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ELECTROPHYSIOLOGY OF MOUSE PAROTID ACINI: EFFECTS OF ELECTRICAL FIELD STIMULATION AND IONOPHORESIS OF NEUROTRANSMITTERS

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SUMMARY

1. Intracellular micro-electrode recordings of membrane potential and input resistance were made from surface acini of mouse parotid glands placed in a Perspex tissue bath through which oxygenated physiological saline solutions were circulated. The acinar cells were stimulated by microionophoresis of both acetylcholine (ACh) and adrenaline (Ad) from extracellular micropipettes, and by electrical field stimulation via a pair of platinum electrodes.

2. The acinar cells had a mean resting membrane potential of $-64{\cdot}9$ mV $\pm$ 0·6 S.E. The input resistance of the unstimulated cell was 4·63 MΩ $\pm$ 0·19 S.E. In a number of cells spontaneous miniature depolarizations were observed, associated with synchronous reductions in input resistance.

3. The responses to ionophoresis of both ACh and Ad and the response to supramaximal field stimulation were identical. Stimulation always evoked a marked decrease in input resistance associated with an initial potential change, generally followed by a delayed hyperpolarization during which the input resistance returned to normal.

4. Field-stimulation responses could be evoked to single shock (1–2 msec) and to low frequency (1–4 Hz) stimulation. The latency for this response was 245 msec $\pm$ 12 S.E.

5. The field-stimulation response was shown to be susceptible to blockade of nerve conduction in sodium-free or tetrodotoxin- (TTX-) containing media; and to blockade of neurotransmitter release in calcium-free media.

6. The field-stimulation and ACh responses were recorded at different levels of membrane potential within the same cells by applying either hyperpolarizing or depolarizing direct current through the recording electrode. The membrane potential at which the initial potential change undergoes reversal, i.e. changes from a depolarization to a hyperpolarization, is known as the equilibrium or reversal potential, E_{FS} and E_{ACh} respectively. The field-stimulation (FS) and ACh responses underwent simultaneous reversal at about -60 mV, i.e. $E_{FS} = E_{ACh}$.

Equilibrium potentials were also determined indirectly by analysis of the responses evoked by each stimulus in the manner described by Trautwein & Dudel (1958).

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Using this technique the equilibrium potentials of the responses to all three stimuli, field stimulation, ACh and Ad, were again about -60 mV, i.e. $E_{FS} = E_{ACh} = E_{Ad}$.

7. Both the field-stimulation and ACh responses were abolished by atropine (10^{-6} M) while the response to Ad persisted. Atropine also abolished all spontaneous activity. The α-adrenergic blocker phentolamine (10^{-5} M) abolished the response to Ad but left the field-stimulation response unaffected.

8. Electrical field stimulation of isolated segments of salivary gland evoked release of endogenous neurotransmitter as a consequence of neural excitation. The technique of field stimulation thus makes it possible to investigate the functional innervation of a gland using the *in vitro* preparation. In the mouse parotid gland the field stimulus response was mediated by ACh released from parasympathetic nerve endings.

INTRODUCTION

In vitro preparations have been used extensively in the electrophysiological investigation of the mechanisms of autonomic, particularly cholinergic, receptor activation in salivary glands. The advantages of the isolated preparation lie in its inherent simplicity and in the ease with which the tissue lends itself to pharmacological or ion substitution experimentation. It has, however, suffered from the disadvantage that stimulation has been achieved by application of exogenous autonomic agonists, either in the superfusion media or locally by microionophoresis (Nishiyama & Petersen, 1974; Roberts, Iwatsuki & Petersen, 1978; Roberts & Petersen, 1978). While such pharmacological stimuli enable one to investigate receptor mechanisms they reveal nothing of the role of the intrinsic innervation in activation of these receptors.

Formerly, any investigation of the functional innervation of the salivary glands has necessitated the use of an *in vivo* preparation, release of endogenous neurotransmitter being achieved by electrical stimulation of dissected nerve trunks (Lundberg, 1955, 1958; Kagayama & Nishiyama, 1974; Emmelin, Grampp & Thesleff, 1980). Such an *in vivo* preparation is however difficult and has inherent limitations. It does not provide a stable recording system, and the necessary consideration for the general systemic condition of the animal limits the subsequent pharmacological experimentation. Also it cannot be applied to all species and most studies have utilized the larger experimental animals, cat or dog, not necessarily representative of other species, in particular man (Mandel, Zengo, Katz & Wotman, 1975).

In this study we describe the application of the very simple technique of electrical field stimulation as a means of evoking release of endogenous neurotransmitter in the *in vitro* preparation. This technique has been used extensively by physiologists in the investigation of the intrinsic innervation of smooth muscle. Although House (1973) reported that field stimulation could evoke responses in the cockroach salivary gland its application to mammalian exocrine glands has only recently been described (Nishiyama, Katoh, Saitoh & Wakui, 1980). A preliminary report of this work has already been published (Gallacher, 1979).

METHODS

Segments of parotid gland were removed from adult male mice killed by cervical dislocation. Segments of these glands were secured to a Perspex platform in a tissue bath (20 ml.) through which a physiological salt solution warmed to 37 °C flowed at about 15 ml. min^{-1}. The standard 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 glutamate, 4·9; Na fumarate, 2·7. It was gassed with 95% O_2, 5% CO_2. All solutions were routinely checked for osmolality (290 m-osmole kg^{-1}). In Ca-free solutions $CaCl_2$ was omitted and EGTA (ethylene glycol-bis(β-amino ethyl ether)-*N*, *N'*-tetra acetic acid) was added (10^{-4} M). The Na-free solutions were Tris buffered, NaCl was entirely replaced by Tris base and the substrates were added as the acid rather than the sodium salt. pH was adjusted to 7·4 with HCl and Tris base and gassed with 100% O_2.

