Characterization of $K^+$ Currents Underlying Pacemaker Potentials of Fish Gonadotropin-Releasing Hormone Cells

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Abe, Hideki and Yoshitaka Oka. Characterization of $K^+$ currents underlying pacemaker potentials of fish gonadotropin-releasing hormone cells. J. Neurophysiol. 81: 643–653, 1999. Endogenous pacemaker activities are important for the putative neuromodulator functions of the gonadotropin-releasing hormone (GnRH)-immunoreactive active terminal nerve (TN) cells. We analyzed several types of voltage-dependent $K^+$ currents to investigate the ionic mechanisms underlying the repolarizing phase of pacemaker potentials of TN-GnRH cells by using the whole brain in vitro preparation of fish (dwarf gourami, Colisa lalia). TN-GnRH cells have at least four types of voltage-dependent $K^+$ currents: 1) 4-aminopyridine (4AP)-sensitive $K^+$ current, 2) tetraethylammonium (TEA)-sensitive $K^+$ current, and 3) and 4) two types of TEA- and 4AP-resistant $K^+$ currents. A transient, low-threshold $K^+$ current, which was 4AP sensitive and showed significant steady-state inactivation in the physiological membrane potential range (−40 to −60 mV), was evoked from a holding potential of −100 mV. This current thus cannot contribute to the repolarizing phase of pacemaker potentials. TEA-sensitive $K^+$ current evoked from a holding potential of −100 mV was slowly activating, long lasting, and showed comparatively low threshold of activation. This current was only partially inactivated at steady state of −60 to −40 mV, which is equivalent to the resting membrane potential. TEA- and 4AP-resistant sustained $K^+$ currents were evoked from a holding potential of −100 mV and were suggested to consist of two types, based on the analysis of activation curves. From the inactivation and activation curves, it was suggested that one of them with low threshold of activation may be partly involved in the repolarizing phase of pacemaker potentials. Bath application of TEA together with tetrodotoxin reversibly blocked the pacemaker potentials in current-clamp recordings. We conclude that the TEA-sensitive $K^+$ current is the most likely candidate that contributes to the repolarizing phase of the pacemaker potentials of TN-GnRH cells.

INTRODUCTION

The terminal nerve (TN)-gonadotropin releasing hormone (GnRH) cells show endogenous pacemaker activities and project widely in the brain (Oka and Matsushima 1993). Such characteristics of TN-GnRH cells may be relevant for simultaneous regulation of the target neuron’s excitability in a wide variety of brain regions, depending on the animal’s physiological conditions. We consider that TN-GnRH system may function as a neuromodulator that is involved in the regulation of many long-lasting changes in animal behaviors, such as changes in motivational or arousal states (Oka 1992a,b, 1997; Oka and Matsushima 1993). Because TN-GnRH cells of the dwarf gourami (Colisa lalia, a freshwater tropical fish) form morphologically distinctive clusters immediately beneath the ventral meningeal membrane (Oka and Ichikawa 1990, 1991), they can be easily identified in the whole brain in vitro preparations (Oka 1995, 1996; Oka and Matsushima 1993). This gives an obvious experimental advantages over peptidergic neurons of other vertebrates because they are small and scattered, so that it was extremely difficult to study the physiology of single peptidergic neurons. Interestingly, recent studies suggest that the monoamine neurons, which were also suggested to be involved in neuromodulation, share some anatomic and physiological characteristics in common with TN-GnRH cells (e.g., dopaminergic neurons in the substantia nigra and ventral tegmental area) (Grace 1988, 1991). Thus the study of TN-GnRH cells may give insight to the cellular mechanisms of neuromodulation in general.

As for the mechanisms of the generation of pacemaker potentials, we have already shown by voltage- and current-clamp analyses that a tetrodotoxin (TTX)-resistant persistent Na$^+$ current, $I_{Na(slow)}$ plays an important role in the generation of pacemaker potentials of TN-GnRH cells (Oka 1995, 1996). We investigated the voltage-dependent outward currents [$K^+$ current(s)] that should be involved in the repolarizing phase of pacemaker potentials by using the whole cell patch-clamp technique in in vitro whole brain preparations. We did not study the calcium-dependent potassium currents because we have previously shown that calcium currents may not be the primary component(s) that generate the depolarizing phase of pacemaker potentials of TN-GnRH cells (Oka 1995).

