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Membrane Potential and Resistance Changes Induced in Salivary Gland Acinar Cells by Microiontophoretic Application of Acetylcholine and Adrenergic Agonists

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Summary. The effects of microiontophoretic applications of catecholamines and acetylcholine on parotid acinar cell membrane potential and resistance were investigated using intracellular microelectrode recording in superfused segments of mouse parotid or rat submandibular glands. Short pulses of acetylcholine and α-adrenergic agonists had similar effects, consisting of a marked decrease in membrane resistance accompanied by an initial depolarization or hyperpolarization depending on the level of the resting membrane potential. This initial response was followed by a slow hyperpolarization occurring at a time when the resistance was increasing towards the prestimulation level. The equilibrium potential for the initial potential change caused by excitation of the cholinergic receptors was investigated directly by setting the membrane potential at different levels by injecting direct current and stimulating the same cell repeatedly with equal doses of acetylcholine. The equilibrium potential was found to be about −55 mV. The delayed hyperpolarization could not be reversed by passing hyperpolarizing current, but actually increased in size with higher membrane potentials. The minimum latency of the effect of acetylcholine or α-adrenergic agonists was 200–500 msec.

Excitation of β-adrenoceptors caused, after a long latency of several seconds, a small depolarization. Epinephrine induced a combined α- and β-adrenergic response, with the α-component predominating. Blocking the α-adrenoceptors with phentolamine revealed the β-adrenergic depolarization, while blocking the β-adrenoceptors with propranolol caused the components of the α-adrenergic response to become more pronounced. All three receptors (α- and β-adrenoceptors and cholinergic receptors) were present in individual acini.

Intracellular microelectrode recordings from salivary acinar cells have shown that there are three distinct receptors influencing the membrane potential; α- and β-adrenoceptors and cholinergic receptors (Petersen & Pedersen, 1974). β-Adrenoceptor excitation causes a small de-

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polarization accompanied by a modest resistance reduction, while α-adrenoceptor and cholinergic receptor activation in some glands have complex effects (Kagayama & Nishiyama, 1974; Nishiyama & Petersen, 1974*a*; Petersen, 1976*a*, *b*).

In previous studies, stimulation of the salivary glands has been either by electrical excitation of the parasympathetic or sympathetic nerve supply to the glands *in vivo* (Lundberg, 1955; Creed & Wilson, 1969; Kagayama & Nishiyama, 1974) or by adding drugs to the bathing solution of gland segments *in vitro* (Petersen, 1973; Petersen & Pedersen, 1974; Nishiyama & Petersen, 1974*a*; Petersen, 1976*a*). *In vivo* experiments have in general not given very stable recording conditions nor allowed appropriate pharmacological blocking experiments to be carried out, while previous *in vitro* experiments have suffered from the inability to give precisely reproducible, short-lasting stimuli. A number of problems therefore remain unresolved: (i) What are the true minimum latencies for the stimulation-evoked membrane changes? (ii) What is the precise relationship between the various phases of stimulation-evoked potential changes and the resistance changes? (iii) Is there a reversal potential for the stimulant-evoked potential change? (iv) Are there important qualitative differences between responses to stimulation in parotid and submandibular glands? Using the powerful technique of local application of stimulants by micro-iontophoresis, recently employed successfully in studies on pancreatic acinar cells (Nishiyama & Petersen, 1975; Iwatsuki & Petersen, 1977), we have been able to answer these questions.

Materials and Methods

Parotid glands removed from female mice or segments of submandibular salivary gland from female rats were secured to a perspex platform in a perspex tissue bath (20 ml) through which Krebs-Henseleit solution flowed at a constant rate (10 ml/min). The composition of the Krebs-Henseleit solution was (mM): NaCl, 103; KCl, 4.7; $CaCl_2$, 2.56; $MgCl_2$, 1.13; NaH_2PO_4, 1.15; $NaHCO_3$, 25; D-glucose, 2.8; Na fumarate, 2.7; Na pyruvate, 4.9; Na glutamate, 4.9. This solution was equilibrated with 95% O_2 and 5% CO_2 and warmed to 37 °C.

Measurements of membrane potential and resistance in superficial cells of the gland segments were made by the methods previously described (Nishiyama & Petersen, 1974*b*). One microelectrode was used and current was injected through the recording electrode. Microelectrodes filled with 3 M potassium chloride and 10 mM potassium citrate and having resistances between 20 and 50 MΩ were used. In many of the experiments described here the microelectrodes used had been bevelled using a Sutter Instrument Company beveller, model BV-10.

Agonists were applied by iontophoresis from glass micropipettes containing either 2 M acetylcholine chloride, 1 M epinephrine bitartrate, 1 M isoproterenol hydrochloride or 1 M phenylephrine hydrochloride. Isoproterenol hydrochloride and phenylephrine hydrochloride solutions were adjusted to pH 3.5, using hydrochloric acid, before use. In all cases, micropipettes containing a glass fiber and filled directly were used. The iontophoretic currents were passed for 500 msec, the retaining currents ranged from 5 to 20 nA, the ejecting current for acetylcholine ranged from 40 to 100 nA, and for catecholamines it ranged from 80 to 250 nA. Currents passed through the iontophoretic electrode were measured as the current flowing to ground, using a W-P Instruments model 180 Virtual Ground.

When antagonists were used, they were included in the superfusion fluid. Atropine sulphate was used at 1.4×10^{-6} M, phentolamine mesylate at 10^{-5} M, and propranolol hydrochloride at 5×10^{-6} M.

