Calcium-dependent prepotentials contribute to spontaneous activity in rat tuberomammillary neurons

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1. Intracellullar recordings from histaminergic neurons of the tuberomammillary (TM) nucleus reveal subthreshold depolarizing potentials (DPs) which persist in the presence of tetrodotoxin.

2. Block of hyperpolarization-activated current by 1–4 mM Cs^+ failed to reduce spontaneous activity or DPs.

3. In the presence of tetrodotoxin DPs are voltage dependent and are depressed by Cd^{2+} and Co^{2+}.

4. Ba^{2+} (100 μM) treatment enhances DP amplitude and converts low-amplitude potentials to tetrodotoxin-insensitive action potentials.

5. In the presence of TTX, DPs are reduced by Ni^{2+}. Spontaneous action potentials are also reduced by Ni^{2+} (100–300 μM). A low-threshold Ca^{2+} current is present which is sensitive to Ni^{2+}. These results indicate the presence of calcium currents, perhaps of the low-threshold type, which contribute to activation of action potentials in TM neurons.

Immunohistochemical techniques have demonstrated diffuse histaminergic projections to virtually all parts of the brain (Schwartz, Garbarg & Pollard, 1986; Airaksinen & Panula, 1988; Tohyama, Tamiya, Inagaki & Takagi, 1991). The source of this neuronal histamine is the tuberomammillary (TM) nucleus, a group of neurons in the ventral posterior hypothalamus (Panula, Yang & Costa, 1984; Watanabe *et al.* 1984; Ericson, Watanabe & Köhler, 1987).

Histaminergic neuronal activity *in vivo* is closely associated with behavioural state. TM neurons fire tonically during waking, little during slow wave sleep and not at all during rapid eye movement (REM) sleep (Lin, Sakai, Vanni-Mercier & Jouvet, 1989). This association with behavioural state has led to interest in the role of the histaminergic system in control of wakefulness or attention (Wada, Inagaki, Yamatodani & Watanabe, 1991).

Histaminergic neurons fire *in vitro* at rates similar to their activity during waking *in vivo*. TM neurons exhibit a persistent Na^+ current (Uteshev, Stevens & Haas, 1995) which may contribute to spontaneous firing (Llinas & Alonso, 1992) but a role for this current in the approach to threshold has not been demonstrated. In tonically firing central neurons, a variety of pacing events have been proposed, including low-threshold Ca^{2+} currents (Buhrlis & Aghajanian, 1987; Grace & Onn, 1989), hyperpolarization-activated current (McCormick & Pape, 1990; Akasu, Shoji & Hasuo, 1993) and TTX-sensitive persistent Na^+ current (Grace & Onn, 1989; Llinas & Alonso, 1992). Using intracellular recording methods we have examined the events associated with spontaneous and evoked activity of histaminergic tuberomammillary neurons in an *in vitro* brain slice preparation in order to understand better the mechanisms controlling spontaneous firing and thus histamine release.

METHODS

Wistar rats of either sex were killed by cervical dislocation, decapitated, and the brain rapidly removed and placed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl, 124; KCl, 2–4; KH_2PO_4, 1·25; $CaCl_2$, 2·5; glucose, 10; $NaHCO_3$, 26. The solution was bubbled with 95% oxygen–5% CO_2 to establish a final pH of 7·4. The brain was blocked transversely with cuts posterior to the mammillary recess and anterior to the optic chiasm. Transverse slices 300–400 μm thick were prepared from blocks of tissue using a vibratome. Slices were stored at room temperature (18–22 °C) until used and recordings were carried out in slices submerged and maintained at 33 ± 2 °C. Intracellular recordings were carried out using potassium chloride (3 M)- or potassium acetate (4 M)-filled glass microelectrodes with resistances between 60 and 130 MΩ. Cd^{2+} treatment (200 μM) was carried out in ACSF in which the phosphate buffer was omitted. When Ba^{2+} was used at a concentration greater than 200 μM, or when Cd^{2+} was superfused, Ca^{2+} was reduced to 0·2 mM and phosphate was omitted from the solution. In low-Ca^{2+} solutions Mg^{2+} was raised to 3·8 mM except in the case of 2 mM Ba^{2+} solutions in which the magnesium was not altered. All agents were applied as part of the ACSF. Patch clamp recordings were carried out at room temperature using