METHODS

Adult male and female dwarf gourami (Colisa lalia), ~4 cm in standard length, were purchased from a local dealer and kept at 25–27°C until used. The fish were decapitated, and the whole brain was dissected out and pinned ventral side up to the silicone elastomer (Shin-Etsu Silicone No. KE-106, Shin-Etsu Chemical, Japan) base of a small recording chamber. This whole brain preparation was continuously superfused with an oxygenated Ringer solution until the whole cell recording was established. The Ringer solution contained (in mM) 124 NaCl, 5 KCl, 1.2 KH$_2$PO$_4$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, 26 NaHCO$_3$, and 10 glucose (pH 7.4 adjusted with NaOH).

The ventral meningeal membrane was carefully removed with fine forceps. The cluster of TN-GnRH cells could be visually identified under the dissecting microscope. Patch pipettes contained (in mM) 110 KCl, 3 MgCl$_2$, 40 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 5 ethylene glycol-bis(β-aminoethyl ether) N,N,N’,N”-tetraacetic acid (EGTA), and 2 Na$_2$ATP (pH 7.4 adjusted with NaOH). Pipette resistance was ~8 MΩ, and seal resistance was
>10 GΩ. Series resistances as measured from the amplitude of capacitative transients in response to 10-mV pulses were 23 ± 9 (SE) MΩ (n = 27). They were compensated as much as possible. After gigahm seal formation and “break in” for the whole cell recording mode, characteristic spontaneous pacemaker activities were confirmed in the current-clamp mode (see Oka and Matsushima 1993).

Next the solution was changed to the experimental solutions. The standard solution superflushing the cells contained (in mM) 135 choline Cl, 5 KCl, 5 MgCl₂, 5 glucose, 10 HEPES (pH adjusted 7.4 with NaOH). In some experiments, 0.01 mM CaCl₂ was added in the saline, and MnCl₂ was substituted for MgCl₂ in equivalent amount or 5 μM La³⁺ was added in the saline. The experimental solutions were made by modifying the standard solution. 4-aminopyridine (4AP; 5 mM), and TTX (0.75 μM) were added directly in the saline. Tetraethylammonium chloride (TEA; 20 mM)-containing solution was made by adding TEA to the normal saline in equimolar replacement of choline Cl. The temperature of perfusing solutions was maintained at room temperature.

Whole cell voltage- and current-clamp recordings were carried out with the use of CEZ-2300 amplifier (Nihon Kohden, Japan) and pCLAMP software (Axon instruments). The linear leakage currents were digitally subtracted, either automatically with the use of the P/4 protocol, or manually, after measuring ohmic resistance in response to hyperpolarizing command pulses. The data were not corrected for the liquid junction potentials. All data in this report represent means ± SE.

**RESULTS**

Morphological and basic electrophysiological characteristics of TN-GnRH cells were reported in detail elsewhere (Oka 1992a,b, 1995; Oka and Ichikawa 1990, 1991; Oka and Matsushima 1993; Yamamoto et al. 1995). TN-GnRH cells can be identified easily by their characteristic anatomy (location, soma size, and morphology) and regular pacemaker activities. Moreover, the cluster of TN-GnRH cells was visually identified under a dissecting microscope after removing the meningeal membrane. Spontaneous activities of the cells were recorded in the current-clamp mode before the whole cell voltage-clamp experiments, and all recorded cells were identified as TN-GnRH cells (i.e., by the presence of regular pacemaker activities) (see Oka 1995, 1997; Oka and Matsushima 1993).

**Total outward currents in TN-GnRH cells**

In agreement with our previous study (Oka 1996), the whole cell currents in the Ringer solution evoked by depolarizing voltage steps between −100 and +50 mV (10-mV increments, duration 200 ms) from a holding potential of −100 mV were composed of a mixture of inward and outward currents (Fig. 1A). Large and transient inward currents were activated above −40 mV (Fig. 1A, I₅₀.₅₆₆). They are the conventional fast sodium currents because they can be blocked by 0.75 μM TTX (Fig. 1, B and C). In contrast, at −50 mV, small but persistent inward currents were observed (Fig. 1A, I₅₀.₅₆₆). They are the TTX-resistant persistent Na⁺ current, I₅₀.₅₆₆ (Oka 1996). The I₅₀.₅₆₆ currents were masked by large fast sodium currents and outward currents in response to larger depolarizing command pulses. To block the inward currents (I₅₀.₅₆₆, I₅₀.₅₆₆, and Na⁺ currents), Na⁺- and Ca²⁺-free solution was used that was made by equimolar substitution of NaCl and CaCl₂ by choline Cl and MgCl₂, respectively. In addition, 5 μM La³⁺ was added in this Na⁺- and Ca²⁺-free solution. When the current responses were measured in a Na⁺- and Ca²⁺-free solution containing 0.75 μM TTX, only outward currents were recorded (Fig. 1, B and C).