Measurements of cell membrane potential and input resistance were carried out using one intracellular micro-electrode for recording and current injection as previously described (Nishiyama & Petersen, 1974). All glass micro-electrodes were made from Theta glass, filled with 3 M-KCl + 10 mM-K citrate and had a tip resistance of 10–30 MΩ. The electrodes were not bevelled. Impalements of surface acinar cells were achieved using a stepping-motor microadvance (AB Transvertex, Stockholm).

Electrical field stimulation was achieved via a pair of platinum electrodes (6·5 mm platinum wire) brought into light contact with the intact surface of the parotid segments. The distance between these electrodes did not prove to be critical but was generally 2–4 mm. A calibrated stimulator (Devices, Type 2533) triggered by a Devices Pulse Generator provided square-wave stimulation in which the parameters of pulse width, amplitude and frequency could be varied.

The agonists acetylcholine and adrenaline were applied locally by microionophoresis from extracellular micropipettes filled with either 2 M-acetylcholine chloride or 1 M-adrenaline bitartrate (BDH Chemicals Ltd) (See Roberts & Petersen, 1978). The duration of both electrical field stimulation and ionophoresis was controlled by a Devices Digitimer. When antagonists were used they were present in the superfusion fluid. Phentolamine mesylate was included at 10^{-5} M (10^{-7} in one experiment), propranolol hydrochloride at 5×10^{-6} M and atropine sulphate at 10^{-6} M. In one experiment concentrated atropine was added directly to the tissue bath to give a final concentration of 10^{-6} M. The neurotoxin, tetrodotoxin (Sigma) was added to the tissue bath to give a final concentration of 10^{-6} M.

RESULTS

Resting membrane potential and input resistance

The resting membrane potential of the mouse parotid acinar cells was $-64·9$ mV $\pm$ 0·6 S.E. ($n = 262$). This is in agreement with previously published studies (Pedersen & Petersen, 1973; Nishiyama, Katoh, Saitoh & Wakui, 1980). The input resistance of the cells at resting membrane potential was 4·63 $\pm$ 0·19 MΩ S.E. ($n = 135$).

Many of the cells studied exhibited spontaneous changes in input resistance, often associated with spontaneous miniature depolarizations (Fig. 1) of up to 15 mV amplitude. These miniature depolarizations have been described in cat submandibular gland (Lundberg, 1955) and in exocrine pancreas (Dean & Matthews, 1972) and have been compared to the miniature end-plate potentials found at the neuromuscular junction (Fatt & Katz, 1952).

Effects of field stimulation on membrane potential and input resistance

Responses to field stimulation were evoked in 212 cells from sixty-seven preparations. The field-stimulation response was characterized by a marked decrease in input resistance associated with a potential change. At higher values of resting

membrane potential this potential change was a depolarization while at less negative resting potentials the change was hyperpolarizing in nature.

Fig. 1 demonstrates an acinar cell response to supramaximal field stimulation. The threshold for stimulation was generally between 2 and 5 V, showing little gradation thereafter, with no evidence of recruitment beyond 10 V. 15 V was considered supramaximal in terms of stimulus amplitude.

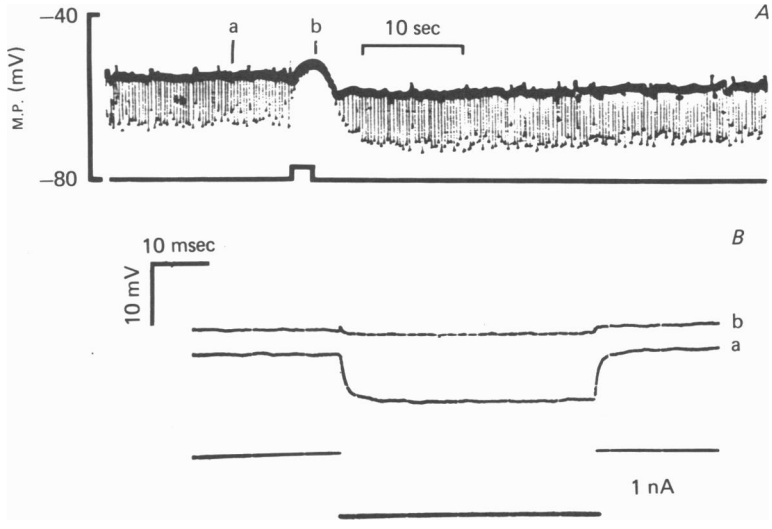


Fig. 1. *A*, recording of membrane potential and input resistance from an acinar cell. The vertical bars superimposed on the recording of membrane potential (M.P.) are due to repetitive injection of hyperpolarizing current pulses (1 nA, 50 msec). The amplitude of these electrotonic potentials corresponds to the input resistance of the cell. Note the spontaneous miniature depolarizations and synchronous reduction in input resistance. At the point indicated on the event marker a supramaximal field stimulus was applied (2 msec, 15 V, 40 Hz, 2 sec). There is a marked reduction in input resistance, associated with a depolarization. The input resistance returns to normal during a period in which the membrane potential becomes more negative than the resting potential, i.e. delayed hyperpolarization. *B*, oscilloscope picture showing the field-stimulation-induced change in input resistance. Time course and amplitude of current pulse induced membrane hyperpolarizations at times a and b in record *A*. The lowest trace is the monitor for injected current.

