Tetrodotoxin-resistant persistent Na^+ current underlying pacemaker potentials of fish gonadotrophin-releasing hormone neurones

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1. Gonadotrophin-releasing hormone (GnRH)-immunoreactive terminal nerve (TN) cells show endogenous regular beating discharges, which may be related to their putative neuromodulator functions. The ionic mechanism underlying the pacemaker potential was studied using intracellular and patch-pipette current clamp recordings from a whole brain *in vitro* preparation of a small fish brain.

2. The pacemaker potentials were resistant to 1·5–3 μM tetrodotoxin (TTX) and were not affected by Ca^{2+} channel blockers (amiloride, Ni^{2+}, Co^{2+}, Cd^{2+}) or in Ca^{2+}-free solution. In contrast, the pacemaker potentials were readily blocked by substituting tetramethylammonium or choline for Na^+ in the perfusing solution, and the resting membrane potential became more hyperpolarized than the control level.

3. The present results suggest that the TTX-resistant persistent Na^+ current, $I_{Na(slow)}$, supplies the persistent depolarizing drive and plays an important role in the generation of pacemaker potentials in TN GnRH cells.

It has previously been shown that the gonadotrophin-releasing hormone (GnRH)-immunoreactive terminal nerve (TN) system of a small fish brain serves as an excellent general model system for studying neuromodulator functions in vertebrate brains (Oka, 1992*a*, *b*; Oka & Matsushima, 1993). The TN GnRH cells project widely in the brain and are structurally and functionally distinct from the preoptic GnRH cells, which project to the pituitary and facilitate gonadotrophin release (Oka & Ichikawa, 1990; N. Yamamoto & Y. Oka, unpublished observations). They show spontaneous regular beating discharges, which are dependent on their endogenous pacemaker properties. These morphological and physiological characteristics are thought to be important for their putative neuromodulator functions (Oka, 1992*a*, *b*; Oka & Matsushima, 1993). In the present study, advantage has been taken of the whole brain *in vitro* preparation of a small fish, in which TN GnRH cells are easily identified as a distinctive structure of cells clustered immediately beneath the ventral meningeal membrane, to examine the ionic mechanism underlying the pacemaker potentials by using pharmacological methods in intracellular and patch-pipette current clamp recordings. The underlying ionic currents may be the target for modulation by hormones and/or transmitters that change levels according to the physiological conditions of the animal. Whereas the low-voltage-activated Ca^{2+} current often plays a major role in the production of neuronal oscillations (Llinás, 1988), it was found in the present study that the TTX-resistant persistent Na^+ current, $I_{Na(slow)}$, plays an important role in the generation of pacemaker potentials.

METHODS

Adult male and female dwarf gouramis (*Colisa lalia*), about 4 cm in length, were purchased from a local dealer and were kept at 27 °C until used. The fish were chilled by immersing them in crushed ice. They were then decapitated and the whole brain was dissected out and pinned ventral side up to the Sylgard base of a small recording chamber. This 'whole brain preparation' was continuously superfused with standard oxygenated Krebs–Ringer solution (Llinás & Sugimori, 1980) containing (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1·2; $CaCl_2$, 2·4; $MgSO_4$, 1·3; $NaHCO_3$, 26; glucose, 10 (pH 7·4). The temperature of the perfusing solution was regulated at 20 °C by placing the chamber on a thermoregulator device.

The intracellular recording electrodes were filled with 2 M KCl. Electrode resistances were usually 30–45 MΩ. The electrical recordings were digitized by an analog-to-digital converter at 5 or 10 kHz unless otherwise noted. Detailed procedures for the intracellular recordings and data analysis are described in Oka & Matsushima (1993). In some experiments, patch pipettes were used instead of sharp microelectrodes. In these cases, the meningeal membrane was carefully removed using fine forceps. The cluster of TN GnRH cells could be visually identified under the dissecting microscope. Patch pipettes contained (mM): KCl, 110; $MgCl_2$, 3; Hepes, 40; EGTA, 3 (pH 7·4). Pipette resistances were 6–10 MΩ, and seal resistances were >10 GΩ. Recordings using microelectrodes and patch pipettes gave similar results. However, the latter method produced more stable recordings, and the recovery from pharmacological

