Journal of Physiology (1988), **399**, *pp.* 633–646
With 6 *text-figures*
Printed in Great Britain

MEMBRANE PROPERTIES OF HISTAMINERGIC TUBEROMAMMILLARY NEURONES OF THE RAT HYPOTHALAMUS *IN VITRO*

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(*Received 4 August 1987*)

SUMMARY

1. Intracellular recordings were obtained from neurones of the tuberomammillary nucleus in an *in vitro* explant of the rat hypothalamus.

2. Tuberomammillary neurones were spontaneously active ($2{\cdot}1 \pm 0{\cdot}6$ Hz) at the resting potential which was around -50 mV. Action potential amplitude was 75 ± 8 mV ($n = 9$); mean mid-amplitude duration was $1{\cdot}8 \pm 0{\cdot}4$ ms ($n = 9$).

3. The mean input resistance of tuberomammillary neurones was 176 ± 42 MΩ ($n = 30$), and the mean membrane time constant was $19{\cdot}8 \pm 5{\cdot}3$ ms ($n = 30$). These neurones exhibited inward rectification with hyperpolarization from the resting potential, and transient outward rectification at the offset of hyperpolarizing electrotonic pulses.

4. Action potentials were followed by an after-hyperpolarization of 300–600 ms duration and 12–18 mV amplitude. This after-hyperpolarization had a reversal potential around -80 mV, was abolished by intracellular loading with caesium, and was reduced but not abolished by bath application of either cadmium, cobalt or nickel.

5. Tetrodotoxin abolished spontaneous action potentials. Further addition of tetraethylammonium ions revealed a regenerative spike which was reversibly blocked by the addition of cobalt.

6. That tuberomammillary neurones exhibiting these properties were indeed histaminergic was confirmed in five cases by intracellular ionophoresis of Lucifer Yellow and subsequent double labelling by immunofluorescent localization of the histamine synthetic enzyme L-histidine decarboxylase.

INTRODUCTION

The tuberomammillary nucleus is populated by neurones which exhibit immunoreactivity to both histamine (Panula, Yang & Costa, 1984; Steinbusch & Mulder,

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1984) and its synthetic enzyme L-histidine decarboxylase (Watanabe, Taguchi, Shiosaka, Tanaka, Kubota, Terano, Tohyama & Wada, 1984; Pollard, Pachot & Schwartz, 1985). At present, the magnocellular neurones of the tuberomammillary nucleus appear to be the sole group of histaminergic neurones in the brain.

While the postsynaptic actions of histamine are well documented (Haas & Konnerth, 1983; Haas & Greene, 1986), very little is known of the physiological properties of histaminergic tuberomammillary neurones. Recent extracellular recordings *in vivo* have demonstrated that these neurones are spontaneously active in anaesthetized rats, and that the conduction velocity of cortically projecting tuberomammillary axons is 0·3–0·5 m s^{-1} (Reiner & McGeer, 1987). Considering the proximity of tuberomammillary neurones to the ventral surface of the hypothalamus (Reiner, Semba, Watanabe & Wada, 1987), we reasoned that they might survive well in an unperfused *in vitro* explant of the hypothalamus, and have carried out a set of experiments aimed at characterizing the active and passive membrane properties of these neurones. These results have been presented in preliminary form (Reiner & Haas, 1987).

METHODS

Tissue preparation. Young adult male rats (75–150 g) were anaesthetized with ether and decapitated. The brain was rapidly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid. The extreme ventral hypothalamus was removed *en bloc* from the interpeduncular fossa caudally to the optic chiasm rostrally, and the explant thus formed placed ventral side up in a recording chamber as previously described (Haas, Schaerer & Vosmansky, 1979). The explant was completely submerged and continuously superfused with artificial cerebrospinal fluid flowing at a rate of 0·5–1·0 ml min^{-1}; temperature of the bath was kept constant at $30 \pm 0{\cdot}5$ °C by a feed-back temperature control system. The tuberomammillary nucleus was easily identified by surface landmarks (Reiner *et al.* 1987), in particular its location just lateral to the mammillary recess.

The preparation was viable for at least 12 h. Viability of tuberomammillary neurones was apparently related to their superficial location on the ventral surface of the hypothalamus as evidenced by the following observations. Neurones with stable resting potentials were rarely encountered more than 200 μm below the surface of the explant. When examined histochemically (below), it was observed that only L-histidine decarboxylase-immunoreactive neurones within 200–300 μm of the surface possessed the typical morphological features of tuberomammillary neurones. Although tuberomammillary neurones located deeper within the explant (i.e. at the dorsolateral edge of the mammillary recess) were immunoreactive, they were invariably pyknotic. The present results are limited to neurones located in the ventral subnucleus of the tuberomammillary nucleus as defined by Staines, Daddona & Nagy (1987).

