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THE ACTIVITY OF IDENTIFIED SUPRAOPTIC NEURONES AND THEIR RESPONSE TO ACETYLCHOLINE APPLIED BY IONTOPHORESIS

BY J. J. DREIFUSS AND J. S. KELLY*

From the Département de physiologie, Ecole de Médecine, Université de Genève, Switzerland, and the Department for Research in Anaesthesia, McGill University, Montreal, Canada

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SUMMARY

1. In urethane/pentobarbitone anaesthetized male rats, the hypothalamus and pituitary stalk were exposed by a transpharyngeal approach. Cells recorded with glass micro-electrodes from the ventral hypothalamus near the bifurcation of the internal carotid artery were identified as supraoptic neurones only when their antidromic action potential evoked at constant latency by stimulation of the pituitary stalk was cancelled by collision with a spontaneously occurring action potential.

2. More than 80% of the identified cells were spontaneously active. The frequency of firing was often slow (under 1 Hz) and rather irregular. More than half the cells, however, had a more distinctive firing pattern which recurred every 1–3 min and consisted of a period of low or absent activity alternating with periods of quite marked discharge which occasionally exceeded 20 Hz. This periodicity was retained when the over-all activity was enhanced by an intracarotid injection of 0·2 ml. 5% (w/v) NaCl.

3. Of fifty-two cells tested, thirty-six were excited by acetylcholine applied by iontophoresis from a multibarrelled micropipette with average ejection currents of 35 ± 5 nA (S.D. of thirty-six observations). Ten of these cells were also excited by nicotine applied with similar currents.

INTRODUCTION

In keeping with the early experiments of Pickford (1939) on the dog, rat hypothalamic neurones located in the region of the supraoptic nucleus can be excited by intracarotid injections of cholinomimetic drugs (Dyball & Koizumi, 1969). The presence of cholinoceptive cells within the supraoptic

* Canadian MRC Scholar and UNESCO/IBRO Fellow.

nucleus has also been inferred from the high levels within the nucleus of choline acetyltransferase (Feldberg & Vogt, 1948) and (acetyl) cholinesterase (Abrahams, Koelle & Smart, 1957; Pepler & Pearse, 1957).

The excitation of supraoptic neurones and the associated release of vasopressin (Dyball, 1971) need not, however, be the primary response to an intracarotid injection of acetylcholine (ACh) or a cholinomimetic but could be secondary to a generalized increase in hypothalamic activity. 'Cholinergic' acetycholinesterase containing nerve fibres (Shute, 1970) and neurones excited and depressed by iontophoretic applications of ACh (Bloom, Oliver & Salmoiraghi, 1963; Oomura, Ooyama, Yamamoto, Ono & Kobayashi, 1969) have been demonstrated in all regions of the hypothalamus.

It therefore seemed necessary to test the response of the supraoptic neurones, whose axons can be shown electrophysiologically to lie within the pituitary stalk, to iontophoretic applications of ACh and nicotine.

Some of these results have been presented at a meeting of the Physiological Society (Dreifuss & Kelly, 1970).

METHODS

The results to be presented in this paper were obtained from the series of experiments described in a previous report (Dreifuss & Kelly, 1971). Action potentials were recorded extracellularly through one channel of a multibarrelled glass pipette (tip outer diameter $< 3\ \mu$) filled with 3 M-NaCl while the other channels were used for iontophoresis and contained Na L-glutamate (1·0 M, Calbiochem), acetylcholine chloride (1·0 M, British Drug Houses) and nicotine bitartrate (1·0 M, British Drug Houses).