Results are presented as the arithmetic mean $\pm$ SEM with the number of observations in the group indicated in parenthesis. Comparisons between groups were performed using Student's *t*-test.

Results

Receptor Types Represented on Parotid Acinar Cells

Using microiontophoresis, we were able to confirm that cholinergic receptors and α- and β-adrenoceptors are present on the one acinus (Fig. 1). All 20 acini tested with acetylcholine and one of the catecholamines responded to both agents. The results in Fig. 1 indicate that epinephrine can activate both α- and β-adrenoceptors of parotid acinar cells, the β-response only becoming obvious in the presence of phentolamine.

Effects of Acetylcholine on Parotid Acinar Cells

As shown in Figs. 1 and 2, acetylcholine induced a biphasic response, but while the last phase was always a hyperpolarization, the first phase could be either a depolarization or a hyperpolarization, varying from cell to cell (Fig. 2). An analysis of 78 consecutive successful impalements showed a relationship between the size and direction of the potential change in the initial phase of the response to acetylcholine and the resting membrane potential of the cell (Fig. 3). These results suggested that the reversal potential for this initial phase was approximately -55 mV.

In a number of cells the initial phase appeared more complex than the pure hyperpolarization or depolarization just described. Examples of these responses are shown in Fig. 4.

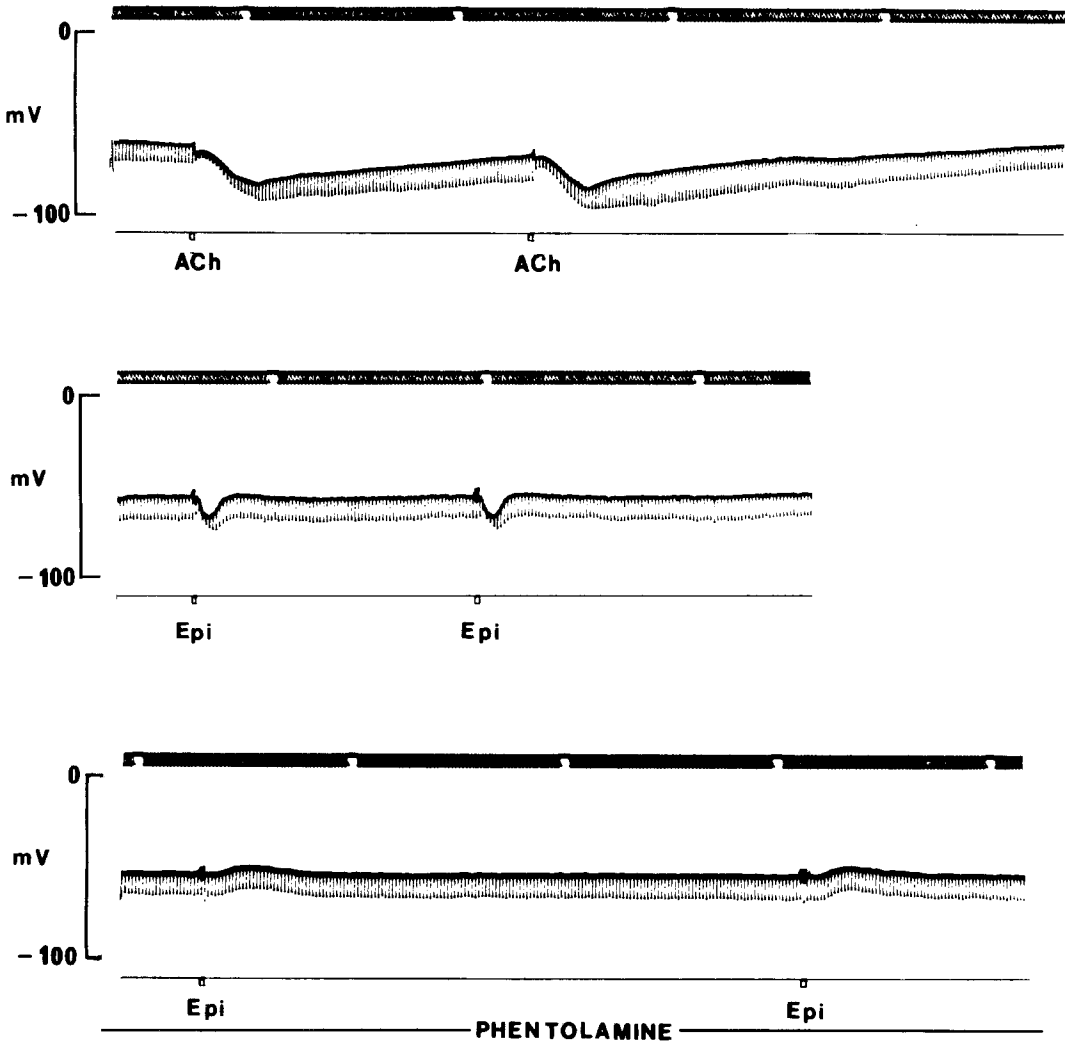

Fig. 1. Membrane potential and resistance in an acinar cell of mouse parotid superfused *in vitro*. These three tracings are sections of a continuous record obtained from one cell. Effects of microiontophoretic application of acetylcholine (ACh, retaining current 20 nA, ejecting pulses 40 nA, 500 msec) and epinephrine (Epi, retaining current 40 nA, ejecting pulses 400 nA, 500 msec) on acinar cell membrane potential and input resistance were measured with an intracellular microelectrode. Rectangular current pulses (2 nA, 100 msec, 1/sec) were injected through the recording microelectrode. Phentolamine (10^{-5} M) was added to the superfusing solution during the period indicated on the lower trace. On the time marker trace, minute intervals are marked by the absence of signal