pipettes with resistances of 3–7 MΩ. Whole-cell recordings of spontaneous activity were carried out with a potassium chloride solution (KCl, 140 mM; Hepes, 10 mM; EGTA, 10 mM; $CaCl_2$, 1 mM; pH 7·3) and recordings of low-threshold Ca^{2+} currents were carried out with similar pipettes filled with the the same intracellular solution with the exception that Cs^+ was added instead of K^+. Substances used were: tetraethylammonium chloride (TEA), $CdCl_2$, $CoCl_2$, tetrodotoxin (TTX), CsCl, $NiCl_2$, $ZnCl_2$ and papain (Sigma). TM neurons were readily identified by the presence of an ensemble of features including transient outward current and hyperpolarization-activated current (I_H; see Fig. 2); and spontaneously occurring, broad action potentials with a shoulder region, a pronounced after-hyperpolarization, and a slow after-hyperpolarization following repetitive activation (Reiner & McGeer, 1987; Haas & Reiner, 1988; Llinas & Alonso, 1992). The primary distinction between histaminergic neurons and other neuronal types encountered in this region is the presence or absence of burst firing. Following a hyperpolarization, return to resting potential strongly activates the transient outward current in TM neurons (Greene, Haas & Reiner, 1990), while in the non-histaminergic neurons of this region, break spikes or bursts follow the return to rest following hyperpolarization. This distinction proved adequate in whole-cell recordings as well, since the transient outward current and hyperpolarization-activated currents persist for a short time even with Cs^+ in the pipette.

RESULTS

The results of this study were obtained during recordings from 121 neurons of the ventral posterior hypothalamus in those regions shown to contain histaminergic neurons (Panula *et al.* 1984; Watanabe *et al.* 1984; Airaksinen & Panula, 1988). TM neurons typically fire in a beating pacemaker pattern although activity is not always monotonous (Fig. 1). During spontaneous firing, occasional misses revealed subthreshold depolarizing potentials (DPs). When the membrane potential was hyperpolarized, action potentials were blocked but DPs persisted, supporting the conclusion that DPs normally initiate action potentials. Further hyperpolarization prevented DPs (Fig. 1*B*, $n = 5$).

Following prolonged hyperpolarizing steps, there is a slow depolarization which activates DPs as well as action potentials (Fig. 2*A*, $n = 8$). Block of sodium-dependent action potentials by the addition of TTX failed to block this slow depolarization (Fig. 2*A*, $n = 5$) indicating that it was not due to non-inactivating Na^+ current (which is blocked in TM neurons following treatment with TTX (Llinas & Alonso, 1992; Uteshev *et al.* 1995)). Since I_H is strongly activated under these conditions, and has been shown to contribute to spontaneous firing in a number of excitable

