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MOUSE PANCREATIC ACINAR CELLS: EFFECTS OF ELECTRICAL FIELD STIMULATION ON MEMBRANE POTENTIAL AND RESISTANCE

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SUMMARY

1. Intracellular micro-electrode recordings of acinar cell membrane potential and resistance were made from the mouse pancreas superfused *in vitro*. The acinar cells under investigation were stimulated by electrical field stimulation using two platinum wire electrodes and by micro-ionophoretic acetylcholine (ACh) application from an extracellular AChCl-filled micro-electrode.

2. Field stimulation evoked membrane depolarization and reduction in input resistance. Maximal effects were observed at 20–40 Hz frequency, 1–2 msec pulse width and 8–20 V amplitude. The mean latency for the field stimulation-evoked depolarization was 900 msec. Field stimulation responses were seen at low frequency levels of stimulation, the majority of cells responding at 5 Hz and some at 2 Hz. The physiological significance of the low frequency stimulation is discussed.

3. The field stimulation effects resembled those induced by ACh ionophoresis and were abolished by atropine. The equilibrium potentials for both field stimulation and ACh ionophoresis were identical at about -15 mV. The field stimulation response was selectively abolished by tetrodotoxin and by superfusion with Na-free or Ca-free media, while the ACh ionophoretic response persisted. Field stimulation therefore initiated nerve action potentials and consequent ACh release.

4. Spontaneous miniature depolarizations observed in some preparations were not abolished by tetrodotoxin and would therefore seem to be a result of quantal release of ACh from nerve terminals.

5. There is no indication from the present studies of the existence of neurotransmitters other than ACh. No inhibitory effects have been observed.

6. All preparations studied to date have responded to field stimulation and it is concluded that all acinar cells are potentially under cholinergic neural influence.

INTRODUCTION

Electrophysiological studies on pancreatic acinar cells have provided useful information on the process of stimulus-secretion coupling (Petersen, 1976). Ionophoresis of neurotransmitter acetylcholine (ACh) produces well defined electrophysiological changes in acinar cells recorded from intracellular micro-electrodes and it seems, therefore, that intracellular recording would be invaluable in studies on the functional innervation of the exocrine pancreas if coupled with suitable methods of nerve stimulation. However, there have been few attempts to develop suitable

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preparations. Dean & Matthews (1972), using intracellular recording techniques, described depolarization of a pancreatic acinar cell following stimulation of the perivascular nerves in an isolated segment of mouse pancreas. Davison & Ueda (1977) have succeeded in recording depolarization of rat pancreatic cells *in vivo* following cervical vagal stimulation. More recently, Nishiyama, Katoh, Saitoh & Wakui (1979) have described a simple technique of electrical field stimulation of isolated pancreatic and salivary gland segments which activates the gland cells, presumably by releasing transmitter from the nerve terminals. Because of its elegant simplicity and because similar methods have proven invaluable in studies on the intrinsic innervation of smooth muscle (Kosterlitz, 1968), we have developed a similar method to study the pancreatic innervation. In this report we present data to show that electrical field stimulation activates pancreatic nerves, leading to release of endogenous ACh and we compare the response of the gland cells to endogenous transmitter and exogenous ACh applied by micro-ionophoresis. A preliminary report of this work has already been given (Davison & Pearson, 1979).

METHODS

Experiments were performed on isolated segments of mouse pancreas. The tissue was secured to a platform, placed in a Perspex bath (30 ml.) and superfused with physiological salt solutions at 37 °C at a flow rate of about 15 ml. min^{-1}. The standard Krebs–Henseleit solution had the following composition (mM): NaCl 103, KCl 4·7, $CaCl_2$ 2·56, $MgCl_2$ 1·13, $NaHCO_3$ 25, NaH_2PO_4 1·15, D-glucose 2·8, Na pyruvate 4·9, Na fumarate 2·7, Na glutamate 4·9; it was gassed with 95 % O_2 and 5 % CO_2. To Ca-free solutions, EGTA (ethylene glycol-bis-β-amino ethyl ether *N*, *N'*-tetra-acetic acid (Sigma) 10^{-4} M) was added. In Na-free solutions, NaCl was replaced by equiosmolar TrisCl; the pyruvate, fumarate and glutamate substrates were added as acids rather than as Na salts and the pH was adjusted to 7·4 with HCl (or H_2SO_4) and Tris (base). All solutions were routinely checked for osmolality (290 ± 5 m-osmole kg^{-1}). Drugs used were: acetylcholine chloride (Merck), atropine sulphate (Merck) and tetrodotoxin (Sigma).