RESULTS

Identification of supraoptic neurones

The response of the neurones in this study to stimulation of the pituitary stalk was described in a companion paper (Dreifuss & Kelly, 1971). Only cells whose antidromic action potential was seen to be cancelled by collision with a spontaneously occurring action potential were classified as identified supraoptic neurones. The term unidentified neurone was reserved for spontaneously active cells found adjacent to identified supraoptic neurones (in the same electrode track). Many of the identified supraoptic neurones were also distinguishable from their spontaneously active neighbours by the shape of their action potential, or the inhibition of their spontaneous discharge evoked by pituitary stalk stimulation. During the isolation of single units, only the action potentials of identified units developed into the 'giant' predominantly positive spikes usually associated with recording from the soma and basal dendrites with small tipped electrodes. Inhibition

of the spontaneous discharge by stimulation of the pituitary stalk at intensities close to threshold for antidromic invasion of identified neurones appeared on all but three occasions to be restricted to identified supraoptic neurones.

Spontaneous activity of identified cells

In twenty-two experiments in which anaesthesia was initiated with a mixture of urethane and pentobarbitone and maintained with pentobarbitone supplements, seventy-two cells out of eighty-eight shown to be invaded by an action potential at constant latency after stimulation of the pituitary stalk proved to be spontaneously active.

Urethane anaesthesia

In fifteen preliminary experiments in which the animals were anaesthetized for the duration of the experiment by a single dose of urethane, forty of one hundred cells shown to be invaded by an antidromic action potential were found to be 'silent' (Cross & Green, 1959). The silent cells are in all probability not restricted to the supraoptic nucleus where they can be detected by the antidromic action potential since the number of unidentified, spontaneously active cells increased from less than 20% of the total number of cells to greater than 30% when the dose of urethane was reduced to one half and supplemented with pentobarbitone.

Silent cells

The amplitude and shape of the antidromic action potential of the silent cells and their stability appeared to be no different from that of the identified spontaneously active cells. Attempts to provoke the silent cells into activity by intracarotid injections of hypertonic sodium chloride (5%, w/v) and by iontophoretic applications of glutamate and acetylcholine were unsuccessful. Short applications of small doses of glutamate by iontophoresis caused a marked reduction in the amplitude of the antidromic action potential even though it was unaccompanied by a spike discharge. A decline in the amplitude of the action potential also occurred in response to glutamate applications when the discharge of spontaneously active cells was accelerated by glutamate iontophoresis.

Periodicity

The firing frequency of the spontaneously active identified cells was often slow (under 1 Hz) and rather irregular. Slightly more than 50% of the cells (thirty-seven of seventy-two) showed, however, a more distinctive firing pattern of which examples appear in Figs. 1, 2 and 6. The pattern recurred every 1–3 min and consisted of periods of low or absent activity

alternating with periods of quite marked activity which occasionally exceeded 20 Hz. This pattern, although difficult to characterize more precisely, was easily distinguished on simple rate-meter records from the slower but more regular background discharge shown in Fig. 4*A*, *B* and *C* and 6*C* and *D* which was recorded from neurones also identified as supraoptic neurones by the same techniques and occupying similar anatomical positions.