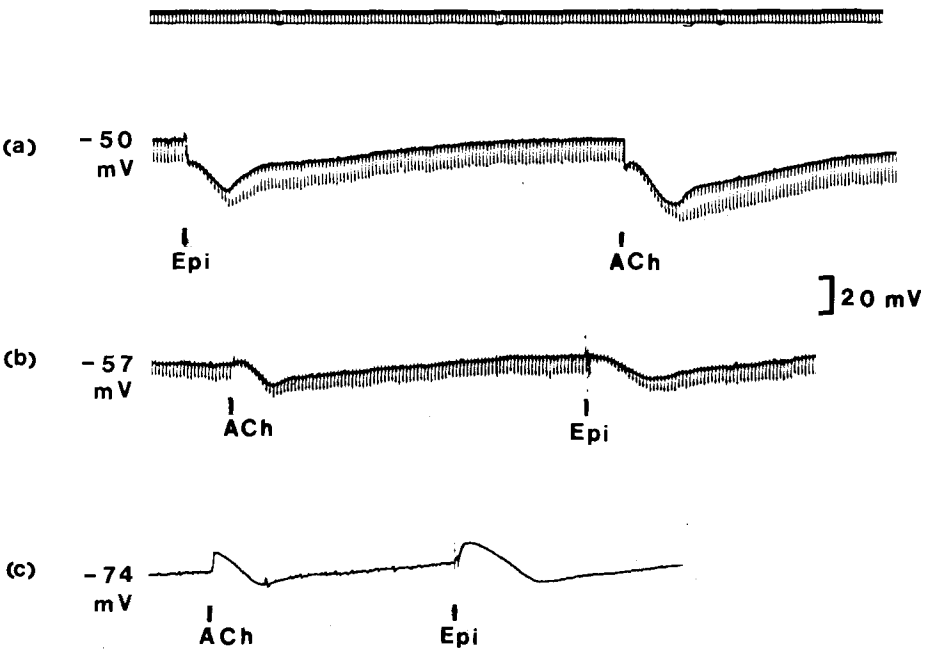

Fig. 2. Effects of acetylcholine (ACh) and epinephrine (Epi), applied by microiontophoresis, on the membrane potential and resistance of 3 parotid acinar cells. The resting potential of each cell is written to the left of its potential recording. In cells (*a*) and (*b*) the pulses on the potential record are produced by the passage of rectangular current pulses (2 nA, 100 msec, 1/sec) through the recording microelectrode. The shorter intervals on the time marker trace represent 1 sec

The effect of acetylcholine on the membrane input resistance is shown in Figs 1, 2 and 4. In this study, the resting input resistance of parotid acinar cells was 5.2 ± 0.4 MΩ ($n=25$). The minimum input resistance seen during the early phase of the acetylcholine response was 0.6 ± 0.1 MΩ ($n=20$). The peak of the delayed hyperpolarization always occurred at a time when the resistance had returned towards that measured prior to stimulation (Figs. 1, 2 and 4). The time constant of the resting membrane was 3 to 8 msec and during the period of acetylcholine action, when the input resistance was reduced, the time constant was reduced to below 2 msec. The reduction in input resistance and time constant in one cell is shown in Fig. 5.

Atropine was effective in blocking the effect of acetylcholine in all 9 experiments where it was used. Phentolamine (3 experiments) and propranolol (5 experiments) did not block the effects of acetylcholine.