Glass micro-electrodes, filled with 3 M-KCl and 10 mM-K citrate, with tip resistances of about 20–30 MΩ were used for measurement of cellular transmembrane potentials. Micro-electrodes with initial tip resistances of above 35 MΩ were bevelled to within the range quoted using a micro-electrode beveller (Sutter Instruments, BV-10). Impalements of surface acinar cells were obtained with a stepping motor micro-manipulator (AB Transvertex, Stockholm). The intracellular micro-electrode was connected to an electrometer amplifier (W-P Instruments, M701) allowing current injections and measurement of membrane potential and input resistance (for a detailed description see Nishiyama & Petersen, 1974).

Electrical field stimulation of the tissue was achieved using a pair of 0·5 mm diameter silver or platinum wire electrodes. They were mounted on a micromanipulator and brought into contact with the tissue on the perspex platform at a distance apart of about 3 mm. The electrodes were connected to a Devices stimulator (Type 2533) which was triggered by a Devices Pulse Generator to provide square-wave stimulation of the required frequency, pulse width, and voltage. To ensure precise control of the duration of field stimulation the pulse generator was triggered by a Devices Digitimer (Type 3290).

ACh was applied topically to the tissue by micro-ionophoresis from an extracellular micro-electrode filled with 2 M-AChCl. A WPM 160 Ionophoresis Programmer was used, triggered by a Devices stimulator in a manner similar to that previously described (Nishiyama & Petersen, 1975). The arrangement of electrodes and tissue is shown schematically in Fig. 1.

Membrane potentials, their electrotonic changes and the injection current, were displayed on a Tektronix storage oscilloscope screen and recorded with a Devices M2 pen-recorder.

RESULTS

Electrical field stimulation of the pancreas produced depolarization of impaled acinar cells. Depolarizations were generally seen in response to stimulation with square-wave of 5–20 V amplitude, 5–40 Hz frequency and 1–2 msec pulse duration. Responses have also occasionally been seen at frequencies as low as 2 Hz (see Fig. 1, Davison & Pearson, 1979). The field stimulation-evoked depolarization was accompanied by a reduction in membrane resistance and electrical time constant with maximal effects of about 15–20 mV depolarization occurring at 20–40 Hz, 1–2 msec, 8–20 V (Fig. 2). At the 5 and 10 Hz frequencies of stimulation the rate of rise of the depolarization was slower than that of the 40 Hz frequency of stimulation and it was therefore necessary to increase the stimulus duration to achieve the full depolarizing effect.

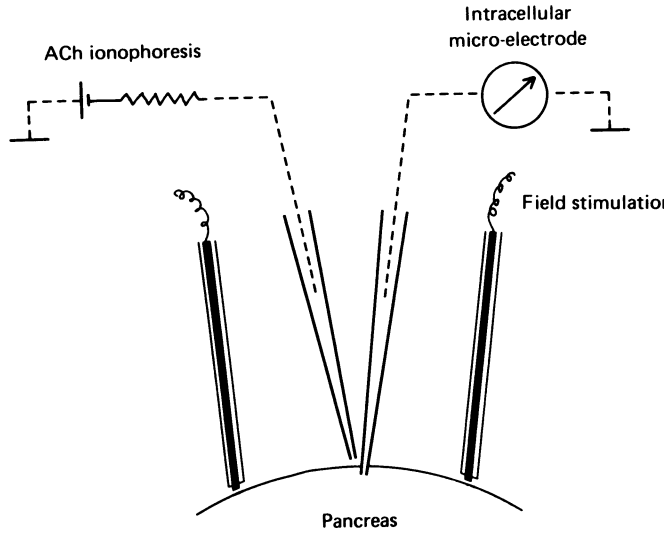

Fig. 1. Schematic representation of experimental arrangement showing intra- and extracellular micro-electrodes and platinum field stimulation electrodes.

Fig. 3 shows how field stimulation parallels the now well established response of acinar cells to ionophoresis of ACh (Nishiyama & Petersen, 1975). Shown in the lower half of the Figure are latency recordings for the two types of stimulation. The two values are similar in this particular cell at about 700 msec. However the latency for the ACh depolarization is somewhat longer than the accepted norm of around 100–300 msec (Nishiyama & Petersen, 1975). This may be due to the depth of impalement which was greater than usual in this case. Indeed, we have occasionally failed to record any effect of ACh ionophoresis in very deep impalements, which did however still respond to field stimulation in the normal manner. The mean latency for the field stimulation-evoked depolarization was 900 msec (range 450–1300, $n = 20$).