The isolated outward current responses to depolarizing command pulses from holding potential of −100 mV are shown in Fig. 1B. The outward currents consisted of transient and persistent current components. When the holding potential was changed from −100 to −60 mV, the total outward current amplitudes were decreased (Fig. 1C). We could not find hyperpolarization-activated (inward rectifier) currents nor M-like potassium currents in our cells.

To define the permeant ion, the reversal potential of the outward current was evaluated by a tail current analysis with three different concentrations of extracellular K⁺ ([K⁺]₀) in Na⁺- and Ca²⁺-free experimental solution containing 0.75 μM TTX (Fig. 2, A and B). After a 200-ms conditioning pulse to +50 mV from a holding potential of −100 mV, tail currents were evoked by a series of subsequent hyperpolarizing voltage steps in 5, 10, and 20 mM [K⁺]₀, which reversed at −78.22 ± 2.21 mV (n = 3), −62.69 ± 3.79 mV (n = 3), and −41.05 ± 0.83 mV (n = 3), respectively (Fig. 2C). When these reversal potentials were plotted as a function of [K⁺]₀ (Fig. 2D), it could be well fitted by a line predicted from Nernst equation (58 mV per log unit changes in [K⁺]₀). Therefore it follows that the channels underlying these outward currents are highly selective for K⁺ ions. We then isolated the individual K⁺ currents from the total outward current and investigated the voltage dependence and kinetics of these currents.

**Isolation of K⁺ current components**

**4AP-sensitive transient K⁺ current.** First, we examined the 4AP-sensitive transient current, which was briefly described in our previous study (Oka 1996). When the current responses were measured in Na⁺- and Ca²⁺-free experimental solution containing 0.75 μM TTX and 20 mM TEA, almost all the inward currents and some portions of the persistent outward currents were eliminated, but large transient currents and smaller persistent outward currents could be recorded in response to a series of 200 ms depolarizing pulses (in 10-mV increments) from a holding potential of −100 mV (Fig. 3A). When 5 mM 4AP was further added to this solution, the transient outward currents were eliminated, but some outward currents could be still elicited by the same depolarizing pulses (Fig. 3A). This residual outward currents and the dose response of TEA blockade will be described in the next section. The subtracted current (4AP-sensitive current, Fig. 3B) are transient K⁺ currents and appeared to be similar to the potassium-A current (Rudy 1988). The peak amplitude of the 4AP-sensitive transient current was dependent on the test potential (Fig. 3, B and C), increasing as the test potential was made more positive. The 4AP-sensitive transient current could be elicited by voltage steps to test potentials more positive than −50 mV (Fig. 3C).

The voltage dependence of activation was examined by converting the current to conductance with the equation \( I_\text{K} = g_\text{K} (E - E_{\text{rev}}) \). \( E_{\text{rev}} \) was calculated by tail current analysis with the \([K^+]_0 = 5 \text{ mM} \) (described in the previous section). The normalized conductance at each membrane potential was determined for each cell tested and then averaged for eight cells. This conductance–voltage relation could be well fitted with a Boltzmann function of the form

\[ \text{normalized conductance} = \frac{1}{1 + e^{(V - V_{1/2})/k}} \]

where \( V \) is the membrane potential, \( V_{1/2} \) is the half-maximum voltage, and \( k \) is the voltage sensitivity.
where $g$ is conductance, $g_{\text{max}}$ is the maximum conductance, $V_m$ is the membrane potential, $V_{1/2}$ is the half-activation voltage, and $k$ is the slope factor describing the steepness of the voltage dependence (Fig. 3D). The average $V_{1/2}$ value was $-22.14 \pm 3.35$ mV, and $k$ value was $15.51 \pm 0.94$ ($n = 8$).

Then the voltage dependence of steady-state inactivation was also examined. A 5-s conditioning pulse to potentials between $-120$ and $+50$ mV was applied to reach its steady-state inactivation level, and the peak current amplitude of the outward current evoked by a subsequent 200-ms test pulse ($+50$ mV) was recorded and plotted as normalized current (Fig. 3D). The relation between the prepulse potential and the normalized current could be fitted with a Boltzmann function of the form

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp([V_{m} - V_{1/2}]/k)}$$

where $I$ is current, $I_{\text{max}}$ is the maximum current when the conditioning pulse is $-120$ mV, $V_m$ is the membrane potential, $V_{1/2}$ is the half-inactivation voltage, and $k$ is the slope factor. The average $V_{1/2}$ value was $-79.44 \pm 3.36$ mV, and $k$ value was $6.15 \pm 0.72$ ($n = 4$).