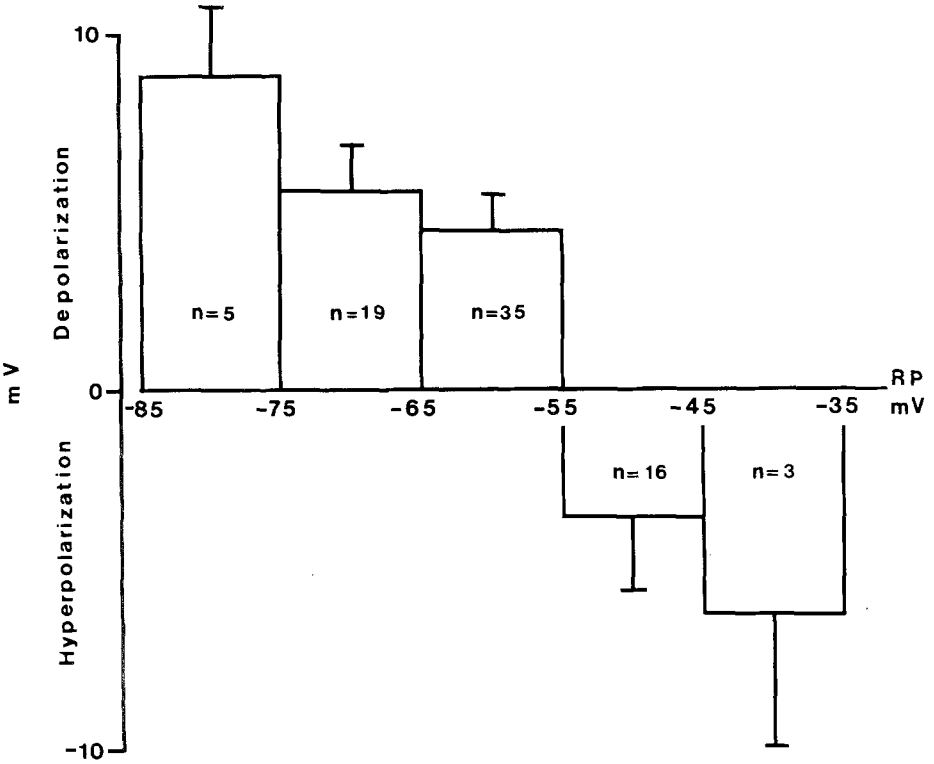

Fig. 3. The distribution of the maximum potential change in the early phase of the response of parotid acinar cells to acetylcholine plotted against the resting membrane potentials. The number within each box represents the number of cells falling within that interval

Effects of Epinephrine on Parotid Acinar Cells

In a number of experiments two iontophoretic micropipettes were brought close to the recording electrode so that the effects of different agonists could be compared on the same cell. The membrane potential changes induced in 3 parotid acinar cells in the same gland by both acetylcholine and epinephrine are shown in Fig. 2. In any one of these cells the response to the two agonists was similar, and in all 13 cells, which were stimulated with both of these agonists, no qualitative differences were seen between the responses to epinephrine and to acetylcholine. In some cells stimulated with epinephrine the different phases of the response were not as clear as those previously described, an example of this being shown in Fig. 6, where there is no distinct delayed hyper-

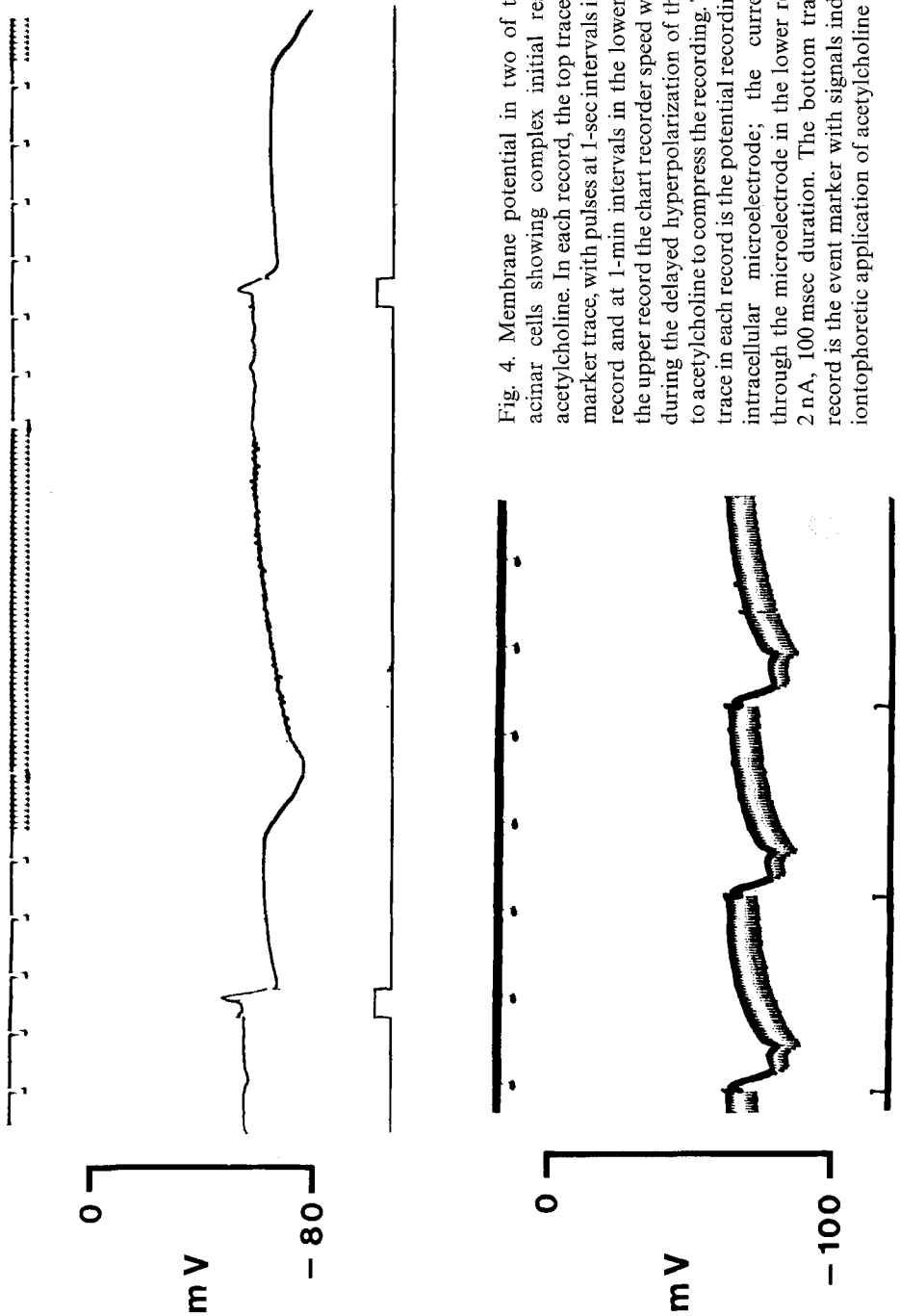

Fig. 4. Membrane potential in two of the parotid acinar cells showing complex initial responses to acetylcholine. In each record, the top trace is the time marker trace, with pulses at 1-sec intervals in the upper record and at 1-min intervals in the lower record. In the upper record the chart recorder speed was changed during the delayed hyperpolarization of the response to acetylcholine to compress the recording. The middle trace in each record is the potential recording from the intracellular microelectrode; the current pulses through the microelectrode in the lower record were 2 nA, 100 msec duration. The bottom trace in each record is the event marker with signals indicating the iontophoretic application of acetylcholine to the cells