The similarity between the responses of acinar cells to externally applied ACh and field stimulation is best seen in Fig. 4*A*, *B*, where the equilibrium potentials for both types of stimulation (E_{ACh} and E_{FS}) are determined. There is a very good correlation

between the two when the potential changes are plotted as functions of resting potential. A range of -10 to -16 mV has been obtained for five such determinations of E_{FS}. This would appear to correlate well with a mean of -15 mV for E_{ACh} in mouse pancreas (Iwatsuki & Petersen, 1977).

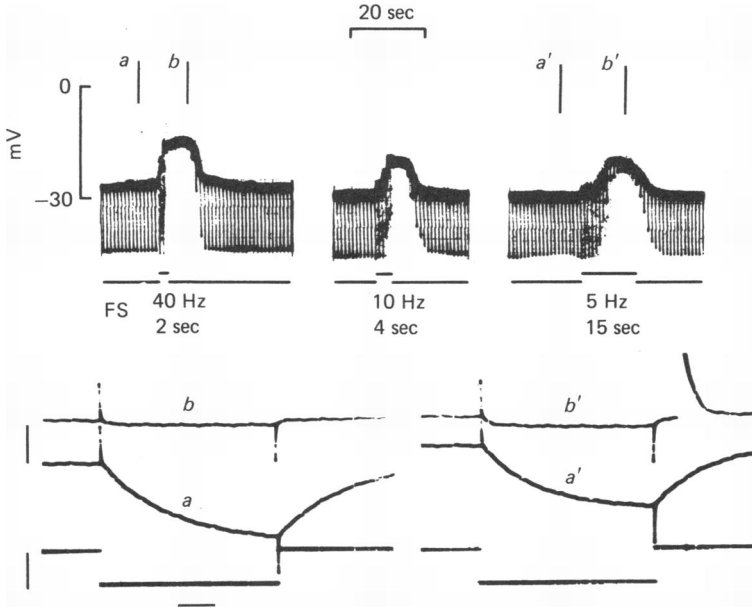

Fig. 2. Effects of electrical field stimulation (FS) on membrane potential and resistance. The top portion shows the intracellular potential recorded with a micro-electrode, through which rectangular current pulses (10^{-9} A, 100 msec) were repetitively injected. These pulses caused short-lasting membrane hyperpolarizations the amplitude of which gave an indication of the cell input resistance. Parameters for field stimulation were 10 V amplitude and 1 msec pulse width (frequencies as in Figure). The breaks in the recording each constitute 2 min intervals. The oscilloscope photographs below show, at a higher time base amplification, the shape of cell membrane hyperpolarizations caused by the rectangular current pulses (shown at bottom of trace). Photographs were taken at rest (a, a') and during field stimulation (b, b'). Calibration: vertical, 10 mV and 10^{-9} A; horizontal, 20 msec.

Atropine ($1{\cdot}4 \times 10^{-6}$ M) abolished the depolarization evoked by both ACh-iontophoresis and field stimulation (Fig. 5). Five experiments of this type were performed.

Tetrodotoxin (10^{-6} M) very quickly abolished the response to field stimulation, leaving the response to ACh ionophoresis largely unaffected (Fig. 6). This total abolition of the field stimulation-evoked depolarization by tetrodotoxin has been shown in a total of seven experiments. In each, as in the experiment depicted in Fig. 6, the response to ACh ionophoresis was slightly reduced in duration after tetrodotoxin application. At present it is difficult to ascertain whether this is in fact due to a direct action of the drug or simply some effect of changing the flow rate through the tissue bath. Fig. 6 also shows that, as expected, atropine blocks the effect of ACh ionophoresis.

Frequently, like previous authors (Dean & Matthews, 1972, Nishiyama & Petersen,

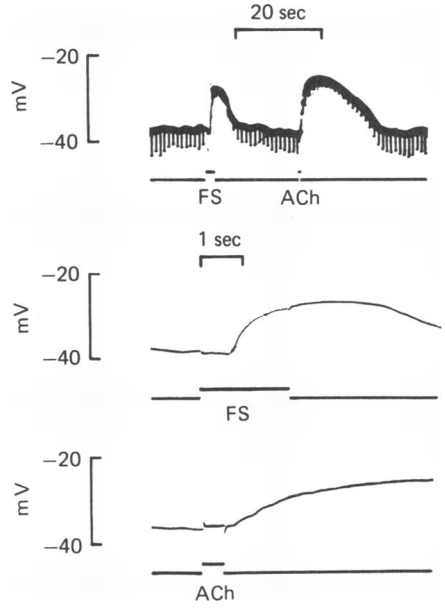

Fig. 3. Effects of field stimulation and ACh applied by ionophoresis. The upper portion shows the resting potential and resistance and the responses to field stimulation (FS) (40 Hz, 2 msec, 20 V, 2 sec) and ACh ionophoresis (90 nA, 500 msec, with a retaining current of 25 nA). Hyperpolarizing current injections were 2×10^{-9} A, 100 msec. The time courses of two subsequent depolarizations in response to field stimulation and ACh are shown below on a faster chart speed.