The magnitude of the response to an above-threshold stimulus was dependent on the frequency and duration of stimulation. Responses could be evoked by single shock stimuli of 1–2 msec duration (Fig. 2). These single shock responses had latencies of between 180 and 300 msec. Fig. 3 demonstrates the effect of increasing frequency on the field-stimulation response. At 1 Hz the response is a train of individual spikes, the time course of which is identical to that of the spontaneous depolarizations. At a frequency of 5 Hz the individual spikes have fused to give a sustained depolarization associated with a reduction in input resistance. At 10 Hz the response becomes biphasic; the initial depolarization and reduction in input resistance are more marked; however, upon cessation of stimulation there is a delayed hyperpolarization during which the input resistance returns to pre-stimulus levels. At 20 and 40 Hz

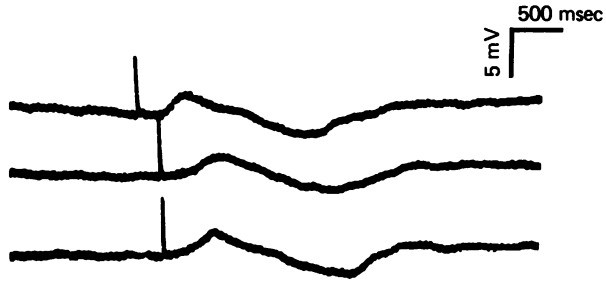


Fig. 2. Oscilloscope picture showing responses to single shock stimuli (2 msec, 15 V). The three responses are from the same cell. The resting membrane potential is −65 mV in each case, although they have been displaced on the screen for presentation. The vertical bars preceding each response are the stimulus artifacts.

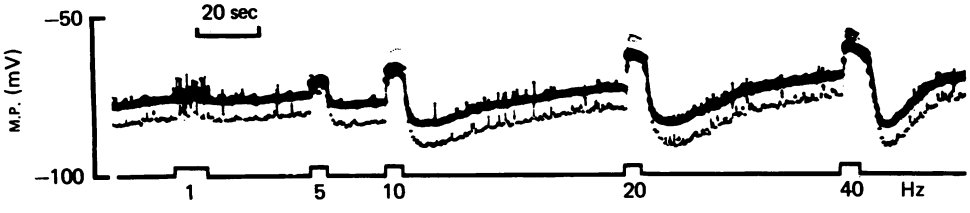


Fig. 3. Continuous recording of membrane potential (M.P.) and input resistance (current pulses 1·5 nA hyperpolarizing, 100 msec). Note the spontaneous miniature depolarizations and fluctuations in input resistance. The response to field stimulation (1 msec, 15 V) at 1, 5, 10, 20 and 40 Hz is shown. At 1 Hz the response is a train of spikes identical to the miniature depolarizations. At 5 Hz the response is a sustained depolarization with a reduction in input resistance. At 10–40 Hz the initial depolarization is followed by a delayed hyperpolarization, increasing in magnitude with frequency, during which the input resistance returns to normal.

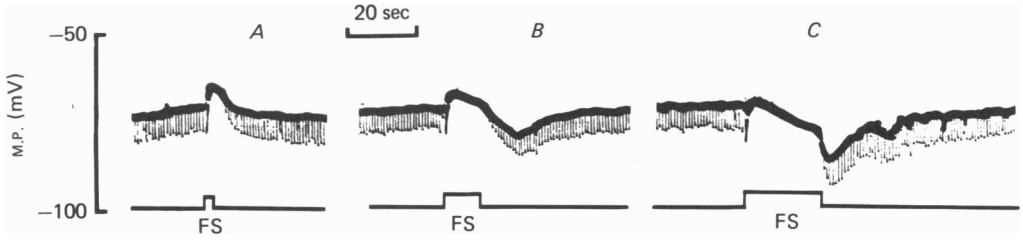


Fig. 4. Sections of a continuous record of membrane potential and input resistance (current pulses 2 nA hyperpolarizing, 100 msec). The field stimulation (FS) (2 msec, 15 V, 40 Hz) responses at 4, 10 and 20 sec duration are shown in *A*, *B* and *C* respectively. In all three there is a marked reduction in input resistance which is sustained throughout the period of stimulation. In *A* this reduction in input resistance is associated with a monophasic depolarization. In *B* there is an initial depolarization which declines throughout the period of stimulation, and is followed on cessation of the stimulus by a delayed hyperpolarization. In *C* the decline of the initial depolarization is more marked and the subsequent hyperpolarization greater.