treatments was more easily obtained. TTX (0·75–3 μM), amiloride (1 mM), $NiCl_2$ (1 mM), and TEACl (5 or 10 mM) were added directly to the standard solution. $CoCl_2$ (2 mM) was added to a solution ('control' solution for Co^{2+}) consisting of (mM): NaCl, 130; KCl, 3; $MgCl_2$, 2; $NaHCO_3$, 26; glucose, 10 (pH 7·4). $CdCl_2$ (0·5 mM) was added to a solution ('control' solution for Cd^{2+}) consisting of (mM): NaCl, 124; KCl, 5; $CaCl_2$, 2·4; MgCl, 1·3; Hepes, 10; $NaHCO_3$, 15; glucose, 10 (pH 7·4). Low-Na^+ solution was made by substituting TMACl or choline chloride for NaCl in the standard solution. Ca^{2+}-free solution consisted of (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1·2; $MgCl_2$, 10; $NaHCO_3$, 26; glucose, 10 (pH 7·4).

For the statistical analysis of the frequency of pacemaker activities, Student's two-tailed paired t test was used. Data are expressed as means ± S.E.M.

RESULTS

The basic morphological and electrophysiological characteristics of TN GnRH cells have already been reported in detail elsewhere (Oka & Ichikawa, 1990, 1991; Oka, 1992*a*, *b*; Oka & Matsushima, 1993). TN GnRH cells can be identified easily by their characteristic anatomy (location, soma size and morphology) and regular spontaneous activities. The cluster of TN GnRH cells can also be visually identified under a dissecting microscope when the meningeal membrane was removed (see Fig. 1 of Oka & Ichikawa, 1991). The regular spontaneous activities of TN GnRH cells have been demonstrated not to be driven synaptically by other pacemaker neurones but to be intrinsic to these cells (Oka, 1992*b*; Oka & Matsushima, 1993). All the cells recorded in the present study showed such characteristic pacemaking activities (Figs 1–4). The pacemaker potentials underlying these activities sometimes failed to reach spike threshold (arrowheads in Fig. 1*A a–c*), but they were robust and could be seen throughout the recordings. Changes in the membrane resistance during pacemaking were estimated (Fig. 1*B a* and *b*) by injecting brief repetitive hyperpolarizing current pulses (0·1–0·2 nA, 1–2 ms duration, 200–500 pulses s^{-1}; examined in 3 cells). In the cell shown in Fig. 1*B*, the membrane resistance of the cell during the rising phase of the pacemaker potentials decreased to 73% of that during the resting phase, as shown by decreased potential deflections in response to brief repetitive pulses (Fig. 1*B b*; 6·4 ± 0·3 to 4·7 ± 0·4 mV, $n = 50$). The other two cells showed a decrease in