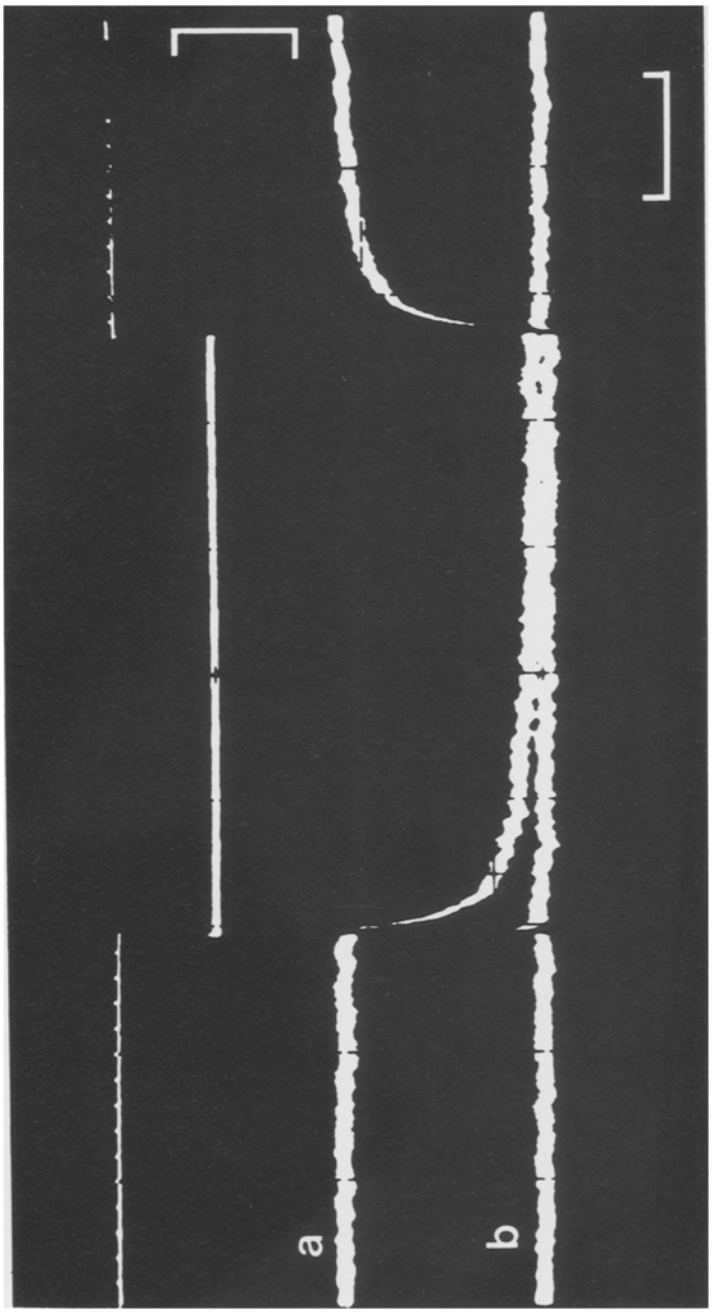

Fig. 5. Acetylcholine-induced change in the membrane input resistance of a parotid acinar cell. The upper trace represents the monitor for current injected through the intracellular microelectrode. The lower traces are membrane potential recordings; (*a*) was recorded from the resting cell and (*b*) was recorded during the initial hyperpolarization produced by acetylcholine. The vertical calibration bar represents 2.5 nA or 10 mV; the horizontal bar represents 20 msec

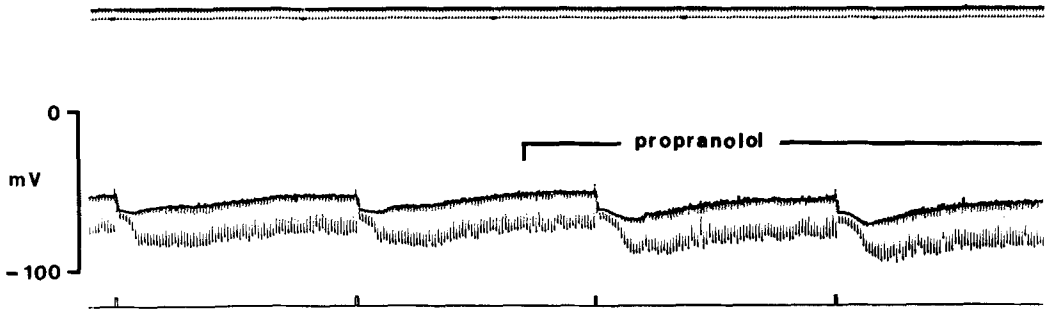

Fig. 6. Membrane potential and resistance changes produced in a parotid acinar cell by the iontophoretic application of epinephrine at the signals in the bottom pen recorder trace. Propranolol (5×10^{-6} M) was included in the superfusion fluid during the period indicated

polarization in the response to epinephrine. Blocking of the β-adrenoceptors by addition of propranolol made the delayed hyperpolarization more obvious (Fig. 6).