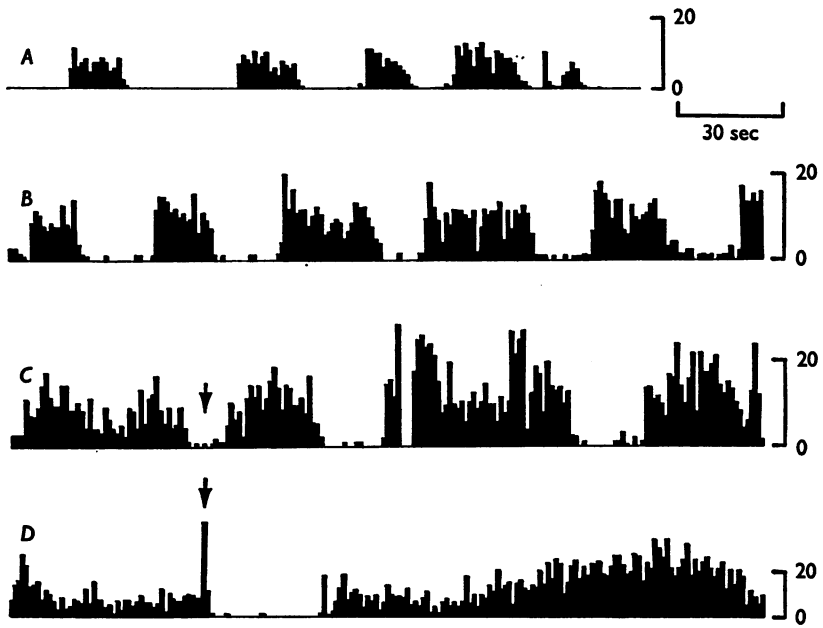

Fig. 1. Background discharge of single units recorded by a pulse counter as firing frequency on a paper chart. *A*, *B* and *C* are from identified supraoptic neurones and *D* from an unidentified neurone. In *C* and *D* the onset of an injection of 0·2 ml. 5% NaCl into the contralateral carotid artery is marked by an arrow.

5% NaCl

The response of a number of identified supraoptic neurones to injections of hypertonic sodium chloride (5% NaCl; 0·2 ml., w/v) into the contralateral carotid artery was similar to that shown in Fig. 2. After a short delay the injection of 5% NaCl was followed by a clear-cut acceleration of the background discharge which lasted only a few minutes. The original discharge of this neurone was slow and rather irregular; however, 5 min after the initial excitation the discharge became distinctly periodic in nature. One hour later the background discharge rate was still increased and the periodic pattern was still well established. In other cells the change

was less obvious as shown in Fig. 1 *C* where the acceleration of the discharge clearly accentuated the periodicity.

It was possible to demonstrate the same response to an injection of 5% NaCl on the neurone whose records appear in Fig. 2 on no less than three occasions some hours apart. More often, however, the response was either difficult to detect or absent and was rarely obtained more than once from the same neurone. The initial response to an injection of 5% NaCl similar

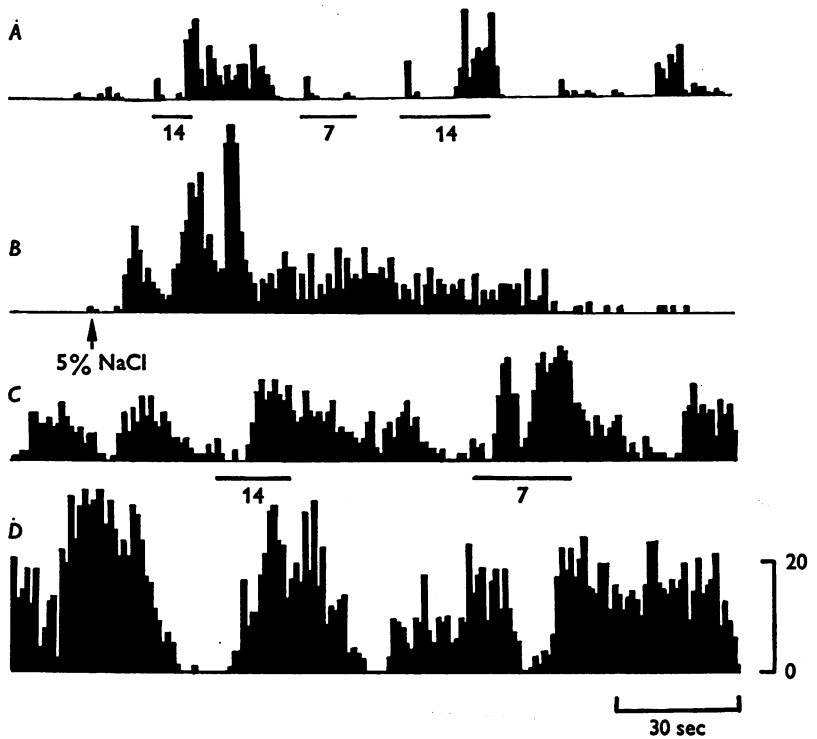

Fig. 2. The response of an identified cell to intracarotid 5% NaCl and iontophoretic acetylcholine. *A*, resting discharge; *B*, *C* and *D* show in turn the immediate response, and records taken 5 and 50 min after an injection of 5% NaCl into the contralateral carotid. In this and subsequent Figures the durations of the ejecting currents are represented by horizontal bars and the current intensity expressed in nA negative or positive with respect to inside of the electrode barrel.

to that shown in Fig. 2*B* was obtained not uncommonly from supraoptic neurones whose discharge pattern did not show any evidence of periodicity. The response could, however, also be obtained from the neighbouring unidentified cells as shown in Fig. 1 *D*. This cell showed none of the features of identified cells discussed in a companion paper (Dreifuss & Kelly, 1971).