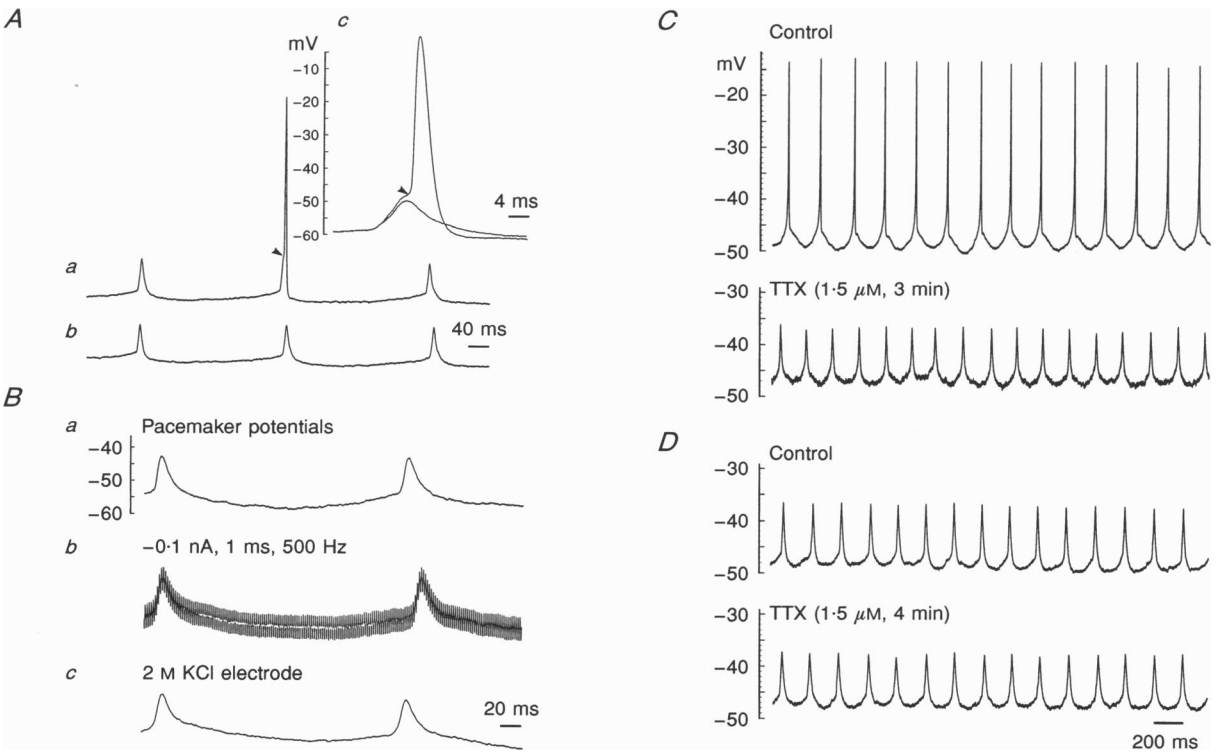

Figure 1. Regular beating discharges of TN GnRH cells recorded intracellularly

Subthreshold pacemaker potentials are shown in *A a–c* (*A c* shows superimposed traces; firing threshold is indicated by arrowheads). Changes in the membrane resistance are measured by injecting brief repetitive hyperpolarizing current pulses (*B b*, 0·1 nA, 1 ms duration, 500 pulses s^{-1}). The pacemaker potentials did not reverse to negative-going ones even after a prolonged intracellular recording with KCl electrodes (*B c*). Bath application of TTX did not affect the frequency of beating discharges (*C*) or the subthreshold pacemaker potentials (*D*). Voltage scale in *A c* also applies to *A a* and *A b*, and that in *B a* to *B b* and *B c*.

membrane resistance to 80 and 90% of that during the resting phase. Negative-going pacemaker potentials were never seen, even after prolonged intracellular recording with 2 M KCl electrodes in which passive diffusion of Cl^- ions is expected to occur (Fig. 1*B c*). Thus, it follows that the increase in conductance of cations such as Na^+ or Ca^{2+}, and not the decrease in conductance of Cl^-, is associated with the generation of pacemaker potentials. Bath application of TTX (1·5 μM) to a regularly beating cell did not affect its frequency, although TTX-sensitive spike components were quickly and completely blocked (Fig. 1*C*). Similarly, TTX did not affect the frequency or amplitude of pacemaker potentials that failed to reach spike thresholds (Fig. 1*D*). These results indicate that the inward current that makes up the rising phase of the pacemaker potential is caused by either a Ca^{2+} current or a TTX-resistant Na^+ current.

The possibility of involvement of Ca^{2+} currents was investigated first. The upper two rows in Fig. 2 show experiments on full spike beating discharges, and the lower ones show those on subthreshold pacemaker potentials. The first column (Fig. 2*A*) shows the effects of Ca^{2+}-free solution (examined in 8 cells). The frequencies of pacemaker activities (beating discharges or pacemaker potentials) before and after perfusion with Ca^{2+}-free solution were 4·5 ± 1·0 and 4·3 ± 1·0 spikes s^{-1}, respectively. Thus, the Ca^{2+}-free solution had no significant effect ($P > 0·2$). The second (Fig. 2*B*) and third (Fig. 2*C*) columns show the effects of the blockers of low-voltage-activated Ca^{2+} currents, amiloride (1 mM, $n = 6$) and Ni^{2+} (1 mM, $n = 6$). The frequencies of pacemaker activities before and after these treatments were 3·9 ± 1·2 and 4·2 ± 1·3 spikes s^{-1} for amiloride (not significant; $P > 0·2$), and 4·0 ± 1·2 and 3·9 ± 1·4 spikes s^{-1} for Ni^{2+} (not significant; $P > 0·5$), respectively. The fourth column (Fig. 2*D*) shows the effects of a blocker of high-voltage-activated Ca^{2+} currents, Co^{2+} (2 mM, $n = 4$). The effects of Cd^{2+} (0·5 mM, $n = 6$) were also examined (not shown). The frequencies of pacemaker activities before and after these treatments were 3·4 ± 0·8 and 3·5 ± 0·9 spikes s^{-1} for Co^{2+} (not significant; $P > 0·5$), and 3·0 ± 0·7 and 3·0 ± 0·7 spikes s^{-1} for Cd^{2+} (not significant; $P > 0·2$), respectively. The concentrations of the blockers were chosen so as to provide supramaximal block of each current. However, none of these treatments affected the frequency of pacemaker activities. Thus it is concluded that the Ca^{2+} currents are not a major component of pacemaker potential.