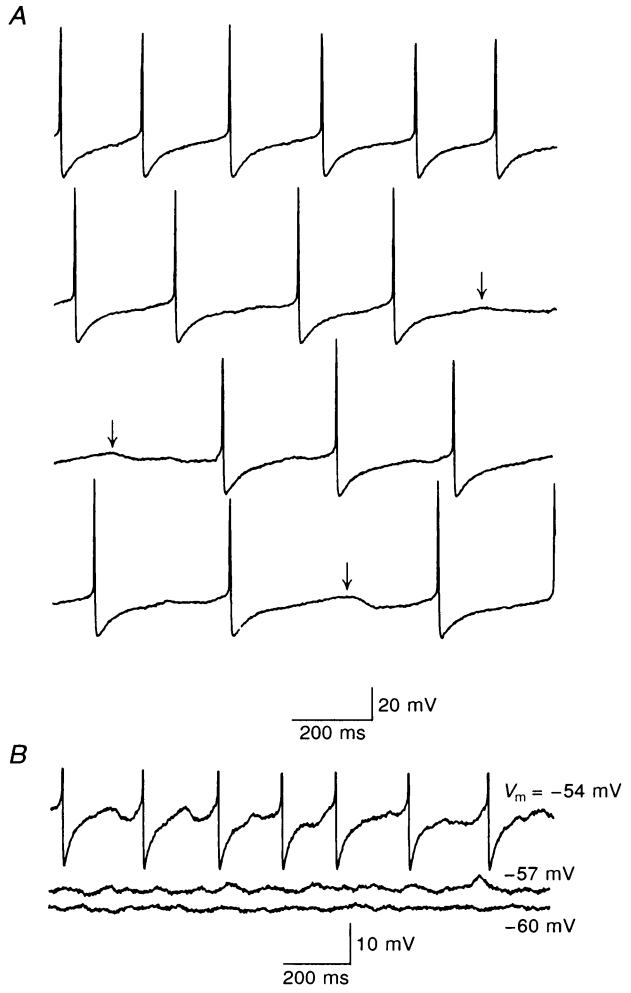

Figure 1. Spontaneous activity of TM neurons includes subthreshold depolarizing potentials

A, recording of spontaneous activity with occasional misses unmasking subthreshold depolarizing potentials (arrows). *B*, hyperpolarization of the membrane potential by current injection (lower traces) blocks action potentials, revealing depolarizing potentials which are reduced by further hyperpolarization (action potentials are truncated).

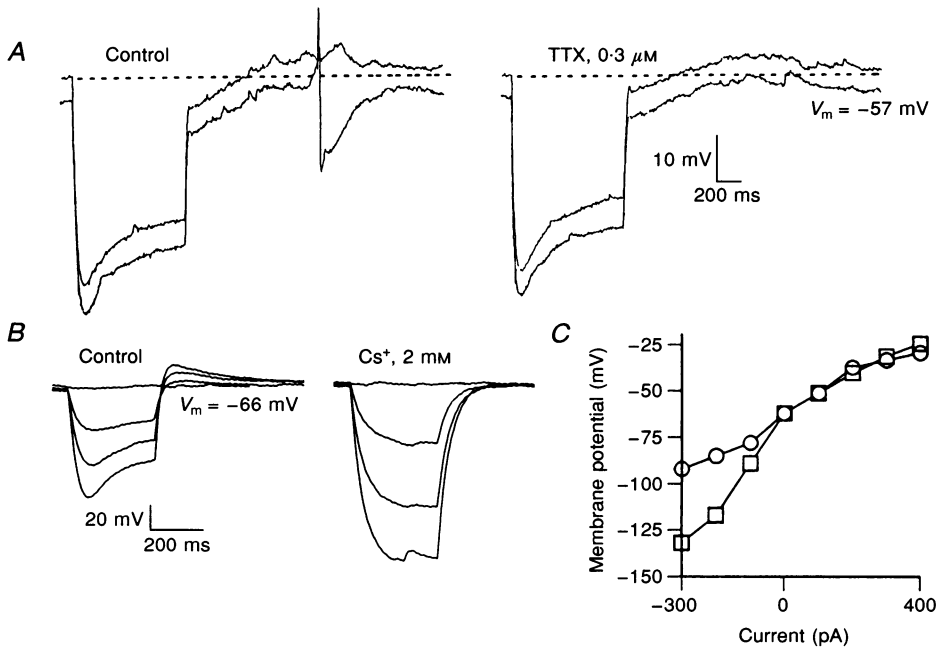

Figure 2. I_H underlies a slow depolarizing potential

A, the slow depolarization following a hyperpolarizing step persists following treatment with TTX, indicating that it is not due to Na^+ channel activation. *B*, with the membrane potential hyperpolarized to reduce I_A activation, this slow depolarizing potential is clearly visible. Treatment with Cs^+ blocks this potential. *C*, the voltage–current relation in control (○) and 2 mM Cs^+ (□) shows the increased slope at hyperpolarized membrane potentials characteristic of I_H block.