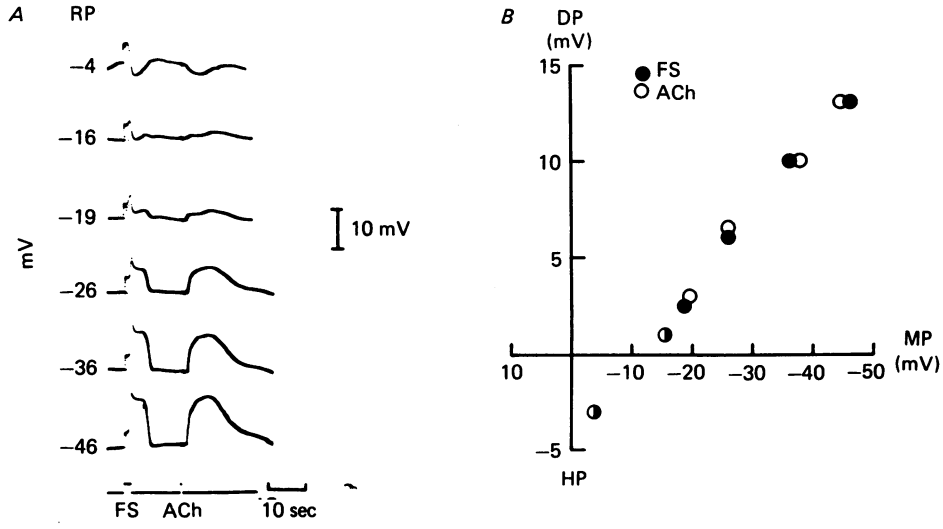

Fig. 4. The effects of field stimulation (FS) and ACh ionophoresis on the membrane potentials (*A*). The true resting potential (RP) was −36 mV. Higher and lower potentials were obtained by passing direct hyperpolarizing (HP) or depolarizing (DP) currents, respectively, through the intracellular recording micro-electrode. In *B*, the field stimulation- and ACh-evoked potential changes shown in *A* were plotted against their respective membrane potentials (MP) (abscissa) before stimulation. (● = field stimulation, ○ = ACh). Stimulus parameters: field stimulation, 40 Hz, 2 msec, 10 V, 2 sec; ACh ionophoresis, 90 nA, 500 msec, 25 nA retaining current.

1974), we have observed spontaneous miniature depolarizations superimposed upon the resting potential. Application of tetrodotoxin (10^{-6} M) in two such preparations abolished the response to field stimulation as expected but did *not* affect the spontaneous activity (Fig. 7). However, application of atropine ($1{\cdot}4 \times 10^{-6}$ M) eliminated these fluctuations. In the latter stage of the experiment, depicted in Fig. 7, caerulein (diethylamine salt) was added directly to the bath. This drug, for which specific

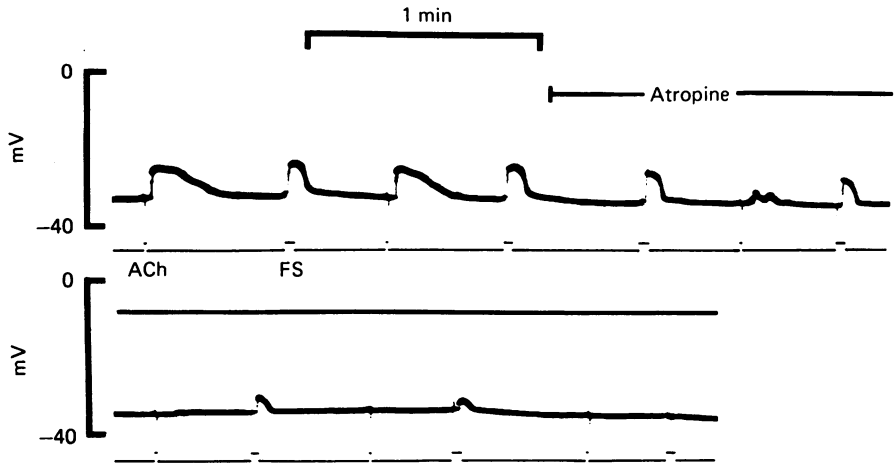

Fig. 5. The effect of atropine ($1{\cdot}4 \times 10^{-6}$ M) on the depolarizations induced by field stimulation and ACh ionophoresis. Stimulus parameters: field stimulation, 40 Hz, 2 msec, 20 V, 2 sec; ACh ionophoresis, 90 nA, 500 msec, 25 nA retaining current.