These results indicate that 4AP-sensitive transient $K^+$ current is activated at potentials more positive than $-50$ mV, which is considered low threshold but almost inactivated at $-60$ to $-40$ mV, which corresponds to the resting membrane potential of the TN-GnRH cells ($-46 \pm 9$ mV, $n = 118$; because $I_{\text{Na(slow)}}$ supplies the persistent depolarizing drive, the resting membrane potential was defined as the base membrane potential during pacemaker activities) (see Oka and Matsu-shima 1993). Therefore this A-like current is considered to contribute little, if any, to the repolarizing phase of subthreshold pacemaker potentials.

**TEA-SENSITIVE CURRENT.** Next we examined the TEA-sensitive current. To define TEA-sensitive current or TEA- and 4AP-resistant current (Fig. 3A2, see next section), we examined the dose dependence of TEA blockade of the persistent $K^+$ current component, which was recorded in Na$^+$- and Ca$^{2+}$-free solution containing 0.75 $\mu$M TTX and 5 mM 4AP.
The current amplitudes were measured at the end of 200-ms test pulses to +50 mV from a holding potential of −100 mV before and after the application of various concentrations of TEA (in the range of 1–60 mM). Application of TEA reduced the current amplitudes in a dose-dependent manner. The persistent K\(^+\) current was reduced 11.6 ± 5.2% by 1 mM TEA (n = 3) and 61.6 ± 4.8% by 60 mM TEA (n = 6). The current amplitudes were normalized to the value in the absence of TEA and were plotted as a function of TEA concentration (Fig. 4A).

The concentration-response curve was best fitted with the form

\[ I/I_{\text{max}} = (1 - U)/(1 + ([\text{TEA}]_o/\text{IC}_{50})) + U \]

where \( U \) is the portion unblocked by TEA and 5 mM 4AP. The IC\(_{50}\) was 8.3 mM, and the \( U \) value was 0.3. This curve suggests that the persistent K\(^+\) current is blocked by [TEA]\(_o\) in a dose-dependent manner, but 30% of the persistent K\(^+\) current is resistant to both TEA and 4AP. In addition, 38% of persistent K\(^+\) current remained in 20 mM TEA. An example of the effect of 20 mM TEA is illustrated in Fig. 4B. However, >80% (92% from the recorded data and 80.5% calculated from the fitted curve) of persistent K\(^+\) current that was sensitive to TEA was blocked by 20 mM TEA. Therefore we defined the persistent K\(^+\) current component that is blocked by 20 mM TEA as TEA-sensitive current and the residual component as TEA- and 4AP-resistant current.

TEA-sensitive current was measured by subtracting the current evoked in a Na\(^+\)- and Ca\(^2+\)-free solution containing 0.75 mM TTX, 5 mM 4AP, and 20 mM TEA from that evoked in a similar solution without TEA. Figure 5 shows the properties of TEA-sensitive current. Persistent outward currents were activated by voltage steps more positive than −30 mV from a holding potential of −100 mV (Fig. 5A). When the holding potential was changed to −60 mV, which is closer to the resting membrane potentials of TN-GnRH cells, the amplitude of persistent current was slightly decreased, but the activation threshold was not changed (−30 mV, Fig. 5, B and D). Steady-state inactivation of TEA-sensitive current was also investigated by measuring the changes in current amplitudes evoked by voltage steps to +50 mV from holding potentials ranging from −120 to +50 mV (Fig. 5C).

The voltage dependence of the activation and steady-state inactivation were examined by measuring the current ampli-