No acceleration of the background discharge was seen when either physiological saline, atropine (200 mg/ml.), nicotine (2 mg/ml.), ACh (10 mg/ml.), or dihydro-β-erythroidine (2·5 mg/ml.) was injected into the contralateral carotid artery in 0·2–0·4 ml. aliquots.

The response to iontophoretic applications of acetylcholine

The photographic records taken from moving film in Fig. 3 are from an identified supraoptic neurone, shown in Fig. 2 to respond to an injection of 5% NaCl into the contralateral carotid. Records *A* and *B* show the excitant action of acetylcholine expelled from the second barrel of a double-

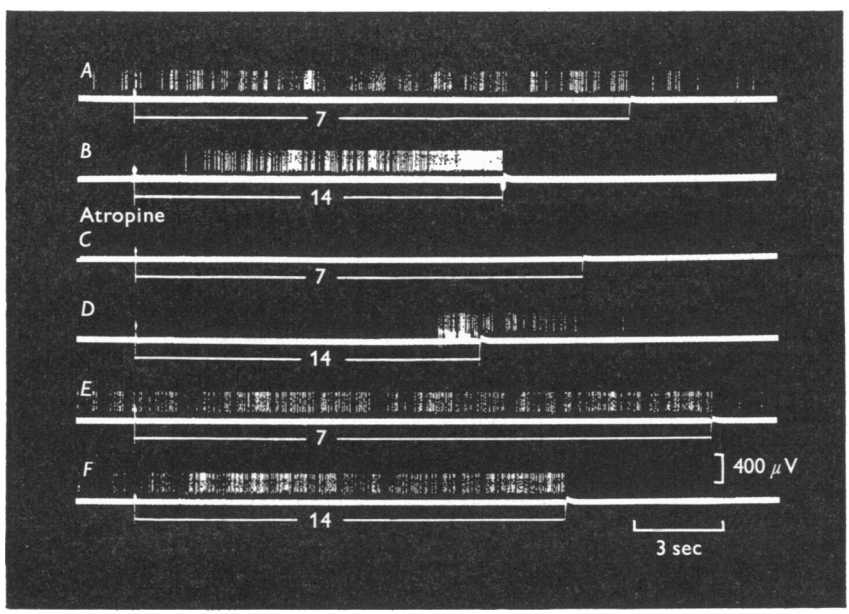

Fig. 3. Effect of iontophoretic ACh on the excitability of the identified neurone shown in Fig. 2 to be excited by 5% NaCl. *A* and *B* are control records showing discharges evoked by acetylcholine released by currents of 7 and 14×10^{-9} A. Records *C–D* and *E–F*, respectively, show the discharge evoked by similar amounts of ACh at approximately 2 and 60 min after an injection of 40 mg of atropine into the contralateral carotid artery.

barrel electrode by positive currents of 7 and 14 nA respectively. Typically the effect had a delayed onset and was fairly reproducible as shown by the polygraphic records from a rate-meter shown in Fig. 2*A*. Since in the majority of experiments satisfactory isolation of the cells appeared easier with double pipettes containing 3 M-NaCl and 1 M-ACh for iontophoresis as opposed to five-barrelled pipettes the exclusion of current effects could

only be carried out indirectly. In this particular cell (Fig. 3) current effects were unlikely to be important, since the currents used were extremely small and the response to ACh took some time to develop.

Further experiments to exclude current effects are shown in Fig. 4.