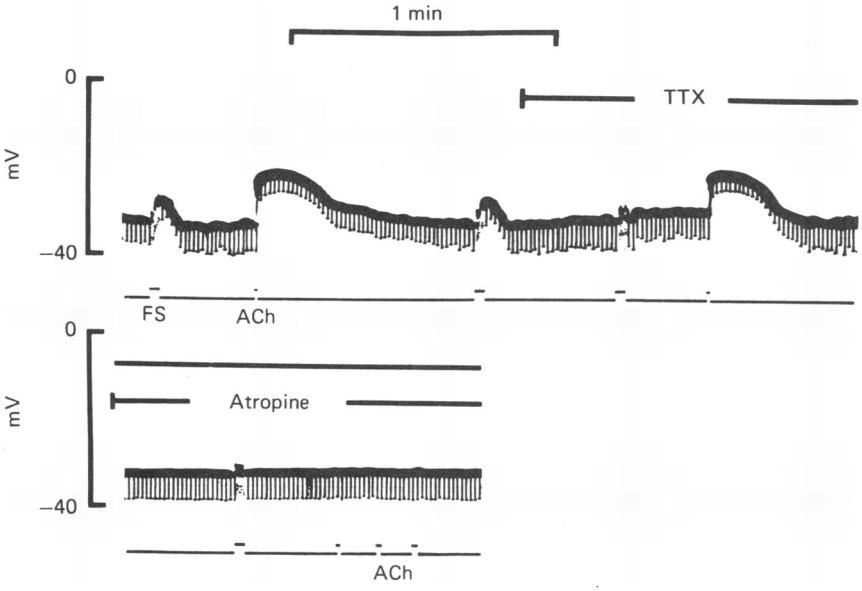

Fig. 6. The effect of tetrodotoxin (TTX, 10^{-6} M) on the depolarizations induced by field stimulation and ACh ionophoresis. Atropine was subsequently applied ($1{\cdot}4 \times 10^{-6}$ M). Stimulus parameters: field stimulation, 40 Hz, 1 msec, 20 V, 2 sec; ACh ionophoresis, 100 nA, 200 msec, 25 nA retaining current. Current injection, 10^{-9} A, 100 msec. The final two ACh ionophoretic applications were for durations of 500 and 1000 msec.

receptors on the acinar cell membrane are known to exist (Iwatsuki & Petersen, 1978*b*), caused a marked depolarization and showed that the preparation was still responsive even in the presence of tetrodotoxin and atropine.

Fig. 8 demonstrates the effects of removal of Na from the perfusing solution. It can be seen that the responses to field stimulation and ACh ionophoresis in this experiment were smaller than normal. This is probably explained by the lower resting potentials recorded in the Tris solutions used during Na-free experiments. However, it is clear that whereas removal of Na abolished the field stimulation response, ACh ionophoresis still produced a response in the Na-free situation, albeit smaller and without so marked a reduction in resistance as in the control state. These changes in the ACh response are, however, consistent with earlier findings in Na-free solutions (Nishiyama & Petersen, 1975). Recovery of the field stimulation response,