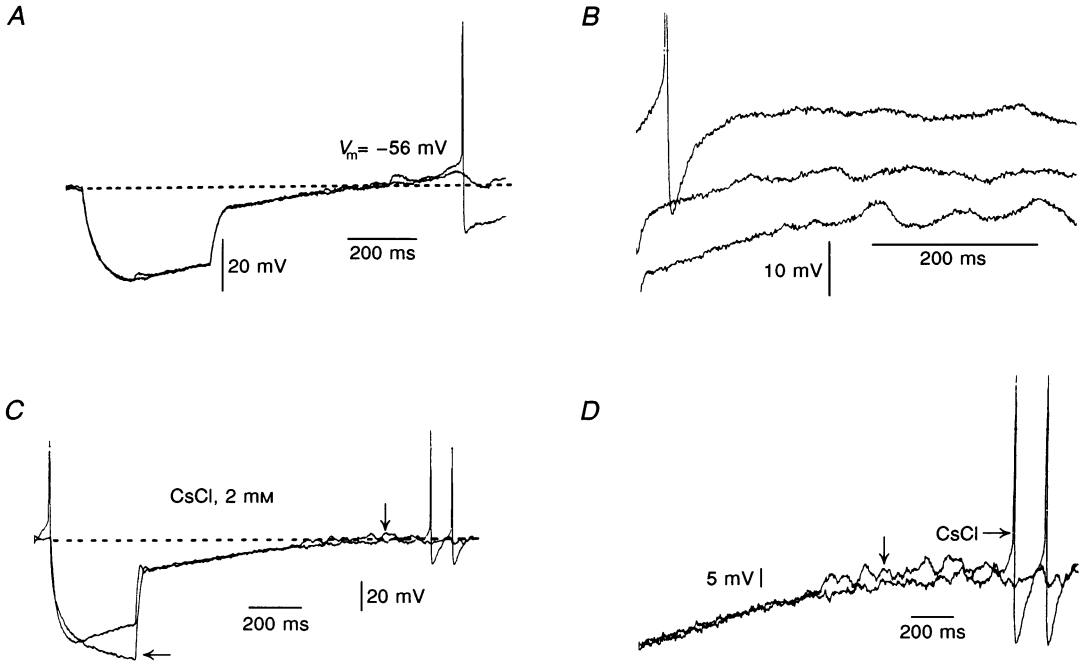

Figure 3. Block of I_H does not reduce DPs

A, control traces showing activation of I_H and return to resting potential. *B*, records of the return to resting potential at higher gain showing activation of DPs. *C*, superimposed control and Cs^+ treatment (arrows) demonstrate block of the sag with no apparent effect on depolarizing potentials. DPs and firing occur during I_H block. *D*, higher resolution traces showing recovery from hyperpolarization. Cs^+ treatment (arrows) failed to decrease activity indicating that I_H does not contribute to spontaneous activity.

cell types (Di Francesco, 1981; McCormick & Pape, 1990; Akasu *et al.* 1993) we examined the role of I_H in the slow depolarization. In the presence of TTX, hyperpolarizing steps were used to activate I_H. With the membrane potential hyperpolarized to reduce activation of the transient outward K^+ current (I_A), a slow depolarization was seen following hyperpolarizing steps. Treatment with Cs^+ blocked this depolarization (Fig. 2*B*, $n = 5$). Thus when strongly activated, I_H can cause an after-depolarization and induce firing in TM neurons.

Cs^+ treatment did not, however, slow firing or hyperpolarize spontaneously active TM neurons ($n = 5$). Similarly, DPs observed during recovery from hyperpolarizing steps (Fig. 3*A* and *B*) were not prevented by Cs^+ treatment which blocks I_H (Fig. 3*C* and *D*, $n = 4$). Though not fully activated, I_H is activated to a greater extent under these conditions than following an after-hyperpolarization.