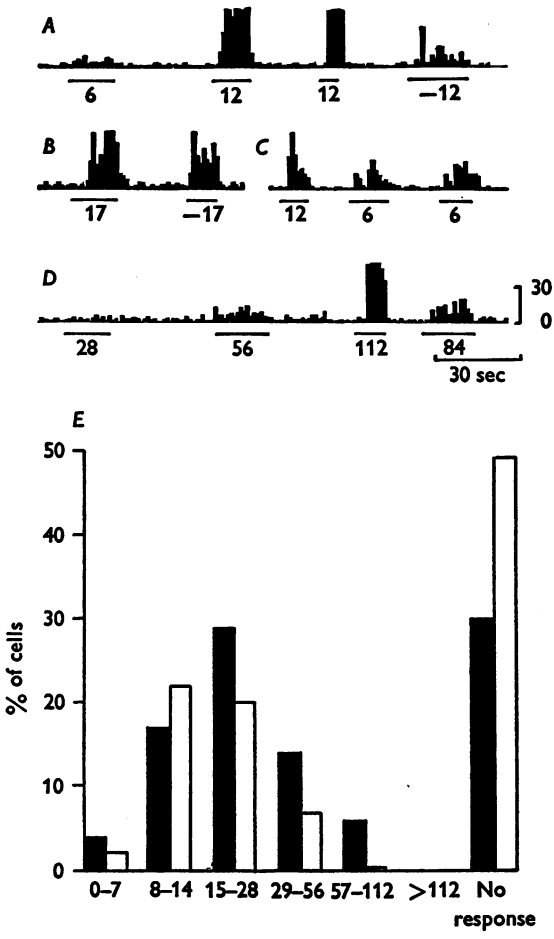

Fig. 4. The response to ACh of four neurones isolated within a few hundred microns of each other. *A*, *B* and *C* are rate-meter records from identified neurones excited by ACh ejected by 6, 12 and 17 nA of positive current. Control tests were made by applying − 12 and − 17 nA of negative current to the same electrode. *D* shows records from an unidentified cell. *E* is a histogram to show the distribution of fifty-two identified neurones (black bars) and forty-five unidentified neurones (open bars) into six classes by either the minimum amount of ACh required to elicit an easily detectable excitation or whether no response was obtained to the highest amount tested (80 ± 10 nA).

Record *A* shows the excitation caused by three applications of ACh resulting from positive currents of 6 and 12 nA applied through the electrode. For the final application the current was reversed; the excitation was immediate in onset rather than delayed as was the case with acetylcholine applied with positive current, the degree of excitation was much less and the excitation did not extend beyond the limit of the application. Similar findings, from another cell, are shown in Fig. 4*B*.

Of fifty-two cells tested, thirty-six were excited by iontophoretic applications of ACh ejected with currents with an average value of 35 nA (S.D. 5). The fourth cell shown in Fig. 4 to be excited by ACh was an unidentified cell isolated during the same electrode tract within a few hundred microns of the other three cells. This particular cell required for excitation rather higher ejection currents than was usually the case. Of forty-five unidentified cells tested, twenty-three were excited by acetylcholine and the amount of current required was 22 nA (S.D. 3). On no occasion was ACh seen to cause a clear-cut lasting depression of the spontaneous discharge although on many occasions it is possible that excitation was preceded by a small depression rather than a simple delay between the start of the ACh application and the onset of excitation.

The responses of both identified and unidentified cells were compared as shown in Fig. 4*E*. The cells in each group have been allotted to six classes, five according to the minimum dose of ACh required to cause clear excitation and a sixth for those showing no clear response; the numbers in each class were expressed as a percentage of the total number in each group. It is apparent from the histogram that the majority of cells excited by ACh in both groups responded to the dose of ACh ejected by less than 28 nA of current from a double-barrel electrode whose over-all tip diameter was smaller than 1 μ. The proportion of cells which failed to respond to ACh was clearly larger when the non-homogeneous population of the unidentified cells was studied.