FIG. 2. Tail current analysis of the total outward current. A and B: tail currents were elicited by hyperpolarizing test voltage step pulses (−100 to 0 mV, 200 ms) after a 200-ms conditioning pulses to +50 mV from a holding potential of −100 mV in the presence of 5 mM (A) or 10 mM (B) external K\(^+\) ions, [K\(^+\)]\(_o\), respectively. The amplitude of tail currents were measured at the time indicated by the arrows. C: plot of the averaged tail current amplitudes (n = 3) as a function of the test membrane potentials. Dashed line indicates the zero current level. Tail currents evoked in 5, 10, and 20 mM [K\(^+\)]\(_o\), reversed at −78.22 ± 2.21 mV, −62.69 ± 3.79 mV, and −41.05 ± 0.83 mV, respectively. D: reversal potentials are plotted as a function of log[K\(^+\)]\(_o\). The line indicates the slope that is calculated from the Nernst equation.
Current amplitude was normalized as described before and was plotted as a function of the test potential (Fig. 5E). Regardless of the holding potential, whether it is −100 mV (Fig. 5E, ■) or −60 mV (Fig. 5E, ○), normalized conductance increased steeply in the test voltage range from −50 to −40 mV, and the curves were almost identical. These plotted data for both activation and steady-state inactivation were fitted with single Boltzmann functions. The average \( V_{1/2} \) and \( k \) values were −13.49 ± 2.70 mV and 13.84 ± 0.73, respectively, for the activation at the holding potential of −100 mV (\( n = 5 \)), −7.53 ± 2.76 mV and 13.95 ± 0.84 for the activation at the holding potential of −60 mV (\( n = 16 \)), and −51.30 ± 2.76 mV and 6.47 ± 0.84 for the steady-state inactivation (\( n = 5 \)).

On the basis of these results, we suggest that the TEA-sensitive current consists of a single component. This current can be activated at relatively less depolarized potentials, and the steady-state inactivation (Fig. 5E) shows that this current is only partially inactivated at −60 to −40 mV, which corresponds to the resting membrane potential of TN-GnRH cells. Therefore it is reasonable to assume that TEA-sensitive current is the most likely candidate that contributes to the repolarizing phase of subthreshold pacemaker potentials.

**TEA- AND 4AP-RESISTANT CURRENTS.** Finally, we examined another outward current component that is resistant to both TEA and 4AP. As already described in the previous section, when the current responses were measured in a solution containing 0.75 \( \mu \)M TTX, 20 mM TEA, and 5 mM 4AP, persistent outward current responses remained (Fig. 3A2). Figure 6 shows the properties of TEA- and 4AP-resistant currents. In response to voltage steps from a holding potential of −100 mV, persistent current was activated more positive than −30 mV (Fig. 6A). However, when the holding potential was changed to −60 mV, which is closer to the resting membrane potential of TN-GnRH cells, the amplitude of the persistent current was decreased, and the activation threshold was shifted to a more positive potential (−10 mV, Fig. 6B). Figure 6D shows the I/V relationships with these two different holding potentials. Steady-state inactivation of TEA- and 4AP-resistant current was also investigated by measuring the change evoked by a voltage step to +50 mV when the holding potential was varied from −120 to +50 mV (Fig. 6C).
FIG. 4. Effects of TEA on persistent K⁺ currents. A: dose-response relationships between the TEA concentration and the persistent K⁺ current amplitude. Currents were measured at the end of a +50-mV test pulse from a holding potential of −100 mV. The numbers in parentheses by the filled squares represent the numbers of cells tested for each TEA concentration. Smooth curve was drawn assuming one-to-one binding relationship with IC₅₀ = 8.3 mM. The dotted line indicates that 30% of the current was not blocked even in higher concentration of TEA. B: effects of 20 mM TEA on persistent K⁺ currents. Current traces before and after drug application are shown.

FIG. 5. Characterization of TEA-sensitive K⁺ current. A and B: current responses (top records) in the Na⁺- and Ca²⁺-free Ringer solution containing 0.75 μM TTX and 5 mM 4AP evoked by 200-ms depolarizing voltage steps (bottom records) from a holding potential (Vₜₚ) of −100 mV (A) and −60 mV (B). C: steady-state inactivation of TEA-sensitive K⁺ current. Current responses in the same solution as in A and B evoked by +50-mV test pulses after 5-s conditioning pulses to membrane potentials ranging from −120 mV to +50 mV. D: I/V curves were constructed by plotting the averaged current amplitudes evoked from Vₜₚ = −100 mV (●) and −60 mV (○) against test potentials. The current amplitudes were measured at the end of 200-ms test pulses. E: activation and steady-state inactivation curves of TEA-sensitive K⁺ current. Activation and steady-state inactivation curves were fitted by the Boltzmann functions g/gmax = 1/[1 + exp((−13.94 − V)/15.17)] (activation, Vₜₚ = −100 mV (●); n = 5), g/gmax = 1/[1 + exp((−7.53 − V)/13.95)] (activation, Vₜₚ = −60 mV (○); n = 16) and I/I₟max = 1/[1 + exp((V − 51.30)/6.47)] (steady-state inactivation (●); n = 5). The TEA-sensitive K⁺ current is only partially inactivated at −60 to −40 mV, which corresponds to the resting membrane potential of TN-GnRH cells.
Voltage dependence of the activation was examined in a similar manner employed for examining other currents (Fig. 6, A–C and E). The relationship between the normalized conductance and test voltage is depicted in Fig. 6E. When the holding potential was −60 mV (Fig. 6E, ●), the activation curve was fitted with a single Boltzmann function, which gave the average $V_{1/2}$ value of 6.35 ± 4.16 mV and $\kappa$ value of 14.58 ± 2.62 ($n = 5$). On the other hand, when the holding potential was −100 mV (Fig. 6E, ■), the activation curve could not be fitted with a single Boltzmann function but was best fitted with a linear summation of two Boltzmann functions