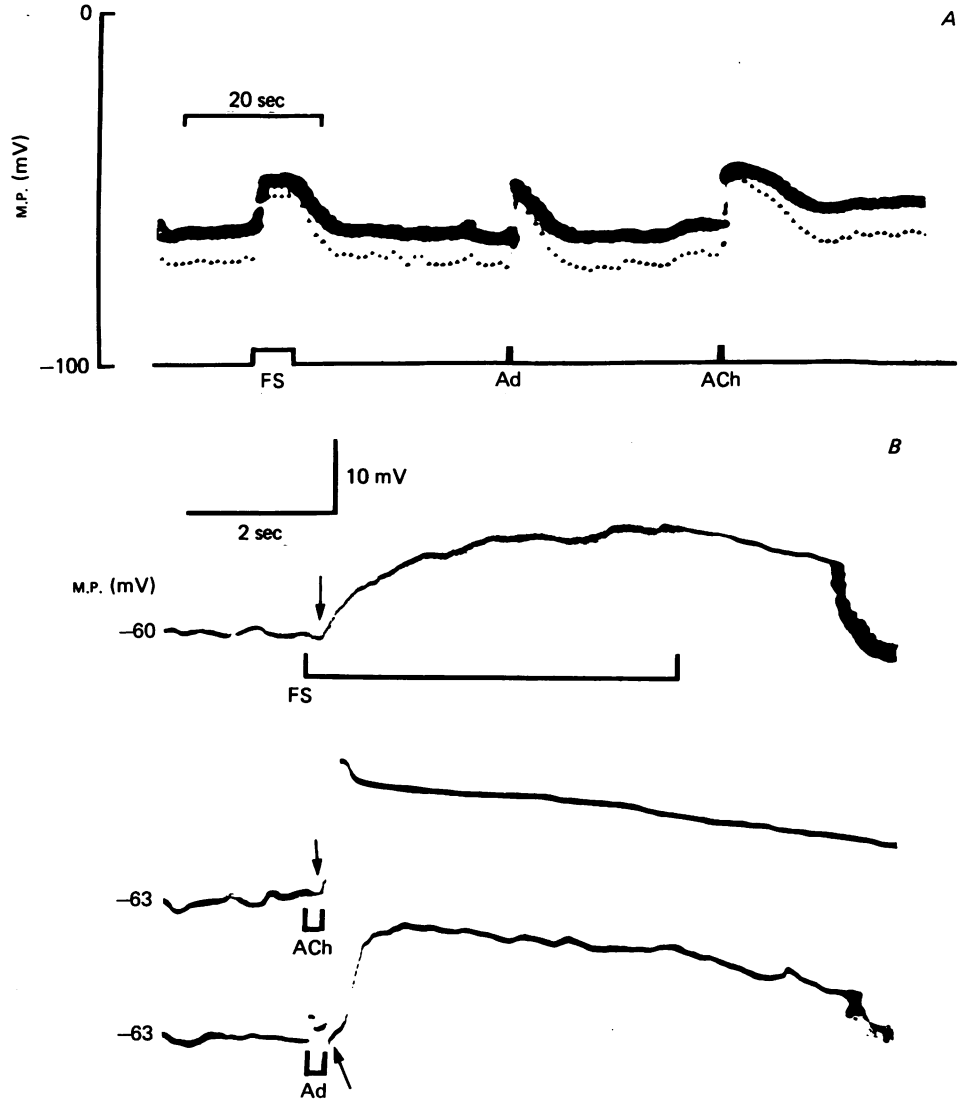


Fig. 5. *A*, recording of membrane potential (M.P.) and input resistance (hyperpolarizing current pulses 2 nA for 100 msec) from a cell responding to field stimulation (FS), adrenaline ionophoresis (Ad) and acetylcholine ionophoresis (ACh). The amplitude of the electrotonic potentials corresponds to the input resistance of the cell. It can be seen that the response to all three stimuli is essentially the same, that is a depolarization associated with a decrease in input resistance (FS, 2 msec pulses of 15 V amplitude at 40 Hz for 5 sec; ACh, 100 nA ejecting current for 250 msec, 34 nA retaining current; Ad, 500 nA ejecting current for 250 msec, no retaining current. *B*, latencies of the response in a single cell to the three stimuli. The latencies are: FS, 200 msec; ACh, 200 msec; Ad, 300 msec. Stimulus parameters were: FS, 2 msec, 15 V, 40 Hz, 5 sec; ACh, 100 nA ejecting current for 250 msec, 34 nA retaining current, Ad, 500 nA ejecting current for 250 msec, no retaining current.

the responses are again biphasic but while there is little increase in the magnitude of the initial depolarization the delayed hyperpolarization becomes more pronounced with increasing frequency.

In Fig. 4 the effects of increasing the duration of field stimulation are shown. A 4 sec duration produced an essentially monophasic response with a marked decrease in input resistance; on extending the duration to 10 sec, the initial response is again a depolarization with reduced input resistance. While the reduction in input resistance is maintained throughout the period of stimulation, the depolarization gradually declines and upon cessation of stimulation there is a marked hyperpolarization during which the input resistance returns to normal. During field stimulation of 20 sec duration the decline in the initial depolarization is more marked and the delayed hyperpolarization on cessation of stimulation more pronounced, and rapid.

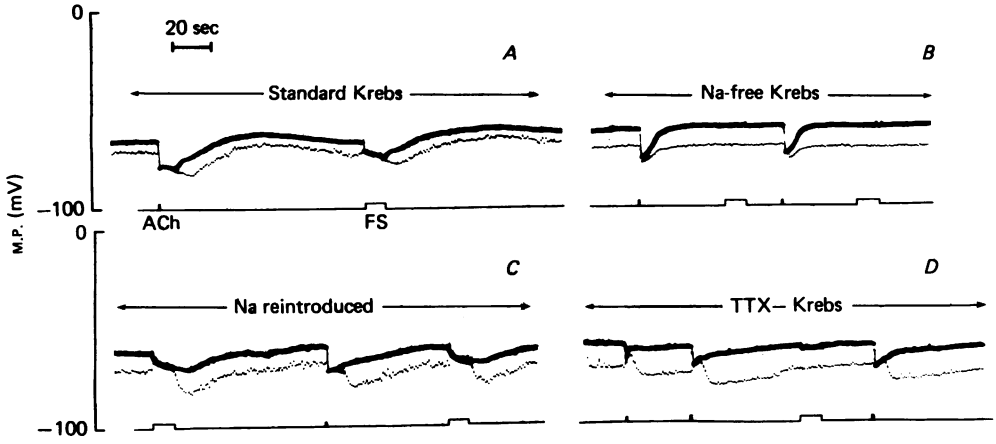


Fig. 6. Sections of a continuous record from a single cell responding to both supramaximal field stimulation (FS) (2 msec, 15 V, 40 Hz, 10 sec) and to ACh ionophoresis (100 nA ejecting current for 250 msec, 30 nA retaining current. *A*, changes in membrane potential (M.P.) and input resistance (hyperpolarizing current pulses 3 nA, 100 msec) to both stimuli in standard Krebs solution. When the solution is changed to a Na-free medium (*B*) the FS response is abolished while the ACh response persists. However, the delayed hyperpolarization is absent from the ACh response. This blockade of FS is reversible upon reintroduction of Na (*C*). When TTX (10^{-6} M) is applied to the tissue bath (*D*) again the FS response is selectively abolished. The ACh response is reduced in magnitude but could be restored by increasing the duration of the ionophoresis from 250 to 500 msec.