Recordings. Intracellular recordings were obtained from neurones in the region of the tuberomammillary nucleus using glass microelectrodes (0·87 mm i.d.) filled with 2 M-KCl (resistance 35–90 MΩ) connected to an Axoclamp 2 amplifier. Following penetration, a hyperpolarizing current of 0·5–1·0 nA was applied for several minutes. Subsequently, hyperpolarizing current sufficient to maintain the membrane just below threshold was applied (usually 10–50 pA), as tuberomammillary neurones were usually spontaneously active. Bridge balance was monitored continuously by applying small hyperpolarizing current pulses and observing the time course of the resultant membrane potential excursions; the marked disparity between the time constant of the membrane and that of the microelectrode was used as criterion for adjustment of the bridge balance which was performed as necessary throughout the course of the experiment. Zero potential was defined as the potential obtained upon rapidly withdrawing the pipette from the cell at the conclusion of the recording. Records were obtained from photographs of oscilloscope traces generated either on line or retrieved from magnetic tape; selected data were analysed using a digital oscilloscope. Measurements are given as mean ± standard deviation.

Solutions. The composition of the oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid was as follows (in mM): NaCl, 124; KCl, 2; KH_2PO_4, 1·25; $MgSO_4$, 1·3; $CaCl_2$, 2·5; glucose, 10; $NaHCO_3$, 26. When cobalt, cadmium or nickel ions were added to the perfusion fluid, the artificial cerebrospinal fluid was modified to eliminate phosphates and sulphates as follows (in mM): NaCl, 124; KCl, 3·25; $MgCl_2$, 1·3; $CaCl_2$, 2·5; glucose, 10; $NaHCO_3$, 26, and membrane properties in this modified solution were compared with those containing the divalent cations. Drugs were applied by adding them at known concentrations (indicated in text) directly to the artificial cerebrospinal fluid. The following drugs were used: tetraethylammonium chloride, tetrodotoxin, 4-aminopyridine, cobalt chloride, nickel chloride and cadmium chloride (all obtained from Fluka Chemicals except tetrodotoxin which was obtained from Sigma).

Intracellular staining and immunohistochemistry. Selected tuberomammillary neurones were marked by intracellular ionophoresis of Lucifer Yellow (1% in 1 M-LiCl) using hyperpolarizing current pulses of 0·5–1·0 s duration and 1·0–1·5 nA intensity delivered at 0·5 Hz for 5–30 min. When using Lucifer Yellow-filled micropipettes, tuberomammillary neurones were penetrated with the aid of depolarizing current pulses only, and no more than one cell was filled per side. At the end of the experiment, the explant was immersed in cold fixative (4% paraformaldehyde in 0·1 M-phosphate buffer, pH 7·4) for 2 h, followed by overnight incubation in cryoprotectant solution composed of 15% sucrose in 0·1 M-phosphate buffer. Serial coronal sections of 20 μm thickness were cut on a cryostat, mounted on gelatin-coated slides, and processed for L-histidine decarboxylase immunofluorescence using an antibody whose specificity has been previously documented (Watanabe *et al.* 1984). The sections were incubated in primary antiserum (rabbit anti-L-histidine decarboxylase) at a dilution of 1:1000 with 0·3% Triton-X 100 and 2% normal goat serum in 0·1 M-phosphate-buffered saline at 4 °C for 48–72 h. Sections were then washed 3 × 20 min in 0·1 M-phosphate-buffered saline, followed by a 1 h incubation in Texas Red conjugated goat anti-rabbit serum (Jackson Labs) at a dilution of 1:200 with 2% normal goat serum and 0·3% Triton-X 100 in 0·1 M-phosphate-buffered saline at room temperature. Following a final set of 3 × 20 min washes in phosphate-buffered saline, sections were air dried, cover-slips applied with paraffin oil and examined using epifluorescence on a Nikon microscope using the following filter combinations: excitation filter 450–490 nm and barrier filter 520–560 nm for Lucifer Yellow fluorescence; narrow bandpass excitation filter of 546 nm and long pass barrier filter above 590 nm for Texas Red immunofluorescence. Examination of singly labelled sections confirmed the selectivity of this filter combination. Identification of double-labelled cells was made by localizing both fluorochromes to either the soma, or when the Lucifer Yellow staining was very intense and thereby obscured the Texas Red immunofluorescence, to the distal dendrites of tuberomammillary neurones.

RESULTS

Intracellular recordings were obtained from fifty-four tuberomammillary neurones studied in twenty-six experiments. Only neurones in which stable recordings (0·5–4·0 h duration, resting potential more negative than −45 mV) were included. At the resting potential (-50 ± 5 mV, $n = 23$), tuberomammillary neurones were spontaneously active, firing at $2·1 \pm 0·6$ Hz ($n = 11$). Action potentials were 75 ± 8 mV in amplitude ($n = 9$, Fig. 1), with a threshold of -52 ± 5 mV ($n = 12$). Mean half-amplitude duration was $1·8 \pm 0·4$ ms ($n = 9$). Action potentials were followed by a pronounced after-hyperpolarization which merged into an interspike plateau. A brief depolarization terminated the interspike period and triggered the next action potential.

Inward rectification

The apparent input resistance of tuberomammillary neurones was determined by holding the membrane potential just below threshold for action potential generation and measuring the voltage responses to 100 ms duration pulses of hyperpolarizing current (Fig. 2*A*). When tested in this manner, tuberomammillary neurones

exhibited input resistances of 176 ± 42 MΩ ($n = 30$). The I–V plots generated by such experiments revealed a progressive fall in resistance with hyperpolarizing current pulses of increasing magnitude. The voltage dependence of this apparent inward rectification was investigated further by applying a standard series of hyperpolarizing current pulses to a neurone while varying the initial holding potential.