Although TTX treatment blocked action potentials, DPs persisted (Fig. 4*A*). The occurrence of DPs remained voltage dependent during TTX treatment. The frequency of DPs at the resting potential was variable and low (4–10 Hz). Addition of Cd^{2+} to the superfusate (200 μM) resulted in a reduction of DPs (Fig. 4*B*, $n = 4$). Co^{2+} treatment reduced Ca^{2+} action potentials by more than 90% ($n = 4$) and also blocked DPs (not shown, $n = 3$).

Ba^{2+} (200 μM) enhanced DPs, which were converted to TTX-insensitive action potentials (Fig. 5*A*, $n = 3$). Raising Ba^{2+} to 2 mM resulted in large depolarizing shifts and plateau potentials (Fig. 5*B*, $n = 4$), which could be blocked by Cd^{2+} or Co^{2+} (not shown). Ba^{2+} (200 μM) treatment also enhanced oscillatory behaviour during prolonged depolarizations (not shown).

The action of Ni^{2+}, which selectively decreases low-threshold Ca^{2+} conductances (Hagiwara, Irisawa & Kameyama, 1986; Fox, Nowycky & Tsien, 1987; Akaike, Kostyuk & Osipchuk, 1989) was examined. In the presence of TTX (0·3 μM), Ni^{2+} treatment (150–300 μM) reduced subthreshold DPs (Fig. 6, $n = 5$). There was no effect of Ni^{2+} (250 μM) on calcium potentials (not shown). In addition to reduction of subthreshold activity, Ni^{2+} prolonged the recovery from hyperpolarizing steps, indicating that an inward current activated during recovery from hyperpolarizing steps may be reduced by Ni^{2+} treatment.

We have attempted to assess further the role of Ca^{2+} currents in normal spontaneous activity of TM neurons. Addition of Cd^{2+} (200 μM) resulted in a reduction of spontaneous activity which was transient and followed by a depolarization and increased firing (not shown, $n = 3$). It is possible that the slowing of firing was unrelated to effects on Ca^{2+} currents. Using whole-cell recording, the effect of

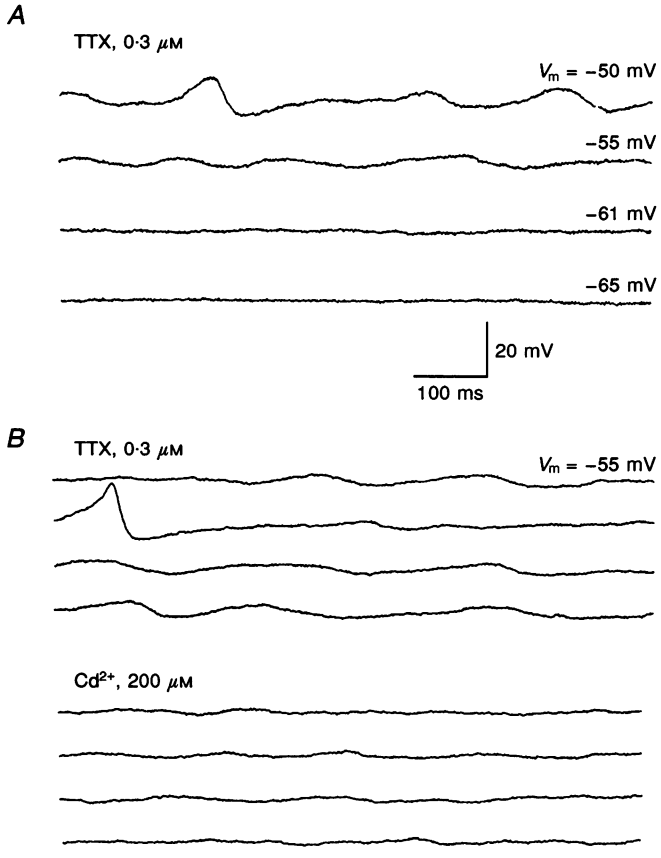

Figure 4. Depolarizing potentials are cadmium sensitive

A, DPs persist following TTX treatment and are reduced by hyperpolarization. *B*, DPs recorded in the presence of TTX are blocked by treatment with Cd^{2+} (200 μM).