Nicotine

Ten of the cells excited by ACh with ejection currents between 14 and 56 nA from four-barrel electrodes were also excited by nicotine applied with similar amounts of positive current. As shown in Fig. 5*A* the excitation caused by nicotine is also delayed in onset and rather less current was required on the average to cause a comparable acceleration of the background discharge.

Further evidence to suggest that ACh and nicotine both cause excitation by interacting with the same receptor was obtained in two experiments by injecting 0·5 mg dihydro-β-erythroidine (Merck, Sharp & Dohme, Ltd.) into the contralateral common carotid artery. This dose effectively blocked

the response to both excitants for more than 3 min, 1 min after the injection was completed. No obvious change occurred in the mean frequency of the spontaneous discharge.

Atropine

The excitatory response evoked by ACh was abolished by atropine sulphate injected into the common carotid artery only in amounts which were at least 100 times greater than the intravenous dose required to block specifically the excitation of cortical neurones (Krnjević & Phillis, 1963*a*, *b*; Crawford & Curtis, 1966). As shown in Fig. 3, a massive dose of atropine sulphate (40 mg dissolved in 0·2 ml. saline; British Drug Houses) caused only a marginal decrease in ACh sensitivity; sufficient ACh to cause excitation could still be expelled with as little as 14 nA.

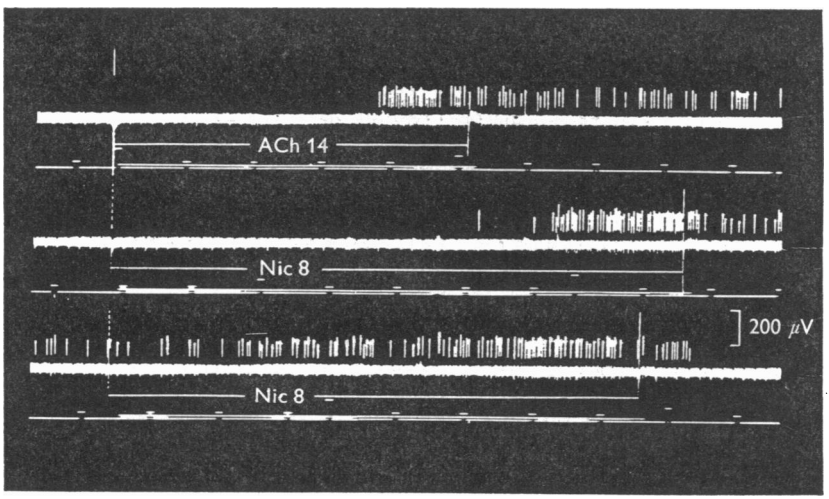

Fig. 5. The response of an identified neurone to ACh and nicotine. Photographic records from moving film to compare the excitation evoked by acetylcholine (ACh 14) and nicotine (Nic 8). Time calibration is 1 sec.

When compared with dihydro-β-erythroidine the antagonism of ACh and nicotine by atropine not only required much larger doses but also required a much longer time to develop. The rate-meter record in Fig. 6*E* began $3\frac{1}{2}$ min after the injection of 40 mg of atropine sulphate was complete and when the response to both ACh and nicotine was unaltered. A further 3 min elapsed before both cholinomimetics became ineffective at approximately the same time. Earlier the response of the same cell to both ACh and nicotine had been effectively blocked for about 3 min, 1 min after an injection of 0·5 mg of dihydro-β-erythroidine.

Invariably, atropine was responsible for a marked decrease in the frequency of the background discharge. Often the return of the background discharge to its control level after approximately 60 min was accompanied by the restoration of ACh sensitivity. Clearly a reduction in ACh sensitivity associated with a reduction in cell excitability cannot be regarded as a specific interaction of atropine with muscarinic receptors.

