosphodiesterase will reduce the amount of intercellular communication.

The calcium hypothesis of Loewenstein (Loewenstein, 1967*a*, *b*; Loewenstein *et al.*, 1967) might fit in this view concerning regulation of intercellular communication. Low intracellular concentrations of divalent ions – Ca^{++} and Mg^{++} ions – establish a high adenyl-cyclase activity (Bradham, Holt & Sims, 1970) resulting in an increase of the cAMP concentration. The increased cAMP level within the cell may now serve different functions: it activates intercellular communication and on the other hand it mobilizes

intracellular Ca^{++} ions (Friedmann & Park, 1968; Borle, 1974). An increase in the cytoplasmic Ca^{++} level may act as an inhibitor of further adenylcyclase activation (Bär & Hechter, 1969; Rasmussen, 1970; Bradham *et al.*, 1970; Kelly & Koritz, 1971; Rubin, Carchman & Jaanus, 1972) and will thus reverse the cell system to a lower communicative state. In this way cAMP and Ca^{++} are mutually involved in negative feed-back control of each others concentrations, while the same feed-back system now regulates the degree of intercellular communication.

These conceptions do not contradict the observation that energy is required for the maintenance of normal cell communication (Politoff *et al.*, 1969). ATP is needed as a substrate for the adenyl cyclase, while, furthermore the effects of cAMP are possibly mediated by protein kinases present in the cell cytoplasm which are activated by phosphorylation. For the phosphorylation reaction ATP is also needed.

Finally, the question remains how cAMP induces the conversion in permeability of the junctional and nonjunctional membrane parts. In a previous paper (Hax, van Venrooij, Denier van der Gon & Elbers, 1973), it was demonstrated that lysophospholipids may act as an inductor for sites of low-resistance junctions between cells. As a consequence it was postulated that the concerted action of a phospholipase A and (an) acyltransferase (s) situated on the contact area of two adjacent cells, could regulate the permeability properties of the junctional membrane part by changing the lysophospholipid content of that particular part of the membrane. There is evidence now in general for a direct or indirect activation of the enzyme phospholipase A through cAMP (Chiappe de Cingolani, Van den Bosch & Van Deenen, 1972; Imre, 1972; Hax, Demel, Spies, Vossenberg & Linnemans, 1974). If one supposes a causal relation between the observed increase in permeability of the low-resistance junction and stimulation through cAMP of the postulated phospholipase A, situated at that junction, then the results found fit in rather nicely: the incorporation into the junctional membrane part of the lysophospholipids formed will facilitate intercellular communication (Hax *et al.*, 1973). Reacylation of the lysophospholipids by acyltransferases will decrease cell communication.

In conclusion, the experiments suggest cAMP to play a prominent part in the control of cell coupling. A high intracellular cAMP level increases the cell metabolism, while in that situation intercellular communication is enhanced. Thus, cAMP may induce metabolic cooperation between adjacent cells in multicellular masses and organisms. Viewed in that light, the results may also be a basis to explain the involvement of cAMP in contact-inhibition of cell proliferation.

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