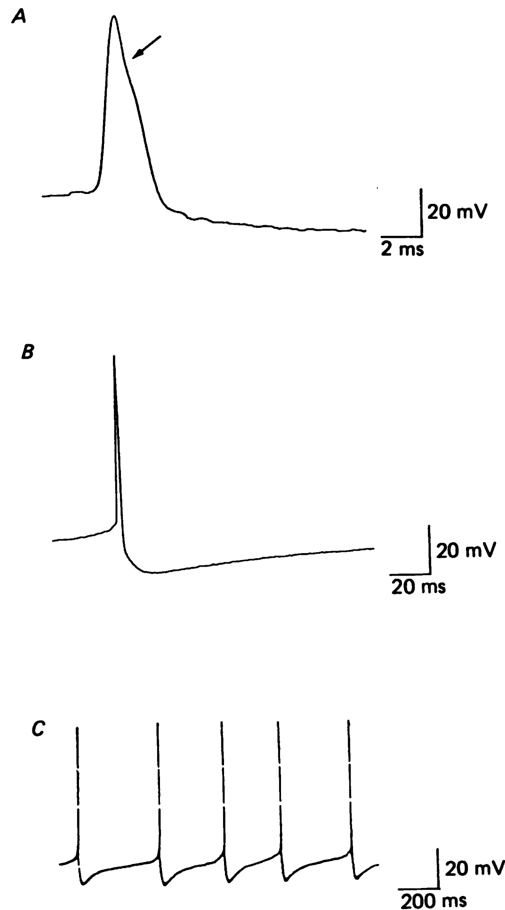

Fig. 1. Spontaneous action potentials. A, action potentials of tuberomammillary neurones are of long duration, frequently with a notch in the descending limb (arrow). B, at slower sweep speed, the action potential can be seen to be followed by a long-lasting after-hyperpolarization. C, the slow regular firing of tuberomammillary neurones is evident, as is the after-hyperpolarization which merges into an interspike plateau.

When tested in this fashion (Fig. 2B), the I–V curves increasingly became linear with more negative initial membrane potentials ($n = 6$). Thus, when the neurone was hyperpolarized to a level at which inward rectification was fully activated, hyperpolarizing current steps produced linearly dependent voltage changes. The time to complete activation of inward rectification varied considerably among tuberomammillary neurones, ranging from 200 to 1000 ms. Inward rectification was

apparently non-inactivating as demonstrated by applying 5–10 s duration hyperpolarizing current pulses (Fig. 2*C*).

Transient outward rectification

When hyperpolarizing current pulses were applied to tuberomammillary neurones near the resting potential, marked asymmetry in the time course of the

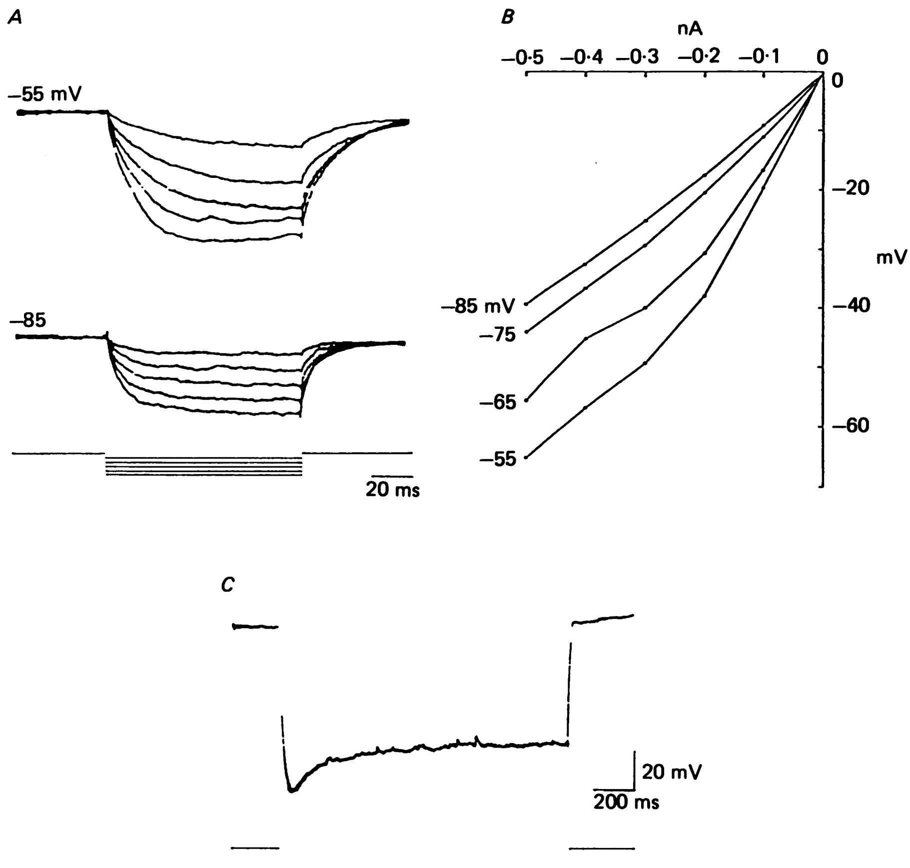

Fig. 2. Inward rectification. *A*, membrane responses to a series of hyperpolarizing current pulses (0·1–0·5 nA, 100 ms duration) at −55 and −85 mV. In the upper trace (−55 mV), the largest pulses evoke a slowly developing inward rectification which appears as a 'sag' in the electrotonic potential. *B*, *I–V* plots of change in membrane potential *vs.* injected current at four different holding potentials. As the cell was hyperpolarized, the *I–V* plot became increasingly linear, suggesting that anomalous rectification was completely activated at the most hyperpolarized levels. *C*, anomalous rectification reaches a steady state after about 500 ms and is not inactivated for the duration of the pulse.