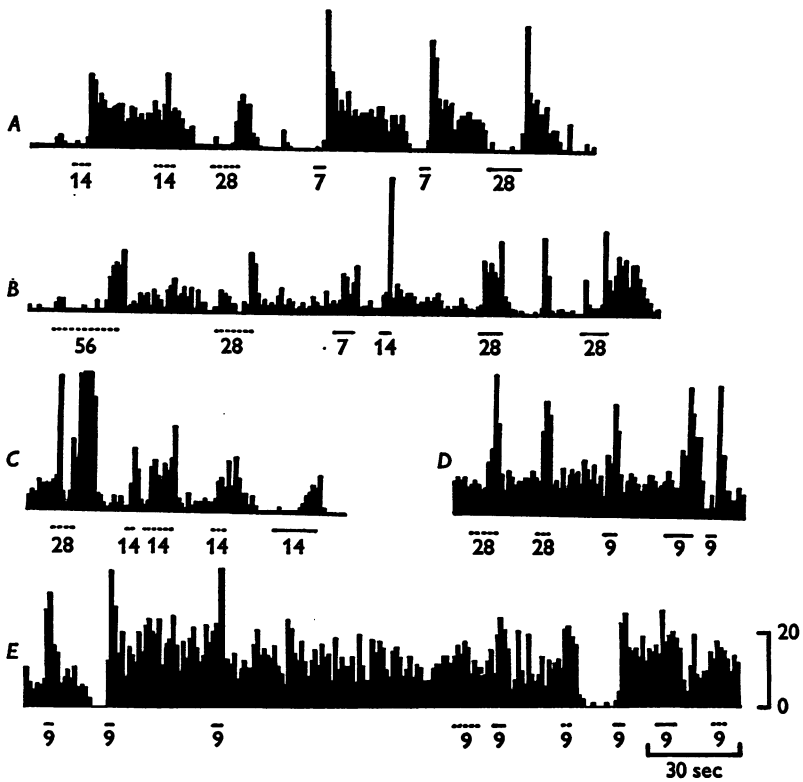

Fig. 6. Rate-meter records from five neurones which were excited by ACh and nicotine; interrupted bars and solid bars show the ejection of ACh and nicotine respectively. *E* is a record begun some 3 min after a 40 mg injection of atropine into the contralateral carotid.

The individual sensitivity of supraoptic neurones to ACh may, however, be related to their level of excitability. 'Silent' cells could not be provoked into activity by iontophoretic applications of ACh. The neurones, characterized by an episodic discharge pattern, were readily excited by ACh both during periods of activity and quiescence (Fig. 6). In Fig. 6*A*, a brief application of ACh or nicotine appeared to trigger an episode of activity.

On several occasions the response to iontophoretic applications of ACh seemed to be enhanced after the excitability of the cell had been raised by an intracarotid injection of 5% NaCl (Fig. 2).

DISCUSSION

Spontaneous activity

The spontaneous activity of identified supraoptic neurones closely resembled that of other cells belonging to similar regions of the hypothalamus. Both 'silent cells' (cf. Cross & Green, 1959) and cells whose discharge is characterized by alternate periods of silence and activity have been described in the rat (Dyball, 1971) and in the unanaesthetized rabbit (Findlay & Hayward, 1969) and monkey (Hayward & Vincent, 1970). A comparable proportion of identified paraventricular neurones exhibiting an episodic pattern was found by Wakerley & Lincoln (1971) in rats spared the extensive surgery necessary to expose the base of the hypothalamus.

It has been proposed that both the silence of some neurones and the episodic behaviour of others occurs as a result of a functionally active negative feed-back loop from intensely excited supraoptic neurones (Hayward & Vincent, 1970; Dyball, 1971). Negative feed-back could be mediated synaptically by the recurrent inhibitory pathway to supraoptic neurones described in the companion paper (Dreifuss & Kelly, 1971). Alternatively the episodic discharge need not be dependent on recurrent synaptic pathways but a consequence of cyclical changes of the cell membrane excitability.

Acetylcholine

Both antidromically identified and adjacent non-projecting neurones from the region of the supraoptic nucleus are excited by iontophoretic application of ACh. The proportion of ACh excited cells was very much greater than was predicted from the earlier studies of the hypothalamus by Bloom *et al.* (1963), who found less than 20% of cells excited by ACh.