Therefore, the possibility of involvement of Na^+ currents was examined. Low-Na^+ external solution was prepared by substituting TMACl or choline chloride for NaCl (examined in 14 cells). $NaHCO_3$ was left to retain buffering action. In

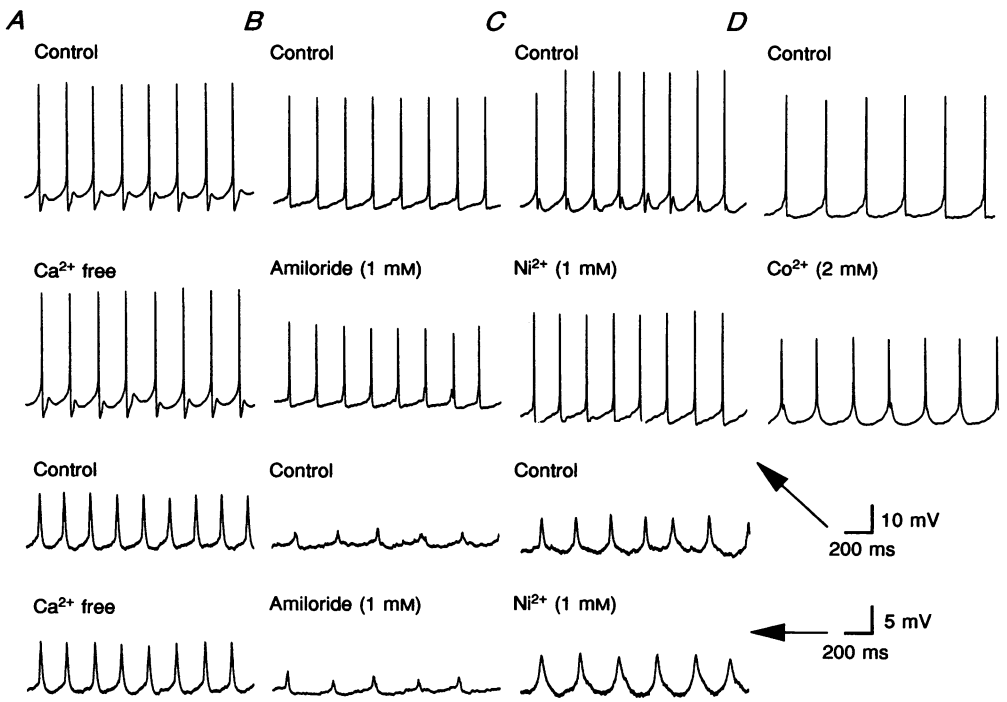

Figure 2. Blockage of calcium currents does not affect the pacemaker potentials

The upper two rows show experiments on full spike beating discharges, while the lower ones show those on subthreshold pacemaker potentials. *A*, effects of Ca^{2+}-free solution. *B* and *C*, effects of blockers of low-voltage-activated Ca^{2+} currents, amiloride and Ni^{2+}, respectively. *D*, effects of Co^{2+}, a blocker of high-voltage-activated Ca^{2+} currents. None of these treatments affected the frequency of pacemaker potentials and the ensuing beating discharges.