onset and offset of the electrotonic potential was observed (Fig. 3*A*). Semilogarithmic plots of the charging and discharging profiles of tuberomammillary neurones to such manipulations revealed the charging profile to be defined by a single-exponential process with a time constant of 19·8 ± 5·3 ms ($n = 30$); similar

plots of the discharging time course showed that it did not conform to a single-exponential process (time constant = 409 ± 230 ms, $n = 13$). The additional component of the discharging profile of tuberomammillary neurones is presumed to be transient outward rectification.

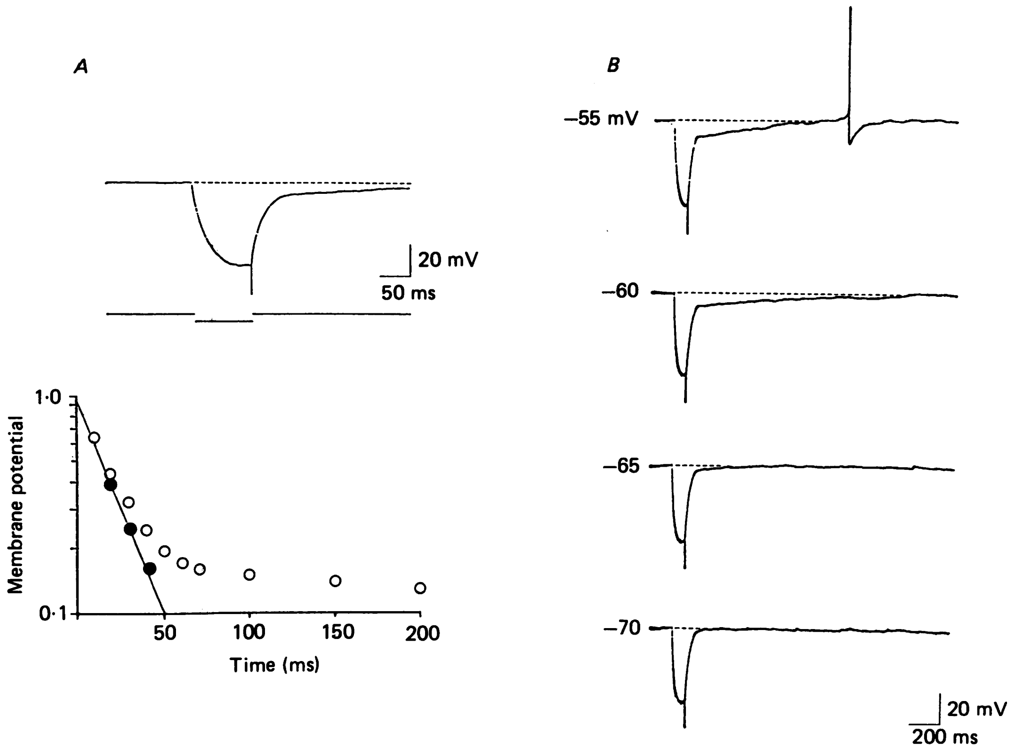

Fig. 3. Transient outward rectification. *A*, the membrane response to a hyperpolarizing current pulse (0·3 nA, 100 ms) is shown at the top. Tuberomammillary neurones typically had long membrane time constants (20 ms for this neurone), and invariably showed transient outward rectification during recharging. Semi-logarithmic plot of charging and discharging membrane potentials is shown below. ● represent the charging profile $[(V_{max} - V_t)/V_{max}]$; ○ represent the discharging profile $[V_{max}/V_t]$, where V_{max} is the final steady-state membrane potential and V_t is the potential at time t. Although charging the membrane appears to be a single-exponential process, the discharging profile clearly is not, reflecting the presence of transient outward rectification. *B*, the voltage dependence of transient outward rectification is shown by applying the same hyperpolarizing current pulse (0·3 nA, 100 ms) while varying the holding potential. Transient outward rectification was of greatest magnitude but of steepest slope at −55 mV (resting potential), and ultimately terminated with an action potential. At −60 mV, transient outward rectification is of longest duration, is minimal at −65 mV, and absent at −70 mV.