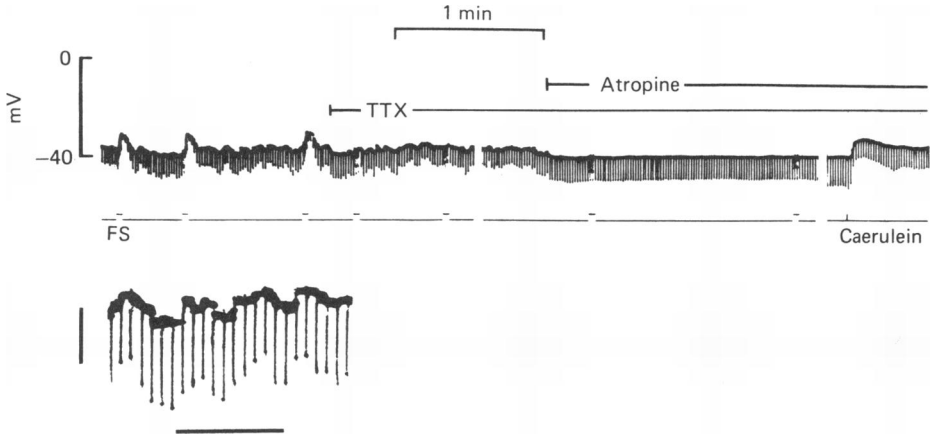

Fig. 7. Effects of tetrodotoxin (10^{-6} M) and atropine ($1{\cdot}4 \times 10^{-6}$ M) on a preparation showing marked spontaneous miniature depolarizations. Field stimulation parameters: 40 Hz, 2 msec, 10 V, 2 sec. Current injection: 10^{-9} A, 100 msec. One drop of a 20 μg/ml. solution of caerulein (diethylamine salt) was applied topically to the tissue in the bath (volume 30 ml.). The breaks in the record constitute 2 and 4 min intervals respectively. Inset: enlargement of spontaneous depolarizations. Calibrations: horizontal, 10 sec; vertical, 5 mV.

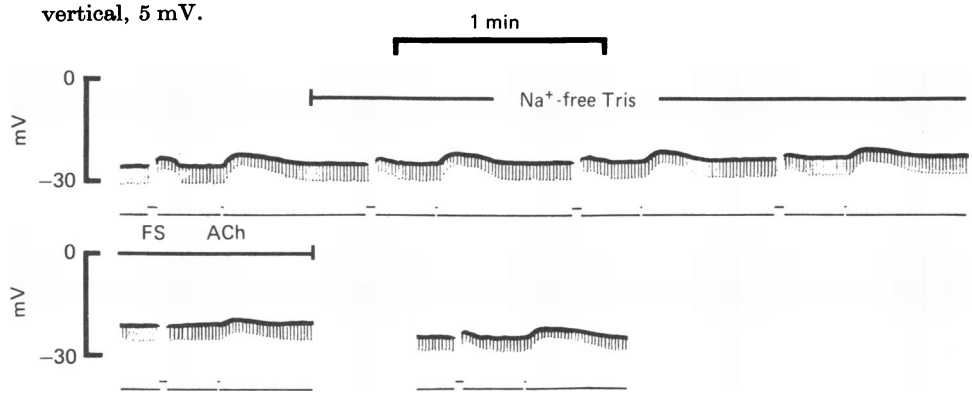

Fig. 8. The effect of Na removal from the superfusion solution on the depolarizations induced by field stimulation and ACh ionophoresis. Stimulus parameters: field stimulation, 40 Hz, 2 msec, 20 V, 2 sec; ACh ionophoresis, 90 nA, 500 msec, 25 nA retaining current. Current injection: 1 nA, 100 msec. The break in the lower part of the trace constitutes a 15 min interval.

albeit not complete, was seen on returning to control Tris solution. The complete blockade of the field stimulation response by removal of Na has been successfully achieved in five other preparations.

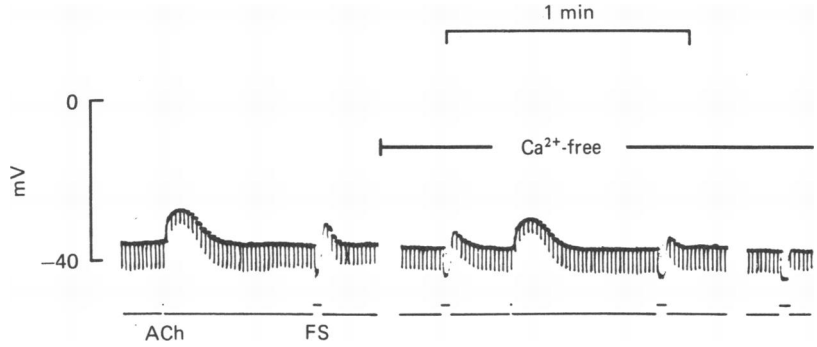

Fig. 9. The effect of Ca removal from the superfusion solution on the depolarizations induced by field stimulation and ACh ionophoresis. 10^{-4} M-EGTA was present in the Ca-free superfusion fluid. Stimulus parameters: field stimulation, 40 Hz, 2 msec, 20 V, 2 sec; ACh ionophoresis, 100 nA, 200 msec, 30 nA retaining current. Current injection: $1{\cdot}5 \times 10^{-9}$ A, 100 msec. The two breaks in the record constitute 5 and 2 min intervals respectively.