such solutions, the Na^+ channels are impermeable to TMA or choline ions. After their addition, the action potentials dependent on fast Na^+ current, $I_{Na(fast)}$, disappeared within about 1–2 min (Fig. 3*A b*). Subsequently, the pacemaker potential was completely blocked (Fig. 3*A c*), and the resting membrane potential became more hyperpolarized than the control level (compare Fig. 3*A a* and *c*; the potential level of *A c* is more hyperpolarized than that of *A a* by about 20 mV). The pacemaking activity and level of the resting potential recovered after washout of low-Na^+ solution (Fig. 3*A d*). These effects of low-Na^+ solution are clearly shown in a trace on a slower time scale (Fig. 3*A e*). They are most reasonably explained by assuming that the removal of permeant Na^+ ions from the external solution severely decreased the inward current that supplies the persistent depolarizing drive during pacemaking activity. The pacemaker potentials were partially blocked by prolonged treatment with a higher concentration of TTX solution (Fig. 3*B a–c*). In this cell, subsequent perfusion of low-Na^+ solution quickly blocked the pacemaker potentials, and the resting potential became progressively hyperpolarized (Fig. 3*B d*). Similar results were obtained when half of the NaCl was replaced by TMACl (examined in 5 cells, not shown). These results indicate that a TTX-resistant persistent Na^+ current that is active at or near the resting potentials, $I_{Na(slow)}$, plays an important role in the generation of pacemaker potentials.

In order to examine the possible involvement of potassium currents in the falling phase of the pacemaker potentials, a K^+ channel blocker, TEA, was added to the bath (examined in 10 cells). After the addition of 5 or 10 mM TEA, the pacemaker potentials were slowed down (8/10 cells, see Fig. 4*A* and *B*) and even stopped in one case (Fig. 4*A*). Moreover, the baseline DC level was shifted to a level more depolarized (range 5–25 mV) than that of the control (Fig. 4*A* and *B*, second traces). The DC level and the pacemaking activity recovered to control levels after