The voltage dependence of transient outward rectification was examined by applying identical hyperpolarizing current pulses to the neurone while varying the initial holding potential ($n = 14$). The neurone shown in Fig. 3*B* was typical: when the membrane was held just below threshold, the duration of the transient outward rectification was greatest, and was progressively reduced and finally abolished at successively more hyperpolarized holding potentials. Interestingly, at the resting

potential the magnitude of the transient outward rectification was greatest, but returned to baseline with the steepest slope, suggesting that other (undefined) conductances were also contributing to the return to the resting potential. Voltage clamp studies have shown that transient outward rectification exhibiting the properties described above is due to the presence of a potassium current termed the A-current (Connor & Stevens, 1971*a*; Neher, 1971) which in many but not all systems is selectively abolished by 4-aminopyridine (Thompson, 1977; Gustafsson, Galvan, Grafe & Wigström, 1982). Transient outward rectification was unaffected by bath application of 4-aminopyridine in concentrations up to 2 mM ($n = 5$), a concentration which appeared to block non-specifically other potassium conductances in tuberomammillary neurones (as evidenced by increased amplitude and duration of spontaneous action potentials). Thus, transient outward rectification in tuberomammillary neurones is highly voltage dependent, but is not sensitive to 4-aminopyridine. Transient outward rectification was also unaffected by bath application of cadmium (1 mM, $n = 3$), suggesting that it is not calcium dependent.

After-hyperpolarization

Spontaneous action potentials were followed by after-hyperpolarizations of $13{\cdot}9 \pm 2{\cdot}7$ mV ($n = 11$) amplitude and 300–600 ms duration. The reversal potential of the after-hyperpolarization was determined by applying a series of hyperpolarizing current pulses both immediately following and 800 ms following a conditioning depolarizing pulse. The reversal potential, as estimated by the intersection of the resultant $I–V$ plots, was $80{\cdot}1 \pm 4{\cdot}8$ mV ($n = 5$, Fig. 4*B*). When the membrane was hyperpolarized to within this region, clear reversal of the after-hyperpolarization following depolarization-induced action potentials was obtained (Fig. 4*A*).

As KCl-filled microelectrodes were utilized in the present set of experiments, it can be assumed that the chloride equilibrium potential was considerably shifted towards zero. In support of this assumption, virtually all tuberomammillary neurones encountered exhibited spontaneous depolarizing synaptic potentials; in 3/3 cells, these synaptic potentials were reversibly abolished by bath application of 10 μM-bicuculline (H. L. Haas & P. B. Reiner, unpublished observations). While this suggests that the after-hyperpolarization might be mediated by an increased potassium conductance, the reversal potential of the after-hyperpolarization is somewhat less negative than that predicted by the Nernst equation for a pure potassium conductance.

None the less, support for the involvement of a potassium conductance in generation of the after-hyperpolarization derives from experiments in which 2 M-CsCl-filled electrodes were used, as intracellular caesium loading is reported to block potassium channels (Eckert & Tillotson, 1981; Puil & Werman, 1981). Immediately following penetration of a tuberomammillary neurone with such an electrode, marked after-hyperpolarizations were observed; 15–30 min later, the after-hyperpolarization was completely abolished and action potential duration significantly lengthened ($n = 5$, Fig. 4*C*). In order to test if this presumed potassium conductance might be calcium dependent, after-hyperpolarizations were examined in the presence of cadmium (1 mM, $n = 3$), cobalt (2 mM, $n = 2$) or nickel (0·5 mM, $n = 2$). Although the presence of each of these divalent cations reduced the