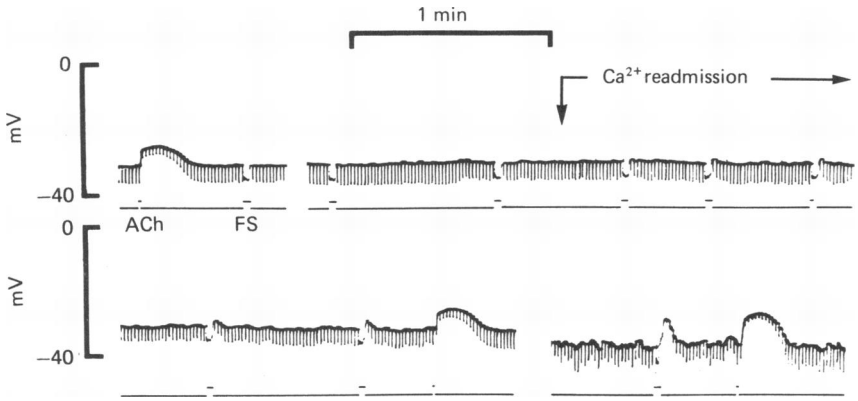

Fig. 10. The effect of Ca readmission on the depolarizations induced by field stimulation and ACh ionophoresis. The tissue had been bathed by Ca-free solution for 15 min before impalement. Stimulus parameters: field stimulation, 40 Hz, 2 msec, 10 V, 2 sec; ACh ionophoresis, 100 nA, 500 msec, 30 nA retaining current. Current injection: 10^{-9} A, 100 msec. The two breaks in the record constitute 1 and 30 min intervals, respectively.

The field stimulation-evoked depolarization is also abolished by replacing control Krebs solution with a Ca-free Krebs containing 10^{-4} M-EGTA (Fig. 9). The ACh-evoked depolarization is unaffected by the replacement. Ca removal was performed on a total of five occasions. Although the response to field stimulation was always blocked without abolishing the ACh response, it was not possible to obtain a good reversal of the effect in the same cell, due to loss of impalement. Therefore it was necessary to reimpale during exposure to Ca-free solution and Fig. 10 shows the results of such a procedure. The tissue had been exposed to Ca-free Krebs with EGTA for 15 min before impalement. Thus, initially, depolarizations are in response to ACh ionophoresis and not field stimulation. On readmission of calcium a response

to field stimulation gradually becomes apparent and some thirty minutes later the response is quite marked. Also worth noting is the fact that, as Ca returns, the frequency and amplitude of spontaneous activity increases.

DISCUSSION

From the present studies on pancreatic cells five main conclusions can be drawn.

1. Electrical field stimulation evoked release of endogenous ACh by initiation of nerve action potentials.

2. The effects were seen at stimulation frequencies which are likely to resemble closely the physiological frequency of discharge in pancreatic nerves.

3. All acinar cells are potentially under cholinergic neural influence.

4. Spontaneous, miniature depolarizations are due to spontaneous, quantal release of ACh from nerve terminals.

5. There is no indication of the existence of transmitters other than ACh.

Nishiyama *et al.* (1979) have recently shown that field stimulation of the exocrine pancreas produces membrane depolarization and reduction in membrane resistance. They showed that the equilibrium potential for the field stimulation-evoked depolarization was between -10 and -20 mV. It was therefore reasonable for them to suggest that their results demonstrated the similarities between the responses of acinar cells to externally applied ACh (Nishiyama & Petersen, 1975; Iwatsuki & Petersen, 1977) and to ACh released from pancreatic nerve endings. Our recent experiments have been conducted to show definitively that field stimulation is indeed effective in releasing ACh and that this is due to the generation of action potentials in pancreatic nerves and not simply a result of direct depolarization of nerve terminals.

The depolarization in response to field stimulation was abolished by the muscarinic antagonist atropine, thus showing that the response was ACh-mediated. Application of tetrodotoxin or removal of Na from the superfusion solution abolished the field stimulation response but not the response to exogenous ACh. Both these procedures prevent action potential initiation (Narahashi, 1974; Hodgkin & Katz, 1949) and would thus block any nerve-mediated ACh release. Removal of Ca has also been shown to completely block the field stimulation response without abolishing the response to ACh ionophoresis. This would again be expected since external calcium is required for the secretion of ACh from nerve terminals (Katz & Miledi, 1967). The effects of tetrodotoxin, the removal of sodium or calcium and the good correlation between E_{FS} and E_{ACh} provides a clear foundation for conclusion (1).