Comparison of membrane effects evoked by field stimulation and by ionophoresis of ACh and Ad

In forty-one experiments the field-stimulation responses were compared with those evoked by ionophoretic application of one or both of the autonomic agonists ACh or Ad. In thirty-nine cells responses were recorded to all three stimuli. In eighty-eight cells responses to both field stimulation and ACh were recorded. The responses to all three stimuli were qualitatively the same, consisting of a marked decrease in input resistance associated with an initial potential change, generally followed by a

delayed hyperpolarization during which the input resistance returned to pre-stimulation level. Such responses to ionophoresis of ACh and Ad have been previously reported (Roberts & Petersen, 1978; Roberts *et al.* 1978). As with field stimulation the direction of the initial potential change was dependent on membrane potential. However, in any one cell the responses to all three stimuli were identical in nature, i.e. all three depolarizing (Fig. 5) or hyperpolarizing (Fig. 6).

It was not possible to evoke ionophoretic responses of a similar short-lasting nature to those induced by single shock or low frequency stimulation. Indeed the ionophoretic responses were matched only by maximal or near maximal field stimulation.

Latencies of the responses

The latency of the field-stimulation response was characteristically short with a mean of 246 ± 12 msec S.E. (n = 30) with a range of only 180–400 msec. This latency corresponds to the shortest latencies recorded in the *in vivo* preparation in response to electrical stimulation of nerve trunks (Lundberg, 1955, 1958; Emmelin *et al.* 1980). In contrast, the latencies of the responses to ionophoresis, in this study and as previously reported, were highly variable, depending on both the proximity of the micropipette to the recording electrode and on the depth of the impalement. The shortest latencies for ionophoresis were 200 and 300 msec for ACh and Ad respectively. The longest latencies were over 1 sec. Fig. 5 shows a cell responding to all three stimuli and their respective latencies.

Susceptibility of the field stimulus response to blockade of nerve conduction and to blockade of neurotransmitter release

Blockade of nerve conduction was achieved by superfusion with either Na-free (eight experiments) or tetrodotoxin- (TTX 10^{-6} M-) containing media. In every case blockade of nerve conduction abolished the field-stimulation response while the response to ACh ionophoresis persisted (Fig. 6). The blockade by Na-free media was reversed upon reintroduction of Na (Fig. 6 *c*). While TTX did not abolish the response to ACh it was reduced in magnitude. This suggests that TTX either modifies the acinar cell response to ACh or that a component of the response to ionophoresis of ACh is susceptible to nerve blockade. Increasing the duration of ionophoresis restored the response, while increasing the parameters of field stimulation could not re-introduce this response.

In three experiments neurotransmitter release was blocked by superfusion with Ca-free media (Katz & Miledi, 1965). Again the field-stimulation response was abolished while the ACh response persisted; it was restored upon reintroduction of calcium (Fig. 7).

Equilibrium potential of the field stimulus response

The equilibrium or reversal potential of the acinar cell response to field stimulation was determined by two different methods. Trautwein & Dudel (1958) and Ginsborg, House & Silinsky (1974) describe how the 'transmitter equilibrium potential' can be determined by analysis of the changes in both membrane potential and input resistance induced by an agonist. This analysis was carried out for the field-stimula-

tion response and for ACh and Ad responses from records of the type shown in Figs. 1, 3, 6 and 9. The field-stimulation equilibrium potential was $-59{\cdot}2 \pm 7{\cdot}9$ mV (S.D.), that for ACh was $-63{\cdot}7 \pm 8{\cdot}5$ mV (S.D.) and for Ad it was $-61{\cdot}9 \pm 8{\cdot}4$ mV (S.D.).

The second method employed was to record responses at different levels of membrane potential, the membrane potential was varied by applying either hyperpolarizing or depolarizing direct current through the recording electrode. The