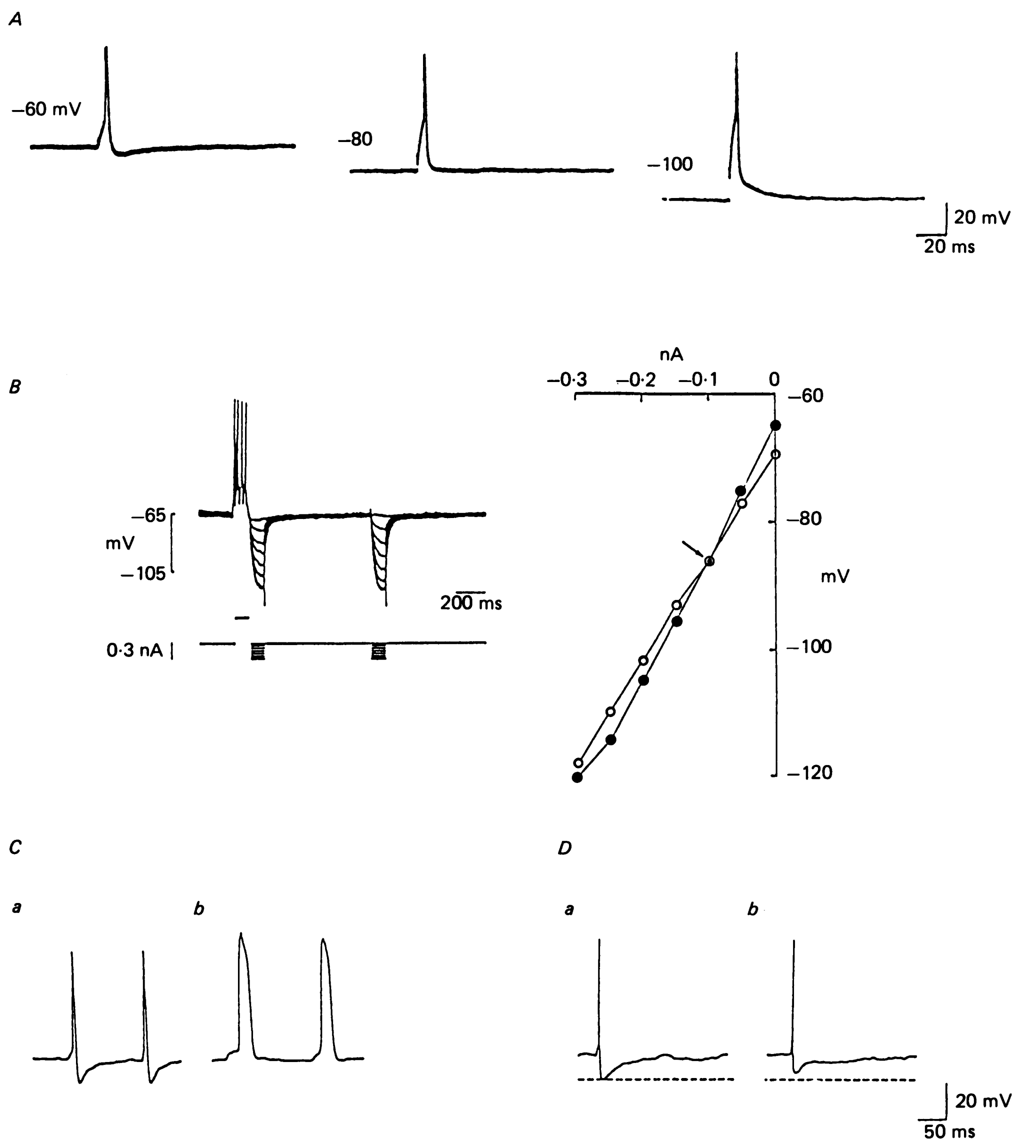

Fig. 4. After-hyperpolarization. *A*, the after-hyperpolarization reversed sign around −80 mV. *B*, a different neurone in which series of hyperpolarizing pulses (0 to −0·3 nA, 100 ms) was applied both immediately and 800 ms after a conditioning depolarizing pulse which triggered a burst of four action potentials and an ensuing after-hyperpolarization. The *I–V* plots for the hyperpolarizing pulses during (○) and after (●) are shown on the right. The reversal potential as estimated by intersection of these two *I–V* curves is −86·5 mV for this neurone. *Ca*, 30 s following penetration of a tuberomammillary neurone with an electrode filled with caesium chloride (2 M), spontaneous action potentials are followed by pronounced after-hyperpolarizations. *Cb*, 16 min later, the action potential has broadened considerably and the after-hyperpolarization is completely abolished. *Da*, control spontaneous action potential in a very slowly firing tuberomammillary neurone. *Db*, reduction of the after-hyperpolarization following addition of 0·5 mM-nickel.

amplitude of the after-hyperpolarization to varying degrees (Fig. 4*D*), in no instance was it abolished by such treatments.

Sodium and calcium spikes

Tetrodotoxin completely eliminated the fast action potentials produced by depolarizing current pulses (Fig. 5). When tetraethylammonium ions were added as well, a long-duration (24 ± 5 ms) regenerative spike appeared in response to small

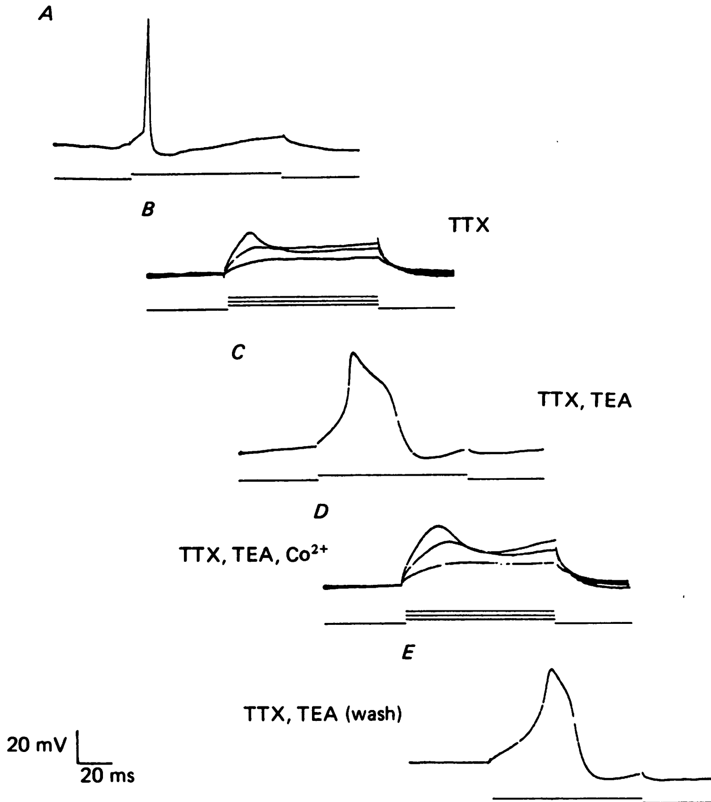

Fig. 5. Sodium and calcium spikes. *A*, control action potential evoked by a small depolarizing pulse (0·1 nA). *B*, after addition of tetrodotoxin (TTX, 3 μM), action potentials are absent even with depolarizing pulses of larger magnitude (0·1–0·3 nA). *C*, after further addition of tetraethylammonium ions (TEA, 10 mM), a long-duration TTX-insensitive spike is revealed in response to a small depolarizing pulse. *D*, addition of cobalt chloride (Co^{2+}, 2 mM) abolishes the TTX-insensitive spike. *E*, after 20 min wash in TTX and TEA, the TTX-insensitive spike returns.

depolarizing current pulses ($n = 6$), and this was reversibly blocked by cobalt (2 mM, 5 min, $n = 4$). Under normal conditions, tuberomammillary neurones often displayed a slight shoulder on the descending limb of the action potential (Fig. 1); this was also abolished by addition of 2 mM-cobalt (data not shown). Thus, both sodium and calcium conductances appear to contribute to the generation of action potentials in tuberomammillary neurones, and a tetraethylammonium-sensitive potassium conductance contributes to its repolarization.