Phentolamine converted the response to epinephrine into a depolarization (Fig. 1). This effect was seen in 3 cells. In all 4 cells where propranolol was added first, the subsequent addition of phentolamine completely blocked the response to epinephrine. Atropine did not affect the responses to epinephrine (7 cells). The resistance changes during the response to epinephrine (Figs. 2 and 6) were similar to those previously described for acetylcholine.

Effect of Phenylephrine on Parotid Acinar Cells

The response to iontophoresis of phenylephrine was observed in 9 parotid acinar cells and in all cases the response was qualitatively similar to that previously described for acetylcholine. In 3 cells the responses to both phenylephrine and acetylcholine were observed and both agonists gave very similar responses.

Effect of Isoproterenol on Parotid Acinar Cells

Iontophoresis of isoproterenol produced a depolarization of 5.7 ± 1.1 mV ($n=9$). The response of one cell is shown in Fig. 7. Propranolol blocked the response to isoproterenol.

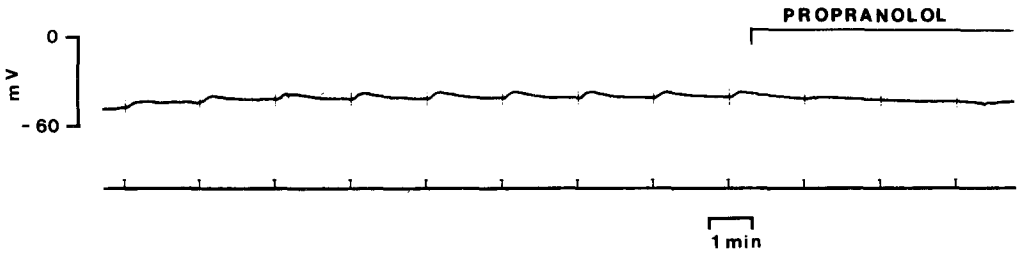

Fig. 7. Membrane potential change in a parotid acinar cell produced by iontophoretic application of isoproterenol, at the signals in the lower trace. Propranolol (5×10^{-6} M) was added to the superfusion fluid during the period indicated

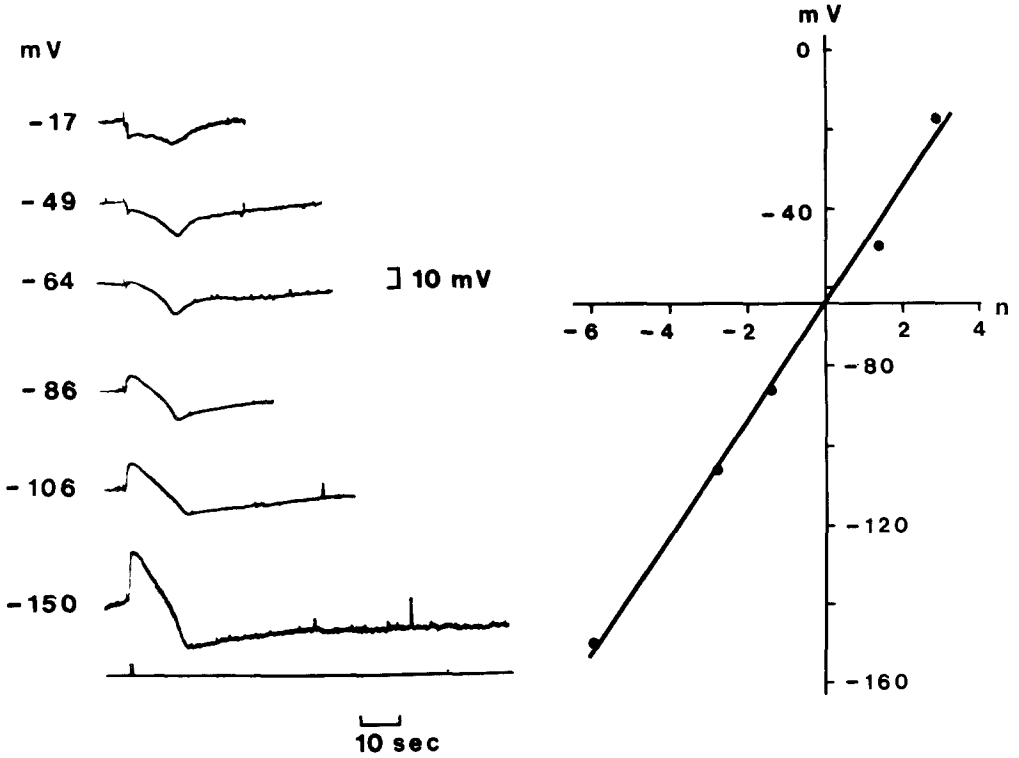

Fig. 8. Membrane potential changes produced by acetylcholine in one parotid acinar cell at different levels of membrane potential, produced by the passage of direct current through the recording electrode. The current-voltage relationship for the resting membrane is shown on the right; the line was fitted by eye

The Response of Parotid Acinar Cells to Acetylcholine at Different Levels of Membrane Potential

Some of the results described above were compatible with the idea that the direction of the early phase of the response to acetylcholine or

epinephrine depended on the potential of the resting membrane. To test this interpretation of the results, the membrane potential was shifted by the passage of hyperpolarizing or depolarizing direct currents through the recording electrode, and the response to acetylcholine recorded at the different potentials. As seen in Fig. 8, the current-voltage relationship for the unstimulated membrane in this cell was linear and the nature of the initial responses did depend on the potential of the resting membrane. This modification of the response on shifting the resting membrane potential was seen in 4 cells. Figure 8 also shows that, as the potential across the resting membrane increased, the size of the delayed hyperpolarization also increased. This was seen in 3 cells. In the other cell, in which current was passed to shift the membrane potential, the polarizing current was turned off after the early phases of the responses.