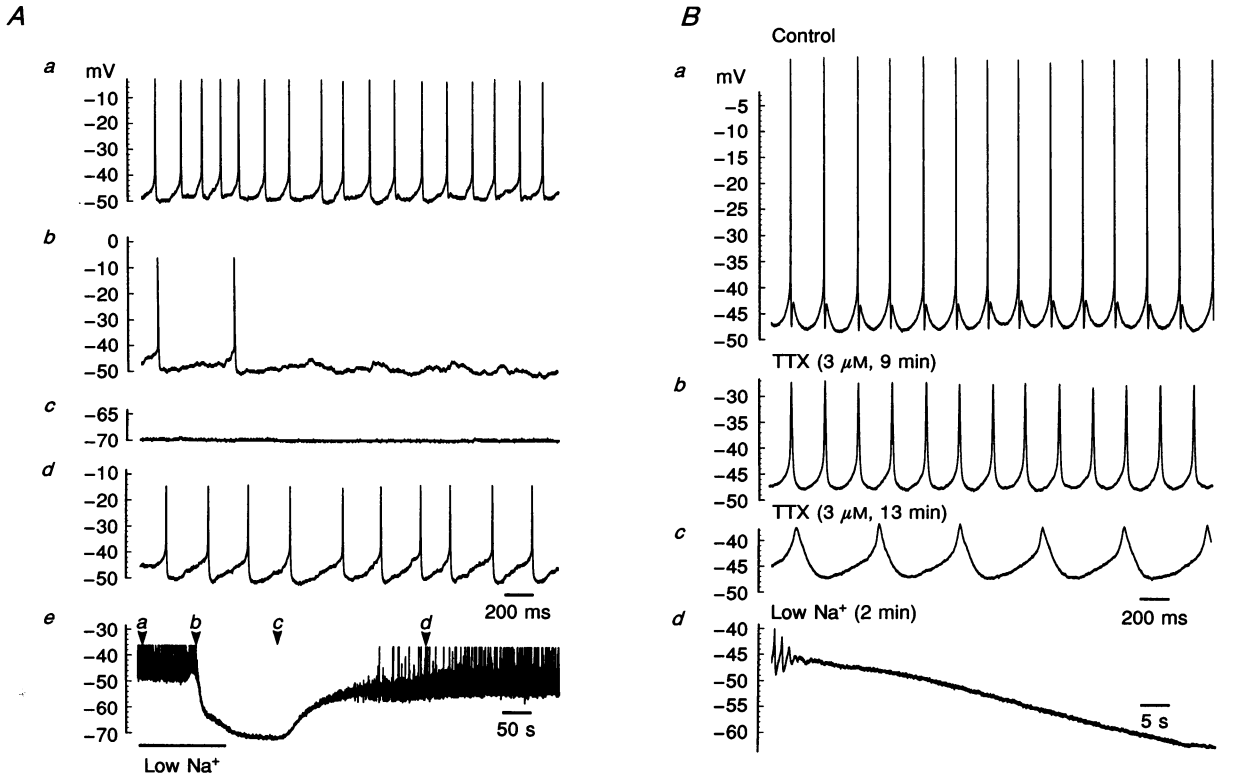

Figure 3. Persistent Na^+ current, $I_{Na(slow)}$, is involved in the generation of pacemaker potentials

Substitution of TMACl for NaCl in the perfusing solution completely blocked the pacemaker potentials, and the resting membrane potential became more hyperpolarized than the control level (*A a–c*). Following washout, spontaneous pacemaker activities recovered (*A d*), and the resting potential returned to the control level. *A e* shows the time course of potential changes on a slower time scale (spikes are clipped). It should be noted in *A e* that the spike activity is not faithfully traced because of the slow A/D sampling rate (20 Hz). The pacemaker potentials were partially blocked by prolonged treatment with higher concentration of TTX solution (*B a–c*). Subsequent perfusion of low-Na^+ solution quickly and completely blocked the pacemaker potentials, and the resting potential became progressively hyperpolarized (*B d*; note slower time scale).

washout ($n = 3$; see Fig. 4*A*, bottom trace). Thus it is suggested that some kind(s) of K^+ current(s) that are at least partially blocked by TEA are involved in the falling phase of the pacemaker potentials.

DISCUSSION

Importance of $I_{Na(slow)}$ in the generation of pacemaker potentials

The present paper indicates that activation of $I_{Na(slow)}$ is essential for the generation of pacemaker potentials in peptidergic modulator neurones, TN GnRH cells. $I_{Na(slow)}$ is necessary because the pacemaker potentials were completely blocked in low-Na^+ solutions in which reduced influx of charge carriers failed to activate the depolarizing drive for the generation of pacemaker potentials. The hyperpolarized resting potentials during perfusion of low-Na^+ solutions (Fig. 3) clearly show the block of persistent depolarizing drive. The block of pacemaker potentials is not a secondary effect of hyperpolarization alone, because the spontaneous pacemaker potentials were not blocked by hyperpolarizing DC current injections (Oka & Matsushima, 1993). The result of low-Na^+ experiments also suggests the non-inactivating or persistent nature of $I_{Na(slow)}$ current. Activation of Ca^{2+} conductances is not necessary for the generation of pacemaker potentials in this system because the pacemaker potentials were not affected at all by supramaximum concentrations of various Ca^{2+} channel blockers or in Ca^{2+}-free solutions (Fig. 2).

The present results also suggest that some kind(s) of K^+ current(s) are involved in the falling phase of the pacemaker potentials. The slowing down of pacemaker potentials probably arises from the prolonged time course of the repolarizing phase because of the block of K^+ currents by TEA. The depolarizing DC potential shift may also be explained by the block of K^+ currents counteracting the persistent depolarizing drive by $I_{Na(slow)}$. However, the nature of the K^+ current remains to be further investigated.

Characteristics of $I_{Na(slow)}$

The Na^+ current $I_{Na(slow)}$, which was suggested to be involved in the generation of pacemaker potentials of TN GnRH cells, was characterized by persistent activation and resistance to block by TTX. Such persistent Na^+ current has been studied in a variety of neurones, including rat nodose ganglion cells (Ikeda & Schofield, 1987), frog sympathetic neurones (Jones, 1987), rat hippocampal neurones (French, Sah, Buckett & Gage, 1990), and rat dorsal root ganglion cells (Elliot & Elliot, 1993; see also Taylor, 1993). It has been characterized by resistance to inactivation during prolonged depolarization and by various degrees of sensitivities to TTX. The persistent nature of $I_{Na(slow)}$ is especially important for the generation of rhythmicity (see Taylor, 1993). In fact, $I_{Na(slow)}$ has been suggested to be involved in the generation of intrinsic oscillatory activities of deep cerebellar nuclear cells of rats (Jahnsen, 1986) and stellate cells of rat entorhinal cortex (Alonso & Llinás, 1989) and neocortex (Stafstrom, Schwindt, Chubb & Crill, 1985; Llinás, Grace & Yarom, 1991). Thus, $I_{Na(slow)}$ may be more widely distributed in the central nervous system of various vertebrate species than has been conventionally thought (Llinás, 1988).