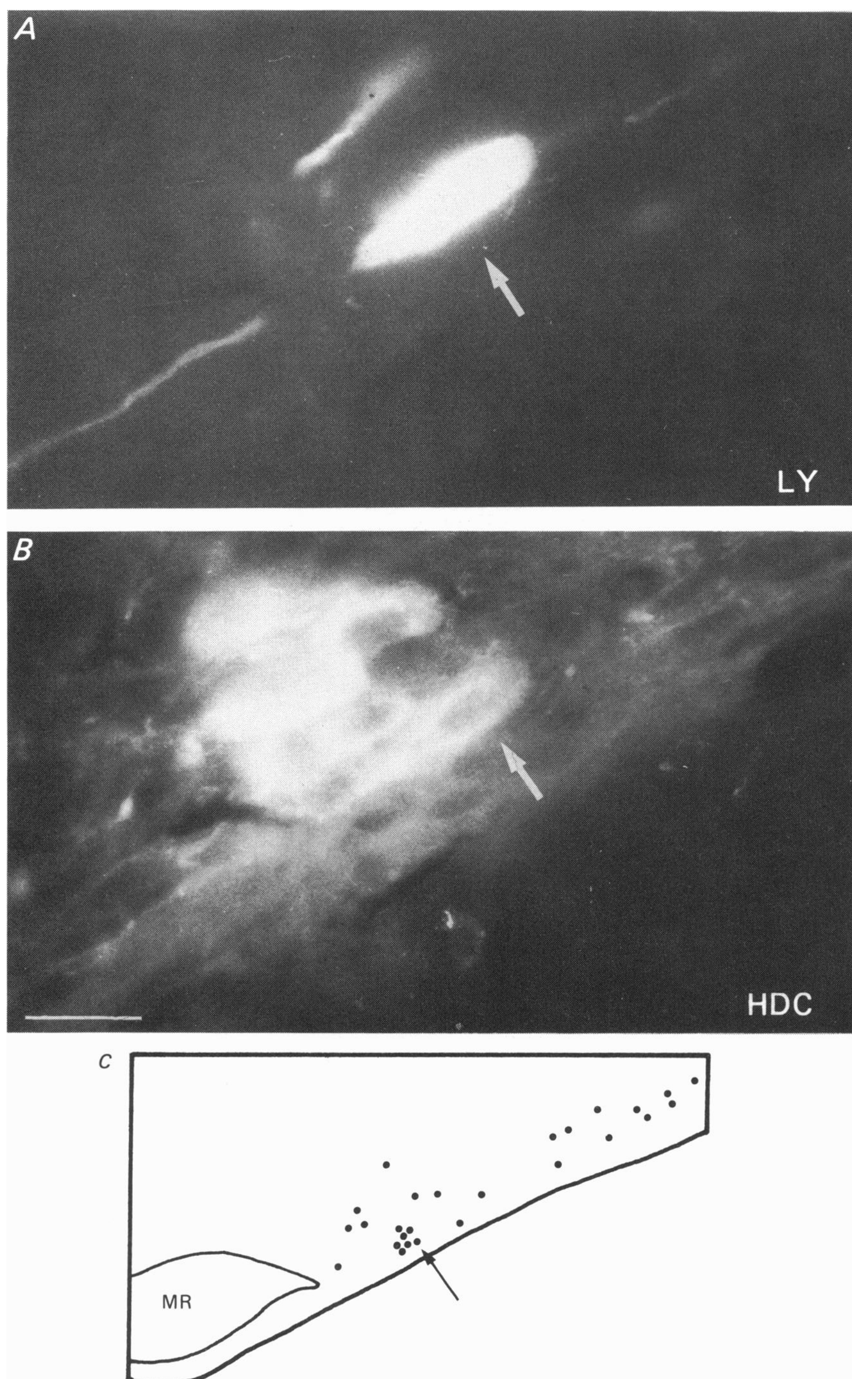

Fig. 6. Transmitter cytochemistry of a physiologically identified tuberomammillary neurone. *A*, Lucifer Yellow (LY) fluorescent cell in the tuberomammillary nucleus. *B*, L-histidine decarboxylase (HDC) immunofluorescence visualized with Texas Red as the fluorochrome. The double-labelled cell is denoted by an arrow. Calibration, 30 μm. *C*, The distribution of L-histidine decarboxylase immunoreactive neurones in the section from which the Lucifer Yellow-filled cell was recovered. MR, mammillary recess.

Atypical tuberomammillary neurones

Two neurones in the tuberomammillary region exhibited active and passive membrane properties which were markedly dissimilar to the results described above. These neurones were distinguished by their marked accommodation of firing in response to depolarizing current pulses, action potential duration less than 1 ms and after-hyperpolarizations of less than 20 ms duration. Furthermore, these neurones showed neither inward nor transient outward rectification. Because of the rarity of these neurones, no attempt was made to mark and double label them (see below) in order to determine if they represent atypical histaminergic tuberomammillary neurones or another class of neurone co-distributed within the tuberomammillary region.