Latency of the Responses

Latency was estimated by recording on an oscilloscope the potential responses to applications of agonists. The iontophoretic micropipette was moved in the region of the intracellular electrode until the position was found which gave a response with the smallest latency. This delay, the minimum latency, was 210 ± 20 msec ($n=9$) in the responses to acetylcholine, 420 ± 40 msec ($n=6$) in the responses to epinephrine and 4.6 ± 0.4 sec ($n=10$) in the isoproterenol responses. The latency of the response to epinephrine was significantly greater than that to acetylcholine ($P<0.01$).

Responses of the Rat Submandibular Salivary Gland to Microiontophoresis of Acetylcholine and Epinephrine

The membrane resting potential of superficial cells in the rat submandibular gland was -57.4 ± 1.1 mV ($n=54$). Acetylcholine produced responses which were very similar to those described above for the mouse parotid gland, consisting of 2 phases. The early phase occurred at a time when the input resistance was markedly reduced, and the direction of the potential change depended on the resting potential of the cell. The late phase was always a hyperpolarization. Epinephrine produced responses similar to acetylcholine (Fig. 9). In 18 cells the effect of both acetylcholine and epinephrine was recorded, and in all cases there were no qualitative differences in the responses to the two agonists.

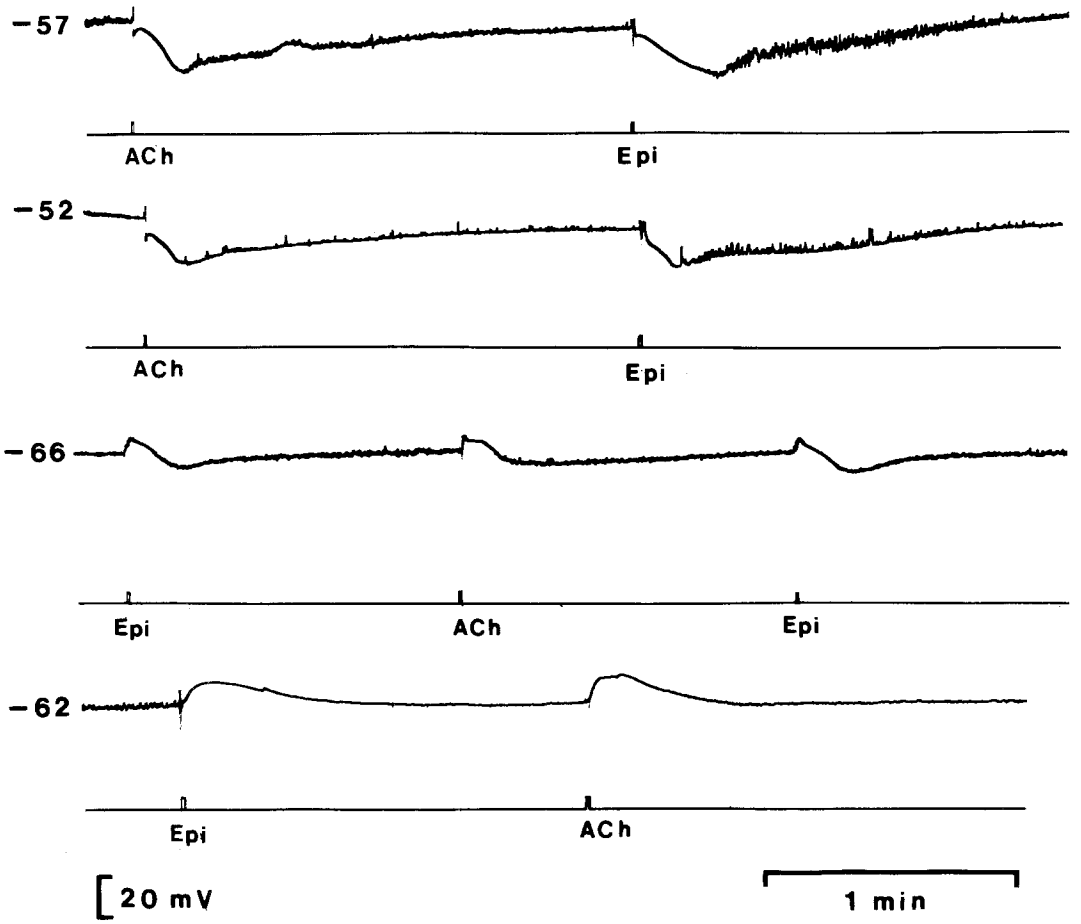

Fig. 9. Rat submandibular gland acinar cells. Membrane potential changes produced in 4 cells by iontophoretic application of acetylcholine (ACh) and epinephrine (Epi) at the event marker signals. The resting potential of each cell is written to the left of the potential recording

Submandibular gland cells resembled parotid cells also in that the initial part of the response to acetylcholine or epinephrine was sometimes more complex than the simple depolarization or hyperpolarization usually seen.