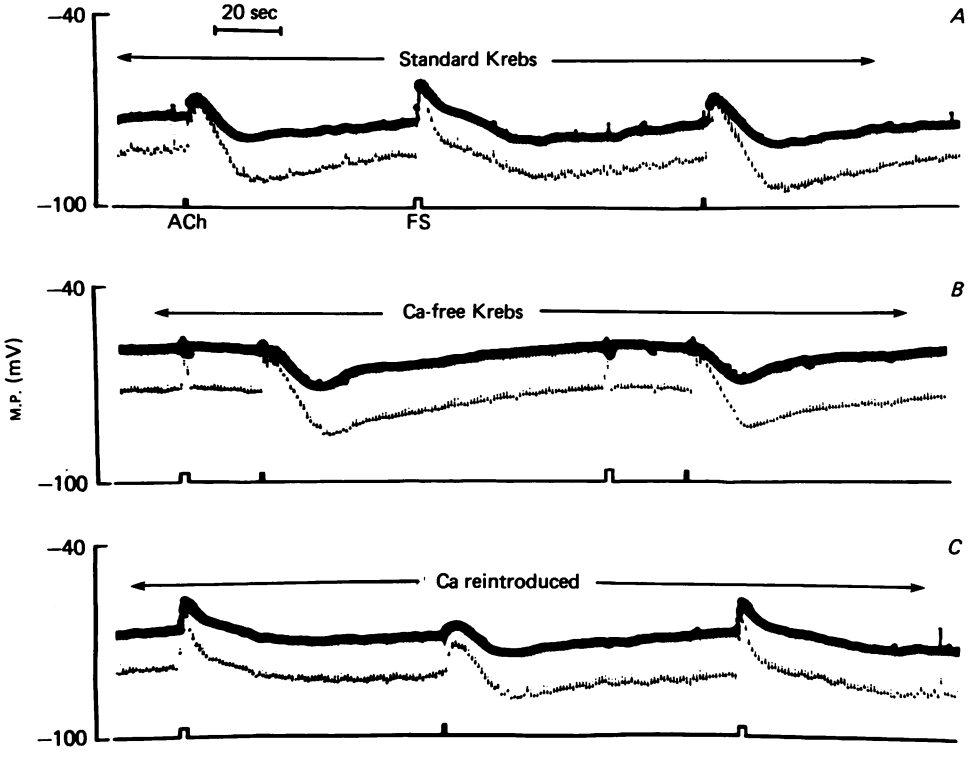
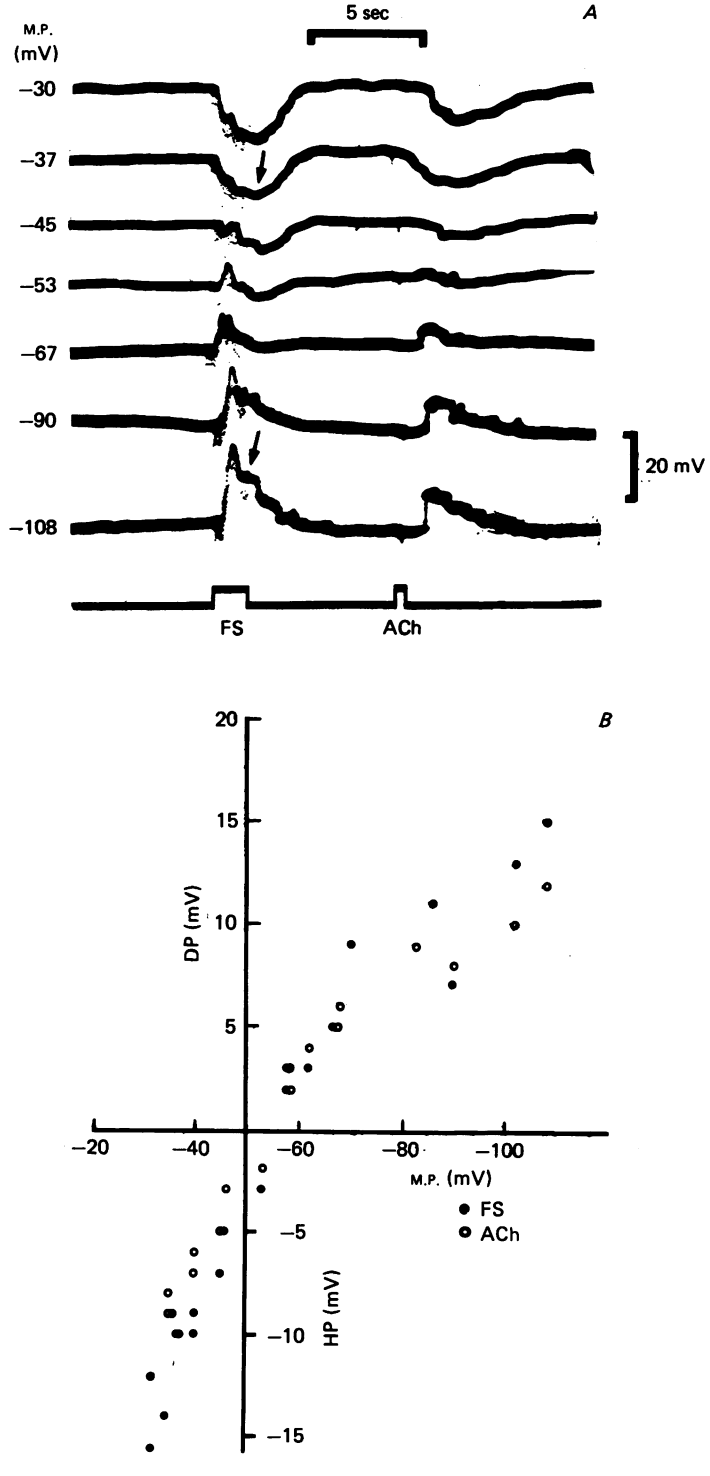


Fig. 7. Sections of a continuous recording of membrane potential (M.P.) and input resistance (hyperpolarizing current pulses 3 nA, 100 msec) from a single cell. In standard Krebs solution (*A*) the cell responds to both ACh ionophoresis (100 nA ejecting current for 500 msec, 20 nA retaining current, and to supramaximal field stimulation (FS) (2 msec, 12·5 V, 40 Hz, 2 sec). When a Ca-free medium is introduced (*B*) the membrane depolarizes slightly, the FS response is abolished, while the ACh response persists. This blockade is reversible and reintroduction of Ca (*C*) restores the FS response.

amplitude of the response is then plotted as a function of membrane potential. The intersection with the abscissa represents the equilibrium or reversal potential. Fig. 8 shows such an experiment in which reversal of both field-stimulation and ACh responses was achieved. Such reversal of the field-stimulation response was observed in four experiments, in three of which the ACh equilibrium potential was also determined. By this method the field-stimulation equilibrium potential was $-58{\cdot}8 \pm 5{\cdot}4$ mV (S.D.) and that of ACh $-58{\cdot}3 \pm 9$ mV (S.D.).