Immunohistochemistry

In order to determine if the physiological properties described herein were indeed those of histaminergic tuberomammillary neurones, five neurones were subjected to intracellular ionophoresis of Lucifer Yellow. These neurones all exhibited the set of physiological properties characteristic of tuberomammillary neurones described above. In each case, double labelling (Fig. 6) of either the soma, or in instances in which Lucifer Yellow fluorescence was very intense, peripheral dendrites, was observed when the tissue was subsequently processed for immunofluorescence using L-histidine decarboxylase as a marker.

DISCUSSION

The present study is the first description of the membrane properties of tuberomammillary neurones. Like aminergic neurones elsewhere in the central nervous system (Crunelli, Forda, Brooks, Wilson, Wise & Kelly, 1983; VanderMaelen & Aghajanian, 1983; Andrade & Aghajanian, 1984; Williams, North, Shefner, Nishi & Egan, 1984; Segal, 1985), tuberomammillary neurones were spontaneously active, their action potentials were followed by a prominent after-hyperpolarization, their apparent input impedances were around 175 MΩ and membrane time constants around 20 ms, and they exhibited transient outward rectification. It is noteworthy that with the exception of two neurones, every neurone in the tuberomammillary region exhibited this same striking set of active and passive membrane properties. Five tuberomammillary neurones with these distinctive properties were positively identified as histaminergic by double labelling combined with L-histidine decarboxylase immunohistochemistry.

Tuberomammillary neurones were spontaneously active *in vitro*, firing at a rate ($2{\cdot}1 \pm 0{\cdot}6$ Hz) virtually identical to that seen in anaesthetized rats *in vivo* ($2{\cdot}0 \pm 1{\cdot}5$ Hz, Reiner & McGeer, 1987). The action potentials of tuberomammillary neurones were abolished by tetrodotoxin, and further addition of tetraethylammonium ions revealed a regenerative spike which was reversibly abolished by cobalt. The after-hyperpolarization which followed spontaneous action potentials appears to be mediated by an increased potassium conductance, as it was abolished by intracellular caesium loading. This conclusion must be considered tentative however, as its reversal potential was somewhat less negative than that predicted by the Nernst

equation. Unlike several other systems (Krnjevic, Puil & Werman, 1978; Hotson & Prince, 1980; Andrade & Aghajanian, 1984; Williams *et al.* 1984), after-hyperpolarization of tuberomammillary neurones appears to be only partly calcium dependent. Taken together, these data suggest that action potentials in tuberomammillary neurones result from the successive activation of a fast sodium and delayed potassium conductance, as well as a high-threshold calcium conductance, followed by an after-hyperpolarization which is generated by a relatively calcium-independent potassium conductance.

On the basis of the current clamp data presented herein, several additional conductances inherent to tuberomammillary neurones may be inferred. Among these are both inward and transient outward rectification. Although transient outward rectification was not abolished by bath application of 4-aminopyridine, a compound to which this conductance frequently displays exquisite sensitivity (Thompson, 1977; Gustafsson, *et al.* 1982), in some systems (i.e. bull-frog sympathetic ganglia) it is refractory to such treatment (Adams, Brown & Constanti, 1982). Furthermore, it has been pointed out that the *sine qua non* of transient outward rectification is not its pharmacology but rather its time course and its activation at voltages straddling the resting potential (Adams & Galvan, 1986). Whether this pharmacological heterogeneity is related to differences in channel structure remains to be determined; however, it is of interest that in *Drosophila* there exist at least two distinct A-type potassium channels which differ in terms of conductance, voltage dependence, gating kinetics, and genetic expression (Solc, Zagotta & Aldrich, 1987).

Regardless of its pharmacology, transient outward rectification in tuberomammillary neurones as determined under current clamp displays properties strikingly similar to that of the so-called A-current (Connor & Stevens, 1971*a*; Neher, 1971) and suggests that transient outward rectification may be an important determinant of firing rate. Although further analysis is required, it is likely that the after-hyperpolarizations exhibited by tuberomammillary neurones produce sufficient hyperpolarization of the membrane to evoke transient outward rectification. Tuberomammillary neurones have been reported to exhibit dramatic changes in firing rate across behavioural states in unanaesthetized cats, firing most during waking, less during slow-wave sleep and falling silent during paradoxical sleep (Vanni-Mercier, Sakai & Jouvet, 1984). It is tempting to speculate that such behaviourally coded modulation of spike train activity in tuberomammillary neurones may be produced via amplification of relatively subtle hyperpolarization by transient outward rectification. Indeed, such a scenario would be very much in keeping with the original predictions of Connor & Stevens (1971*b*) regarding the effects of the A-current upon repetitive firing behaviour.

We thank Drs T. Watanabe and H. Wada for the generous gift of histidine decarboxylase antibody, and Dr R. A. North for a critical reading of an earlier version of the manuscript. These studies were carried out while P. B. R. was on leave of absence from a Medical Research Council of Canada Fellowship and were supported by grants from the Swiss National Science Foundation (3.002.084) and Fonds für Versuchstierfreie Forschung.

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