$$g/g_{\text{max}} = \alpha[1+\exp((V_m-V_{1/2})/\kappa)] + \beta[1+\exp((V_m-V'_{1/2})/\kappa')]$$

where $g$ is conductance, $g_{\text{max}}$ is the maximum conductance at +50 mV, $V_m$ is the membrane potential, $V_{1/2}$, $V'_{1/2}$ is the half-activation voltage, $\kappa$, $\kappa'$ is the slope factor, and $\alpha$, $\beta$ is the coefficient. From the fitted curve, it was calculated that the average $V_{1/2}$ value was 6.32 ± 4.46 mV, $V'_{1/2} = -27.87 ± 2.76$ mV, $\kappa = 12.82 ± 1.98$, $\kappa' = 5.83 ± 0.84$, $\alpha = 0.52 ± 0.05$, and $\beta = 0.48 ± 0.04$ ($n = 5$). Here one of the two Boltzmann functions (right broken line in Fig. 6E) almost completely overlap with the Boltzmann function at the holding potential of −60 mV. These Boltzmann functions were summarized at Table 1.

Therefore we suggest that there are at least two types of TEA- and 4AP-resistant $K^+$ currents in TN-GnRH cells. One of these two $K^+$ currents has a rather low-threshold of activation and show steep voltage dependence (Fig. 6E, left broken line). The other current has higher activation threshold and show steep voltage dependence (Fig. 6E, right broken line). The current responses in the same solution as in A and B evoked by +50-mV test pulses after 5-s conditioning pulses at membrane potentials ranging from −120 mV to +50 mV. D: The I/V curves were constructed by plotting the averaged current amplitudes ($n = 5$) evoked from $V_h = -100$ mV (●) and −60 mV (●) against test potentials. The current amplitudes were measured at the end of 200 ms test pulses. E: activation and steady-state inactivation curves of TEA- and 4AP-resistant $K^+$ currents. Activation and steady-state inactivation curves were fitted by the Boltzmann functions $g/g_{\text{max}} = \{1 + \exp(-(V_h - V_{50})/\kappa)} / 1 + \exp[(6.32 - V_h)/12.82]\} (\text{activation}, V_h = -100 \text{mV}; n = 5)$, $g/g_{\text{max}} = \{1 + \exp((6.35 - V_{1/2})/14.58)\} (\text{activation}, V_h = -60 \text{mV}; n = 5)$, and $I/I_{\text{max}} = \{1 + \exp[(V - -69.12)/9.07]\} (\text{steady-state inactivation} (\bullet); n = 5)$. The activation curve was fitted with a single Boltzmann function when the $V_h$ was −60 mV, but the activation curve was best fitted with a linear summation of two Boltzmann functions when the $V_h$ was −100 mV. Each broken line indicates these Boltzmann functions. One of the two Boltzmann functions almost completely overlaps with the Boltzmann function at the holding potential of −60 mV.