The literature has long predicted the presence of ACh excitable neurones within the supraoptic nucleus and the possible transmitter role that ACh might play. Bisset (1968) has summarized most of the earlier work which showed a correlation between vasopressin release and the injection of acetylcholine, cholinomimetics and anticholinesterases either directly into the nucleus or into its arterial blood supply. In addition, separate studies showed the region of the nucleus to contain ACh and choline acetyltransferase (Feldberg & Vogt, 1948; Hebb & Silver, 1956) and acetylcholinesterase (Abrahams *et al.* 1957).

Several features of the discharge of supraoptic neurones by iontophoretic

ACh can perhaps be attributed to its interaction with post-synaptic membrane receptors located beneath the terminals of cholinergic fibres. Like Renshaw cells, where the role of ACh as a transmitter (Eccles, Fatt & Koketsu, 1954; Eccles, Eccles & Fatt, 1956; Curtis & Eccles, 1958; Curtis & Ryall, 1966*a*, *b*) has been most clearly established, the majority of supraoptic neurones are excited by extracellular ACh concentrations attained with fairly modest currents.

Classification of the ACh receptors as resembling those of Renshaw cells was also supported by the demonstration that ACh and nicotine are equipotent when tested on the same cells. In addition, an intracarotid injection of dihydro-β-erythroidine, which has been shown specifically to block nicotinic receptors of Renshaw cells (Eccles *et al.* 1954; Eccles *et al.* 1956; Curtis & Eccles, 1958; Curtis & Ryall, 1966*a*, *b*) reversibly blocked the action of both ACh and nicotine for several minutes.

Although it is difficult to deny the existence of nicotinic receptors for ACh on the cells of the supraoptic nucleus, some features of the ACh evoked discharge closely resemble the characteristics of ACh effects on cortical neurones usually attributed to muscarinic receptors, reported by Krnjević & Phillis (1963*a*, *b*), and Crawford & Curtis (1966). In Fig. 4 the distribution of supraoptic neurones by the ACh sensitivity closely resembles that of Betz cells of the cerebral cortex where 25% of the cells in anaesthetized cats were totally resistant to ACh and an appreciable number required the use of ACh currents in excess of 40 nA. The onset of the excitation of supraoptic neurones was characteristically slow and began 5–15 sec after the onset of the ACh release and continued for some seconds after its termination.

The cells with highest levels of spontaneous activity responded more readily to ACh than the more quiescent cells. The reduction in the response to ACh after intravenous atropine and its enhancement during the accelerated discharge evoked by injections of hypertonic 5% NaCl into the carotid artery can also be attributed to a dependence of the ACh excitation on the existing excitability of the cell. Since the reduction in ACh sensitivity after intracarotid atropine was accompanied by a marked reduction of the background discharge, there is no evidence of specific muscarinic receptors on supraoptic cells.

Recently, depolarization of the membranes of cortical cells by ACh has been shown by Krnjević *et al.* (1971) to be associated with a decrease in potassium permeability. As a result the ACh sensitivity of a particular neurone would be dependent on both the permeability of the membrane to sodium and the external sodium concentration. It is possible therefore that the differential sensitivity of the supraoptic neurones to ACh and the delayed onset of excitation may reflect some property of the membrane of

these cells which changes during the regulation of the extracellular ionic environment, rather than the nature or distribution of ACh receptors. Earlier we suggested that the different spontaneous firing patterns of supraoptic neurones may also be correlated with the functional status of their membrane excitability.

Since this study was completed, two reports from other laboratories have confirmed that neurosecretory cells are excited by iontophoretic ACh. The spontaneous discharge of almost all paraventricular neurones of the anaesthetized rabbits were found by Cross, Moss & Urban (1971) to be accelerated by ACh; the response was delayed just as described above. In the cat (Barker, Crayton & Nicoll, 1971), excitation of supraoptic neurones required much higher currents than described here and could be prevented by nicotinic antagonists. Still higher amounts led to a depression of the spontaneous discharge.

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