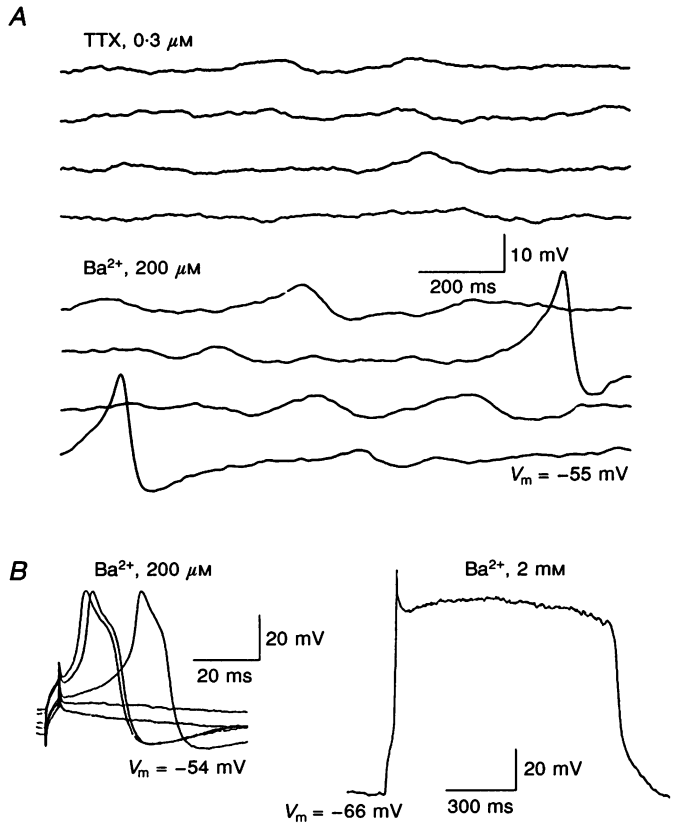

Figure 5. Barium enhances depolarizing potentials

A, subthreshold DPs recorded during treatment with TTX are enhanced by Ba^{2+}. *B*, higher concentrations of Ba^{2+} convert graded potentials to plateau potentials. Ba^{2+} also enhances depolarization-evoked Ca^{2+} potentials.

Ni^{2+} on spontaneous activity was also examined. Without TTX present, Ni^{2+} (150 μM) treatment reduced spontaneous activity in four of five experiments while 300 μM Ni^{2+} reduced firing in four of four cells tested (Fig. 7*A*) consistent with an action on low-threshold Ca^{2+} currents. In the absence of TTX some subthreshold activity can still be seen in the presence of Ni^{2+}. This result suggests that both Na^+- and Ca^{2+}-mediated mechanisms can contribute to subthreshold activity. We have recorded under voltage clamp in the whole-cell configuration in order to determine if a low-threshold calcium current is present in TM neurons. Using Cs^+ as the major intracellular cation in order to suppress outward currents, we find that a TTX-insensitive, transient inward current is present in TM neurons