**TABLE 1. Parameters of the Boltzmann functions that comprise the activation curves of the tetraethylammonium- and 4-aminopyridine resistant $K^+$ currents at $V_h = -100$ mV and $V_h = -60$ mV**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$V_h = -100$ mV</th>
<th>$V_h = -60$ mV</th>
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<tr>
<td></td>
<td>($n = 5$)</td>
<td>($n = 5$)</td>
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<tr>
<td>Coefficient ($\alpha$)</td>
<td>0.52 ± 0.05</td>
<td>0.52 ± 0.05</td>
</tr>
<tr>
<td>Half activation voltage ($V_{1/2}$)</td>
<td>6.32 ± 4.46</td>
<td>6.32 ± 4.46</td>
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<tr>
<td>Slope factor ($\kappa$)</td>
<td>12.82 ± 1.98</td>
<td>14.58 ± 2.62</td>
</tr>
<tr>
<td>Coefficient ($\beta$)</td>
<td>0.48 ± 0.04</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>Half activation voltage ($V'_{1/2}$)</td>
<td>-27.87 ± 2.76</td>
<td>5.83 ± 0.84</td>
</tr>
<tr>
<td>Slope factor ($\kappa'$)</td>
<td>5.83 ± 0.84</td>
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Values are means ± SE.
shows less voltage dependence (Fig. 6E, ● and right broken line). However, data from the steady-state inactivation recordings (Fig. 6E, □) were fitted with a single Boltzmann function, which gave the average $V_{1/2}$ value of $-69.12 \pm 4.95$ mV and $\kappa$ value of $9.07 \pm 0.39$ ($n = 5$). Because the two currents are mostly inactivated at $-60$ to $-40$ mV, which is near the resting membrane potential of these cells, the outward currents that should be evoked by strong depolarization will be smaller. Furthermore, the latter current is activated at membrane potentials more positive than $-20$ mV, which is more depolarized than the peak subthreshold pacemaker potentials. Therefore the contribution of these currents to the repolarization of pacemaker potential should be small, if any.

Effects of TEA on the pacemaker potentials of TN-GnRH cells

From the voltage-clamp experiments, we identified at least four types of K$^+$ currents in TN-GnRH cells. Among these currents, TEA-sensitive K$^+$ current was suggested to be the most likely candidate that contributes to the repolarizing phase of the pacemaker potentials. To confirm this possibility, current-clamp experiments were performed. The left column of Fig. 7 shows the effects of TTX alone. TN-GnRH cells show regular beating discharges in a Ringer solution (Fig. 7A). Bath application of TTX (3 μM, 9 min) blocked action potentials, but subthreshold pacemaker potentials remained intact (Fig. 7A2). The right column of Fig. 7 shows the effect of TTX and TEA. The action potentials were blocked by TTX to check whether TEA affects the subthreshold pacemaker potentials. When 20 mM TEA was added to the Ringer solution together with 0.75 μM TTX, the pacemaker potentials were blocked, and the base membrane potential was shifted to a level more depolarized (10–30 mV) than that of control (Fig. 7B3). These effects are more clearly shown in a trace on a slower timescale (Fig. 7B2). It should be noted in B2 and B4 that the timescales are different from B1 and B3, and the spike activities are not faithfully traced because of the slow A/D sampling rate (50 Hz). The pacemaker potentials returned to normal after washout with normal Ringer (Fig. 7B4).

DISCUSSION

Several components of the voltage-dependent K$^+$ current recorded from TN-GnRH cells of the dwarf gourami were identified on the basis of their voltage dependence, kinetics,
and pharmacology. The identified voltage-dependent K⁺ currents are 1) 4AP-sensitive K⁺ current, 2) TEA-sensitive K⁺ current, and 3) and 4) two types of TEA- and 4AP-resistant K⁺ current.

4AP-sensitive transient K⁺ current

A low-threshold transient K⁺ current was evoked from a holding potential of −100 mV. The current activated at test potentials more positive than −50 mV and was fully inactivated at holding potentials more positive than −40 mV. Also, the transient current was preferentially blocked by 4AP. This transient current therefore resembles the A-current identified in embryonic LHRH (mammalian GnRH) neurons (Kusano et al. 1995) and GT1 cells (Bosma 1993).

However, at the holding potential of −60 to −40 mV, which is near the resting membrane potentials of TN-GnRH cells, 4AP-sensitive transient K⁺ current is almost inactivated, and the membrane potential of TN-GnRH cell usually does not hyperpolarize beyond −60 mV under physiological conditions. In addition, the pacemaker activity was not affected by 5 mM 4AP in current-clamp experiments (Oka 1996). Therefore we suggest that 4AP-sensitive K⁺ current contributes very little to the pacemaker activity of the TN-GnRH cells.

TEA-sensitive K⁺ current

TEA-sensitive K⁺ current was activated during voltage steps to potentials more positive than −30 mV from a holding potential of −100 mV. This current activated slowly and did not inactivate during the test pulse, and the activation curve obtained from current responses was well fitted by a single Boltzmann function. Furthermore, this current showed little voltage-dependent steady-state inactivation at −60 mV, which is closer to the base of the pacemaker potentials of TN-GnRH cells.