Thus the two methods are largely in agreement, with values for the reversal potential of about -60 mV.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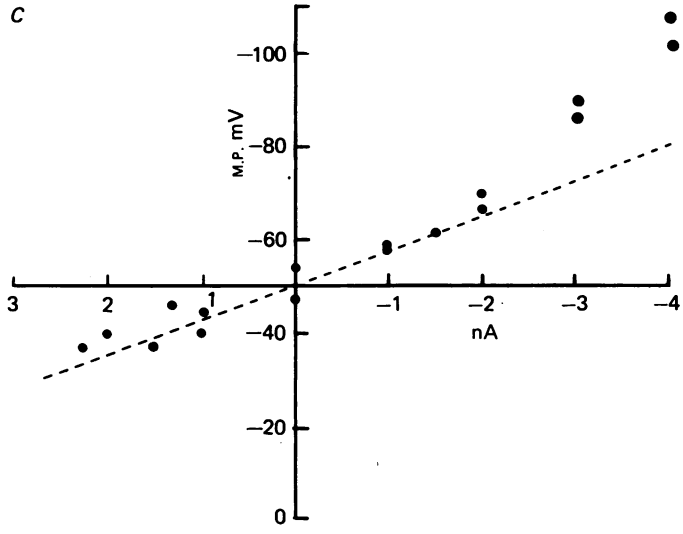

Fig. 8. *A*, family of traces showing potential change evoked by field stimulation (FS; 2 msec, 12·5 V, 40 Hz, 2 sec) and ACh (100 nA ejecting current for 500 msec, 20 nA retaining current) in a single cell at different levels of membrane potential. The resting potential of −53 mV was shifted by applying either hyperpolarizing or depolarizing direct current through the recording electrode. The points of maximal potential change of the responses are plotted in *B* as a function of membrane potential. (The points of maximal potential change after cessation of FS, arrowed, are plotted in *B* to avoid inclusion of stimulus artifact). The plots of both FS and ACh responses are approximately linear, although there is some deviation at the most negative values of membrane potential. This corresponds to the deviation from linearity of the resting current–voltage relationship (*C*). Both the plots in *B* overlap considerably and their intersections with the abscissa occur between −55 and −60 mV. This intersection is the equilibrium or reversal potential (M.P. = membrane potential, DP = depolarizing, HP = hyperpolarizing). *C*, current–voltage relationship for the resting membrane.

Pharmacological blockade of the field stimulus response

The effects of atropine and phentolamine on the field-stimulation response were investigated. In each of ten experiments changing the superfusion medium to one containing atropine (10^{-6} M) simultaneously abolished both field-stimulation and ACh responses. Fig. 9 is a recording from a cell responding to field stimulation, ACh and Ad. At the point indicated concentrated atropine was added directly to the tissue bath to give a final concentration of 10^{-6} M. The effects of the blocker are clearly seen. Both the field-stimulation and ACh responses were abolished as were the spontaneous fluctuations in input resistance, while the Ad response persisted. Atropine also caused a depolarization of the cell. This may have been a consequence of the method of application since it was not a feature of the response when atropine was applied in the more orthodox manner, by inclusion in the superfusion medium. In four experiments phentolamine (10^{-5} M; 10^{-7} M in one experiment) abolished the response to Ad ionophoresis but left the FS response unchanged (Fig. 10).

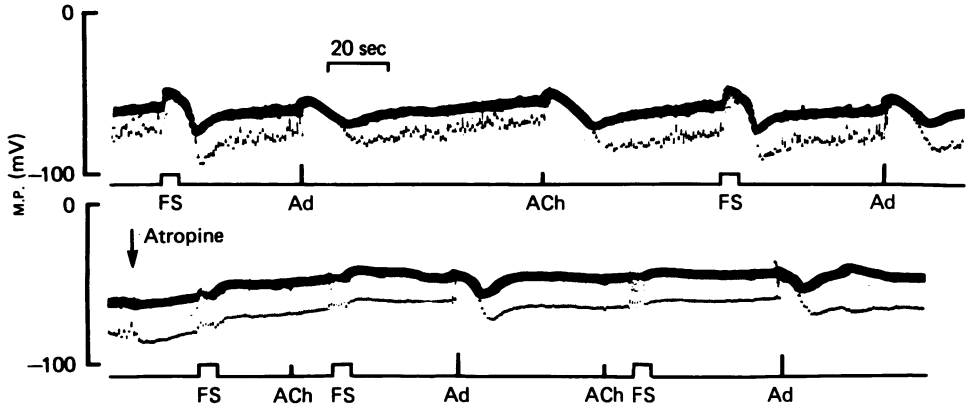

Fig. 9. Continuous recording of membrane potential and input resistance (current pulses 2 nA hyperpolarizing, 100 msec) from a single cell. The upper trace shows the cell responses to supramaximal field stimulation (FS) (2 msec, 15 V, 40 Hz, 5 sec) and to ionophoresis of Ad (500 nA ejecting current for 1 sec, 20 nA retaining current) and of ACh (100 nA ejecting current for 1 sec, 25 nA retaining current). Note also the spontaneous fluctuations in input resistance. The lower trace is a direct continuation of the upper. At the point indicated, atropine is added to the tissue bath to give a final concentration of 10^{-6} M. It is seen that the FS and ACh responses are abolished, as are the spontaneous fluctuations in input resistance. The Ad response persists.