Discussion

Until the present study it was thought that membranes of parotid acinar cells responded in a manner qualitatively different from those of

other salivary glands (Petersen, 1976*b*). In this study it was shown that, when agonists were applied by microiontophoresis, rat submandibular gland acinar cells and mouse parotid acinar cells show apparently identical responses. It no longer appears necessary to propose different response mechanisms in the membranes of acinar cells in different salivary glands, at least in the case of rodents. The resting potentials of rat submandibular gland acinar cells measured in this study were higher than those previously reported for this species (Schneyer & Schneyer, 1965). Petersen (1976*b*) has discussed reasons for considering that the higher values are closer to the true membrane potential.

Petersen & Pedersen (1974) showed that the parotid acini respond electrophysiologically to more than one class of receptor, and a number of studies have demonstrated the functional activation of parotid acinar cells by cholinergic and α- and β-adrenergic agonists (Batzri & Selinger, 1973; Batzri, Selinger, Schramm & Robinovitch, 1973; Schramm & Selinger, 1974; Petersen & Ueda, 1975). The use of microiontophoresis has now provided the first records of individual acini responding to activation of all three of these receptor classes.

The responses of parotid acinar cells to acetylcholine or epinephrine applied by microiontophoresis were more complex than those described in earlier reports where agonists were added directly to the superfusion fluid (Pedersen & Petersen, 1973; Petersen & Pedersen, 1974). The appearance of 2 phases in the responses to these two agonists in the present study was probably explained by the rapid onset and short duration of agonist effect when microiontophoresis was used for drug application.

Cholinergic and α-adrenergic agonists produced similar changes in membrane potential and resistance, suggesting that the way in which agonists of these two classes produce their effects on the membrane of these salivary gland cells may be the same. During the early phase of the responses to acetylcholine and α-adrenergic agents, marked increases in membrane conductance occurred. The reversal potential of the initial phase (E_{ACh}) was approximately -55 mV (Figs. 3 and 8). There has been disagreement on the existence of reversal potentials for the electrophysiological responses in salivary glands. Lundberg (1957) was unable to reverse the hyperpolarization produced by stimulation of the parasympathetic nerves to the cat sublingual gland, while Imai (1974) was able to reverse the hyperpolarization of dog submandibular gland cells, which was produced by electrical stimulation of the parasympathetic nerves supplying the gland. The biphasic responses of mouse sub-

mandibular acinar cells to acetylcholine apparently had a reversal potential of approximately −60 mV for the first phase (Petersen, 1973), and a similar situation has now been convincingly demonstrated for the mouse parotid cells.

It would seem likely that the initial phase of the responses of the acinar cells to acetylcholine and α-adrenergic agonists was produced by Na^+ influx and K^+ efflux. Previous reports have described increased sodium and potassium permeability in submandibular gland acinar cells stimulated with acetylcholine (Petersen, 1970, 1972; Nishiyama & Petersen, 1974*a*; Poulsen, 1974). Recently Poulsen and Bledsoe (1977) have demonstrated that the concentration of K^+ in the interstitial fluid of cat and dog submandibular glands could rise to as high as 18 mM during the period of parasympathetic nerve stimulation. If this were the case in the present study, then the initial potential change of the parotid cells could be explained by increased K^+ efflux, with E_{ACh} equal to E_K. However, it is unlikely that K^+ accumulated in our tissue segments to the same extent as reported by Poulsen and Bledsoe (1977) *in vivo* since Petersen, Gray and Hall (1977) have shown that in the superfused parotid, the rapid flow of bathing fluid prevented any marked rise in $[K^+]$ in the bath effluent. As our recordings were obtained from superficial cells it is likely that K^+ released from the cells was rapidly removed.

The delayed hyperpolarization did not appear to be due entirely to permeability changes as the peak of this response occurred when the membrane conductance had returned towards that seen in the resting cell. In addition, hyperpolarizing the resting membrane by the passage of current increased the delayed hyperpolarization of the acetylcholine response. Hyperpolarizing the membrane by the passage of direct current would increase the electrochemical gradient of Na^+, and more Na^+ influx could then occur in the early phase of the acetylcholine response. It now appears likely that a large portion of the acetylcholine-induced delayed hyperpolarization is due to electrogenic sodium pumps. Roberts and Petersen (*in preparation*) have shown that this delayed hyperpolarization can be reduced by application of ouabain or by the use of K^+-free or Na^+-free superfusion fluids.

The latencies of the responses of the parotid gland to acetylcholine and epinephrine were similar to those reported for the pancreas when acetylcholine was applied iontophoretically and to those measured in cat submandibular gland following stimulation of the parasympathetic nerves (Kagayama & Nishiyama, 1974; Nishiyama & Petersen, 1975). Although the latency of the response to epinephrine was longer than that

to acetylcholine, the physiological significance of this difference is not clear.

Isoproterenol induced a response with a latency more than 10 times longer than that measured with cholinergic and α-adrenergic agonists. Recently Hill-Smith and Purves (1977) reported that the minimum latency for responses of cultured myocardial cells to iontophoretically applied catecholamines is more than 4 sec, a figure similar to that found for the β-adrenoceptor-induced response in this study. The long latency of the β-adrenergic response cannot be accounted for by diffusion delay, as in the same cell epinephrine induced a short latency α-adrenergic response and, after the addition of phentolamine, a long latency β-adrenergic response (Fig. 1). It is possible that the depolarization produced by activation of β-adrenoceptors resulted from some secondary response induced within the cell by the β-adrenergic agonist.

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