Most of the recent studies have been performed using supramaximal levels of stimulation (40 Hz, 2 msec, 20 V, 2 sec). The great majority of cells did however respond at 5 Hz with sizeable depolarizations and on occasions frequencies as low as 2 Hz have evoked responses. Electrical stimulation of the vagus nerve produces conductance changes in the pancreas of the anaesthetized cat with threshold frequencies of about 2 Hz and maximal responses are seen at 7–15 Hz (Greenwell & Scratcherd, 1974). In the same report, vagal stimulation, against a background of secretin stimulation, increased amylase output with maximal effects occurring at only 5 Hz. It appears likely that these low frequencies of stimulation more closely approximate the physiological frequency of discharge in pancreatic nerves.

The fact that all healthy preparations with good resting potentials (i.e. not near

E_{FS}) have responded to field stimulation demonstrates that each acinar cell is potentially under cholinergic neural influence. Indeed it is already known that acinar cells receive a direct innervation from fibres of intrapancreatic ganglion cells which act as terminal stations of vagal fibres (Richins, 1945). However, not all cells are necessarily directly innervated. Indeed, one explanation of the considerable variation in the degree of field stimulation-evoked depolarization could be the lack of direct innervation in some cells. However, since all cells studied responded to field stimulation, it is our belief that a large number of acinar cells receive an indirect innervation by virtue of the fact that they belong to an electrically coupled unit of about 500 cells (Iwatsuki & Petersen, 1978*a*) in which perhaps only a small number are directly innervated. Moreover, it appears that each coupled unit receives a multiple innervation since all such cells, so studied, produced graded responses to increasing voltage with thresholds at 2–5 mV and with maximal effects seen at 8–20 V. This, presumably, is due to the progressive recruitment of nerve fibres of different thresholds, since, as discussed above, the field stimulation is generating nerve action potentials rather than producing graded depolarizations of nerve terminals. Since there are marked differences in latencies of responses to field stimulation, it appears that there may be variations in the closeness of innervation of different cells.

It is possible that the cells with the closest innervation were those exhibiting spontaneous miniature depolarizations. Certainly such cells usually responded with short latencies. Dean & Matthews (1972) were the first to describe these miniature depolarizations in the exocrine pancreas. They suggested that these were cholinergic in origin and were a probable result of quantal release of ACh from the nerve endings resembling those at the neuromuscular junction (Fatt & Katz, 1952). It seemed reasonable to question whether in fact these miniature depolarizations were the result of quantal release of ACh or rather a result of tonic activity in pancreatic nerves. Experiments of the type described in Fig. 6 strongly favour the former possibility since tetrodotoxin effectively blocks the nerves, as shown by the abolition of the field stimulation response, without affecting the spontaneous depolarizations. They are, however, subsequently abolished by atropine. The quantal release of ACh is also suppressed by removing Ca from the superfusate. This would be expected since secretion of ACh from the nerve terminal requires Ca. Spontaneous activity returns as Ca is reintroduced (Fig. 9).

Davison & Ueda (1977), in addition to showing depolarization of acinar cells in response to vagal stimulation, also noted occasional, transient hyperpolarizations possibly suggesting an inhibitory innervation. In our present *in vitro* studies we have never observed hyperpolarizations in response to field stimulation and impalements made in atropinized preparations failed to respond, in any manner, to nerve stimulation, even at supramaximal levels of stimulation. It would therefore seem reasonable to suggest that the transient hyperpolarizations previously recorded *in vivo* were either artifactual or that conditions *in vivo* favour the demonstration of inhibitory effects. Thus we can, at present, provide no evidence for acinar cells receiving any innervation other than cholinergic. It must be said that this does not entirely exclude the possibility that other transmitters than ACh exist.

Our investigations have shown so far that the response to endogenous ACh, released by field stimulation, and the response to ACh ionophoresis are identical. The membrane

depolarization, changes in membrane input resistance and time constant, and the equilibrium potential were similar for both stimuli. Where differences in acinar cell response were observed, for example in latency and in ionic dependence, this was attributable to the mechanism and location of transmitter release rather than to transmitter mode of action. Because of the simplicity of this method, electrical field stimulation may provide a useful additional technique to ACh ionophoresis in studies on stimulus-secretion coupling. Moreover, it provides a simple alternative to *in vivo* extrinsic nerve stimulation for studies on the functional innervation of the exocrine pancreas.

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