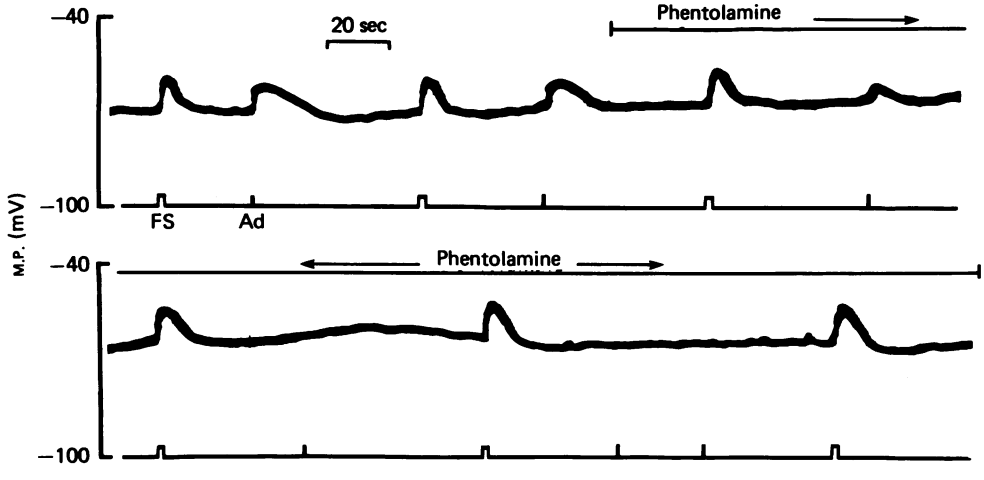

Fig. 10. Continuous recording of membrane potential from a single cell responding to both supramaximal field stimulation (FS; 2 msec, 15 V, 40 Hz, 2 sec) and to ionophoresis of Ad (500 nA ejecting current for 500 msec, no retaining current. At the point indicated the superfusion medium is changed to one containing the α-adrenergic blocker, phentolamine (10^{-5} M). The lower trace is a direct continuation of the upper. It is seen that while the response to Ad is abolished the FS response is unchanged.

DISCUSSION

This study has demonstrated that electrical field stimulation of isolated segments of mouse parotid gland *in vitro* can evoke responses which are characterized by a marked reduction in input resistance associated with a change in membrane potential. The susceptibility of the field stimulus response to blockade of either nerve conduction (by superfusion with TTX-containing or Na-free media) or neurotransmitter release (by superfusion with Ca-free media) confirms that the field-stimulation response is mediated by neural excitation and consequent release of endogenous neurotransmitter from nerve endings within the gland. The sensitivity of the preparation is such that single shock or low frequency stimulation will evoke responses, comparable in nature and latency to those which have formerly only been evoked *in vivo*, following electrical stimulation of dissected nerve trunks.

The field-stimulation responses were identical in nature to those evoked by iontophoresis of either ACh or Ad. It was also demonstrated that all three responses undergo reversal within the same narrow range of membrane potential, i.e. share a common equilibrium potential of about -60 mV. Identical equilibrium potentials for the action of ACh and Ad have previously been reported (Gallacher & Petersen, 1980). This is consistent with the concept that the field-stimulation response is mediated by one or both of the autonomic agonists.

Pharmacological blockade revealed that the field-stimulation response was totally abolished, together with the response to ACh iontophoresis, by atropine blockade of muscarinic receptors. Blockade of α-adrenergic receptors by phentolamine left the field-stimulation response unchanged while abolishing the response to Ad iontophoresis. From this we must conclude that in the mouse parotid the field-stimulation response is mediated by release of ACh from parasympathetic nerve endings. There was no discernible adrenergic component.

It has been reported (Takeda, 1978), on the basis of electronmicroscopic observations, that 70% of the nerve terminals in the mouse parotid are adrenergic in nature. While in the light of such evidence it is surprising that field stimulation failed to elicit any adrenergic component, the anomaly also applies to electrical stimulation of dissected nerve trunks *in vivo* (Babkin, 1950; Richins & Kuntz, 1953; Fritz & Botelho, 1969). The presence of parasympathetic secretomotor fibres can be readily demonstrated in most species in response to nerve trunk stimulation, however the presence of sympathetic secretomotor nerves has been consistently demonstrated in only very few species, classically the cat submandibular (Langley, 1878; Lundberg, 1955; Kagayama & Nishiyama, 1974). Since the morphological evidence suggests that both sympathetic and parasympathetic nerve fibres run in the same bundles within the salivary parenchyma (Eneroth, Hokfelt & Norberg, 1969; Hand, 1972; Bogart & De Lemos, 1973; Alm, 1973), combined with the knowledge that field stimulation can excite sympathetic nerves in other tissues (Blakeley & Cunnane, 1979) it is unlikely that the failure to elicit an adrenergic component reflects an inability of field stimulation to excite sympathetic nerves within the gland. Even at levels of stimulation very much greater than supramaximal there was no evidence of recruitment of another component. It is also important to note that the spontaneous fluctuations in input resistance and the spontaneous miniature depolarizations were

abolished by atropine (Fig. 9). This suggests that all spontaneous activity which is independent of field stimulation is cholinergically mediated.

A hypothesis has been advanced explaining the ionic mechanisms underlying the acinar cell response to ionophoresis of ACh (Petersen, 1970; Roberts *et al.* 1978). The concept is that the initial phase of the response is due to an increase in the passive permeability of the acinar membrane, principally to K and Na. This increased membrane conductance is reflected in the reduction in input resistance. During this period of increased permeability there is a net efflux of K and net influx of Na. After cessation of stimulation the membrane conductance returns to normal. However, the intracellular concentration of Na has been elevated. It is this elevation of intracellular Na which is considered to activate an electrogenic Na pump, giving rise to the delayed hyperpolarization. Such Na-activated electrogenic pumps have been demonstrated in skeletal muscle (Adrian & Slayman, 1966) and in taenia coli smooth muscle cells (Casteels, Droogmans & Hendrickx, 1973). The evidence of this study supports this concept in that the field-stimulation response only became biphasic following high frequency or prolonged stimulation, and that the delayed hyperpolarization increased with intensity or duration of stimulation, the conditions under which Na loading would be greatest. In Fig. 6 it is also seen that in the Na-free medium the delayed hyperpolarization is not evident.

In conclusion, we have demonstrated that the simple technique of electrical field stimulation can evoke release of endogenous neurotransmitter in the *in vitro* salivary gland preparation. Formerly this could only be achieved by electrical stimulation of dissected nerve trunks in the more difficult *in vivo* preparation. The isolated tissue offers not only a much simplified preparation but greatly extends the range of pharmacological and ion substitution experimentation which can be undertaken. Also, it can be readily applied to most species and should do much to broaden our understanding of the functional innervation of the lesser studied species, including man.

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