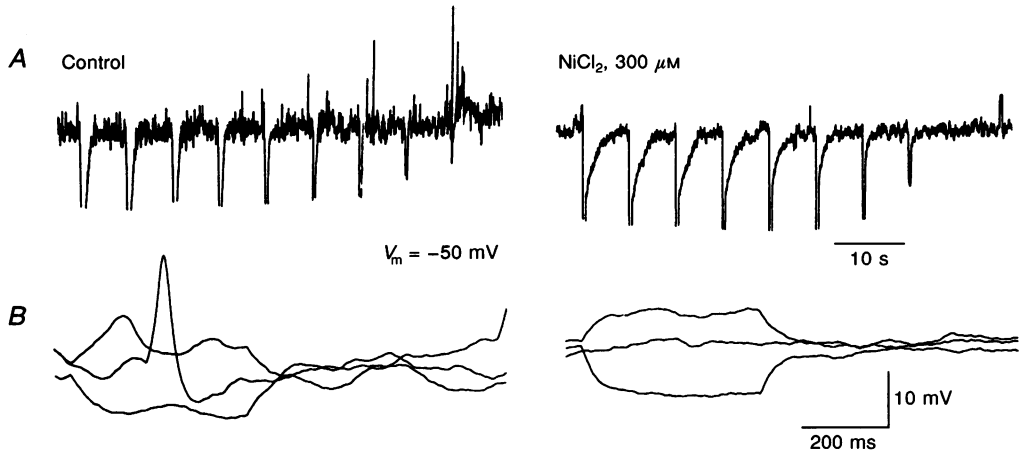

Figure 6. TTX-resistant subthreshold activity is reduced by Ni^{2+}

Chart records of membrane potential before and during 300 μM Ni^{2+} treatment show the reduction of subthreshold potentials by Ni^{2+}. In the presence of Ni^{2+} the recovery from a hyperpolarizing step is prolonged, consistent with the presence of a low-threshold calcium current. *B*, records at faster sweep speed illustrate DPs which are reduced by Ni^{2+} treatment.

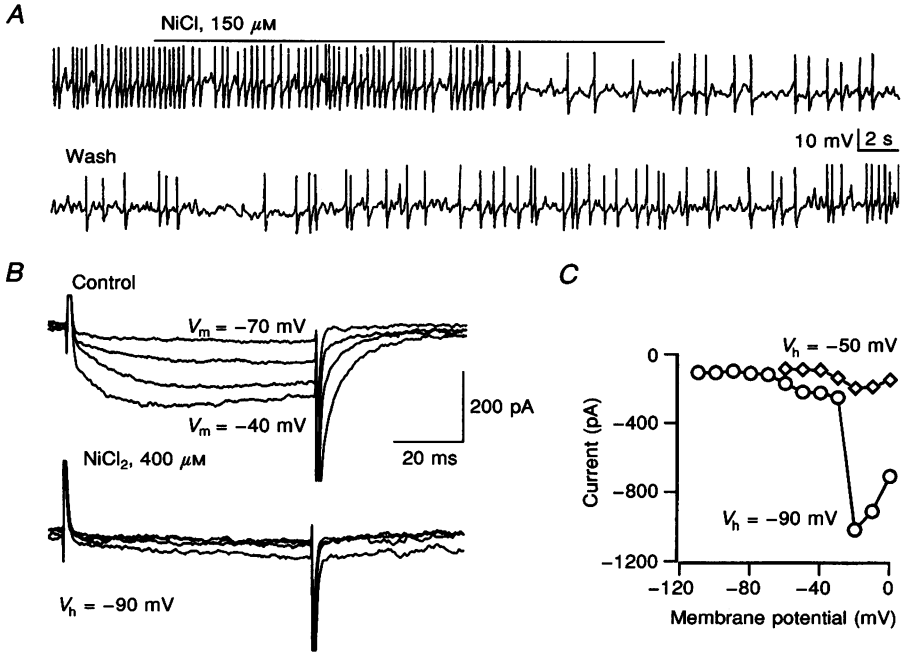

Figure 7. Ni^{2+} reduces spontaneous activity and blocks low-threshold calcium current in TM neurons

A, spontaneous activity is slowed when Ni^{2+} is applied (whole-cell recording, action potentials are truncated by the chart recorder). *B*, voltage clamp recordings from another TM neuron in the presence of TTX show that a transient inward current activates during depolarizing steps and is blocked by Ni^{2+}. *C*, current–voltage relation recorded from holding potentials of −90 and −50 mV show that inward currents exhibit inactivation (TTX present) with low-threshold current activation near −70 mV. V_h, holding potential.

(Fig. 7*B*). This current exhibits inactivation and is completely blocked by 400 μM Ni^{2+}. Current–voltage relations show both low- and high-threshold inactivating components of inward current (Fig. 7*C*, $n = 5$).