Functional significance of $I_{Na(slow)}$ in TN GnRH cells

We have previously proposed a hypothesis that may be relevant to the peptidergic and monoaminergic neuromodulatory systems of vertebrate brains in general (Oka, 1992*b*; Oka & Matsushima, 1993); the modulator neurones have endogenous rhythmic activities that vary according to the animal's hormonal or environmental conditions, and they regulate the excitability of target

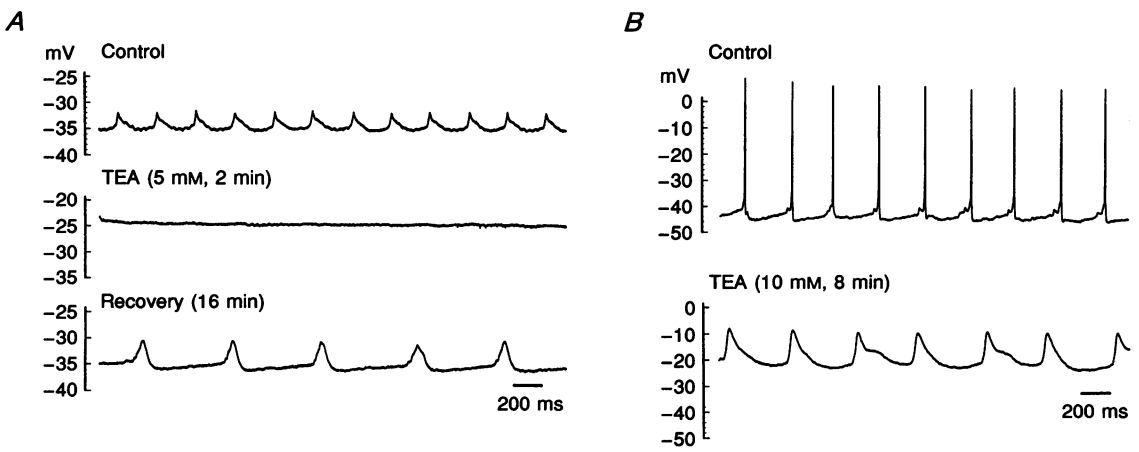

Figure 4. Perfusion of TEA (5 or 10 mM) solutions partially blocked the pacemaker potentials

The pacemaker potentials were stopped (*A*) or slowed down (*B*), and the baseline DC level was shifted to a more depolarized level.

neurones in a wide variety of brain regions simultaneously via multiple axonal branches. In this model, ion channels that are responsible for the generation of endogenous rhythmic activities (pacemaker potentials) may be the target of modulation by hormones or transmitters whose levels are subject to change under different physiological conditions of the animals. Thus, it would be interesting in the future to study possible modulation of $I_{Na(slow)}$ currents by various hormones or transmitters. In this respect, it is important to note that some authors have reported on possible modulation of Na^+ current that is similar to $I_{Na(slow)}$. Alreja & Aghajanian (1991) reported that activation of a cAMP-dependent protein kinase pathway modifies the frequency of spontaneous tonic firing of locus coeruleus neurones and suggested that the substrate may be a kind of persistent sodium channel similar to $I_{Na(slow)}$. They also reported that opiates suppress a resting Na^+-dependent inward current in locus coeruleus neurones (Alreja & Aghajanian, 1993), although the identity of this current remained to be determined. Similarly, Raggenbass, Goumaz, Sermasi, Tribollet & Dreifuss (1991) and Raggenbass & Dreifuss (1992) have reported that vasopressin or oxytocin induces in medullary motoneurones a persistent Na^+ current that is resistant to TTX. Taking these reports into consideration, it is possible that $I_{Na(slow)}$ in TN GnRH cells may be modulated by similar biochemical mechanisms induced by hormones or transmitters.

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