DISCUSSION

Our results demonstrate the presence of subthreshold depolarizations of the membrane potential which contribute to repetitive firing in tuberomammillary neurons. Since DPs were present even when action potentials failed to occur, persisted during hyperpolarization which blocked action potentials and in TTX, and had a lower threshold than TTX-sensitive action potentials, they appear not to be dependent on action potential activity.

The failure of Cs^+ to hyperpolarize or reduce firing in TM neurons indicates that repetitive firing is not dependent upon I_H, in contrast to observations in thalamic neurons (McCormick & Pape, 1990) and in supraschiasmatic neurons (Akasu *et al.* 1993), and consistent with predictions based on voltage clamp analysis of I_H in TM neurons (Kamondi & Reiner, 1991). Increased firing rate and enhanced DPs which occurred during Cs^+ treatment are probably due to non-specific reduction of potassium current.

The persistence of DPs in the presence of TTX indicates that they are not mediated by synaptic activity. Further evidence against a synaptic origin of these events comes from their sensitivity to changes in the membrane potential, as presynaptic events would not be expected to be readily influenced by such manipulations. Similar arguments apply to the possibility that DPs are coupling potentials related to activity in neighbouring neurons. DPs due to electrotonic coupling would not be sensitive to small changes in membrane potential and their incidence would be reduced by TTX.

The sensitivity of the DPs to divalent cations is consistent with a dependence on extracellular Ca^{2+}. The nature of the underlying Ca^{2+} current is unclear. Ca^{2+}-dependent action potentials have been previously reported in TM neurons (Haas & Reiner, 1988). We show evidence for the presence of a low-threshold Ca^{2+} current. The action of Ni^{2+} as well as the potential at which DPs occur are consistent with a role for a low-threshold current in DPs.

The concentrations of Ni^{2+} which alter firing activity are somewhat higher than those reported to block low-threshold currents in chick dorsal root ganglion cells (Fox *et al.* 1987), but much higher concentrations of Ni^{2+} are

required to block low-threshold calcium currents in isolated neurons harvested from the ventromedial hypothalamus (Akaike *et al.* 1989).

Both Na^+ and Ca^{2+} channels may contribute to subthreshold behaviour. A role for Na^+ channels in firing has been suggested by Llinas & Alonso (1992). Persistent activation of single Na^+ channels has been observed in isolated TM neurons (Uteshev *et al.* 1995) and these Na^+ channels will contribute inward current near threshold (Stafstrom, Schwindt & Crill, 1982; Alzheimer, Schwindt & Crill, 1993). Given the relatively high threshold for firing and low resting potentials in TM neurons (Haas & Reiner, 1988; Llinas & Alonso, 1992) this mechanism could be quite important, as is the case in dopaminergic neurons (Grace & Onn, 1989) which are electrophysiologically similar to TM neurons.

DPs in the presence of TTX exhibit a higher frequency, and subthreshold activity is less co-ordinated. The higher rate of activity is probably converted to stable, lower firing rates by the after-hyperpolarization which delays the return to threshold. In addition to limiting firing rate the after-hyperpolarization will enhance the subsequent depolarizing potential, contributing to entrainment of firing.

Our results indicate that spontaneous activity is intrinsic to TM neurons and that Ca^{2+} currents contribute to the activation of action potentials. Calcium-dependent DPs are intrinsic to individual neurons and may be associated with the dendritic release of histamine as well as GABA, which is co-localized in some TM neurons (Senba, Daddona, Watanabe, Wu & Nagy, 1985; Ericson *et al.* 1987). Bicuculline-sensitive, TTX-resistant chloride-mediated postsynaptic potentials occur spontaneously in many TM neurons (Haas, Reiner & Greene, 1991; D. R. Stevens, unpublished observations). DPs are damped by K^+ current and modulation of either conductance by neurotransmitters will probably be an important step in the control of histamine release in relation to behavioural state and during specific behaviours in which histamine release is increased.

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