On the basis of these characteristics, we concluded that the TEA-sensitive K⁺ current consists of a single current and is the most likely candidate that contributes to the repolarizing phase of the pacemakers of TN-GnRH cells. The results of current-clamp experiments in which bath application of 0.75 μM TTX and 20 mM TEA blocked the subthreshold pacemaker potentials strongly support this conclusion.

The characteristics of TEA-sensitive K⁺ current of this study may be similar to the delayed rectifier K⁺ current described in LHRH secretory GT1 cell line (Bosma 1993), embryonic LHRH neuron (Kusano et al. 1995), cultured dorsal root ganglion (I_{KDRG}) (Gold et al. 1996), and many excitable cells (see review by Rudy 1988). These currents activate slowly, do not inactivate during the test pulse, are sensitive to extracellular TEA, and are insensitive to 4AP in concentrations as high as 2 mM (GT1 cell line) or 5 mM (I_{KDRG}). Especially, the steady-state inactivation of these currents occur at relatively positive potential range. However, the voltage dependence of activation of these currents are different; I_{KDRG} of the dorsal root ganglion and TEA-sensitive K⁺ current of the TN-GnRH cell have relatively low activation threshold (approximately −40 mV), but delayed rectifier K⁺ currents of the GT1 cell and embryonic LHRH neuron have relatively high activation threshold (approximately −20 mV).

It may be possible that a kind of Ca²⁺-dependent K⁺ current(s) contribute in part to the sustained outward current because the total sustained outward current was reduced by Ca²⁺-free Ringer solution (Fig. 1). Ca²⁺-dependent K⁺ currents were described in cultured embryonic LHRH neuron (Kusano et al. 1995) and LHRH secretory GT1–7 cell line (Spergel et al. 1996). However, our previous current-clamp study has shown that Ca²⁺-free Ringer solution and Ca²⁺ channel blockers do not affect regular beating pacemaker activities of TN-GnRH cells (Oka 1995). From these data, it is suggested that neither Ca²⁺ nor Ca²⁺-dependent K⁺ current(s) are the primary component(s) that generate regular pacemaker potentials. On the other hand, it was also reported that TN-GnRH cells show different firing modes according to the physiological condition of the fish (regular beating, irregular, and burst firing mode) (Oka and Matsushima 1993). Therefore it may be possible that these Ca²⁺ and Ca²⁺-dependent K⁺ current(s) may be involved in the switching among different firing modes. Thus it may be another very important problem to examine the nature and functional significance of these currents.

TEA- and 4AP-resistant K⁺ currents

Persistent outward K⁺ current was still evoked from a holding potential of −100 mV in Na⁺- and Ca²⁺-free Ringer solution containing 0.75 μM TTX, 20 mM TEA, and 5 mM 4AP. The current activated at test potentials more positive than −30 mV. However, when the holding potential was changed to −60 mV, which is closer to the base membrane potential of TN-GnRH cells, the amplitude of these persistent currents was reduced, and the activation threshold of these currents was shifted to around −10 mV. Furthermore, the activation curve obtained from the current responses was fitted with a linear summation of two Boltzmann functions when a holding potential was −100 mV while it was fitted with a single Boltzmann function when the holding potential was changed to −60 mV. From these data, we conclude that at least two kinds of outward K⁺ currents that is resistant to 20 mM TEA and 5 mM 4AP are present in TN-GnRH cells. The activation threshold of one of them is relatively low (−40 mV), and that of the other one is relatively high (−20 mV). The voltage dependence of activation/steady-state inactivation and pharmacology of these currents are comparable with that of I_{KDRG} and I_{KDRG} in cultured dorsal root ganglion, respectively (Gold et al. 1996). Considering the membrane potential range during the pacemaker activities, we suggest that these currents should contribute very little, if any, to the repolarizing phase of subthreshold pacemaker potentials.

On the other hand, one might argue that these outward currents may belong to a kind of nonselective cation current that was reported in hippocampal pyramidal neurons (Oyama et al. 1991) and mitral cells of rat olfactory bulb (Wang et al. 1996). Alzheimer (1994) reported a similar nonselective cation current from rat neocortical neurons. The channel responsible for this current was not Ca²⁺ dependent, was resistant to block by 4AP and TEA (<35 mM), and was permeable to monovalent cations but not to anions, such as Cl⁻. If the TEA- and 4AP-resistant currents of TN-GnRH cells belonged to a kind of nonselective cation currents, deactivating tail current should be inward, when the membrane potentials are depolarized from depolarizing test potentials to −60 mV. However, in some experiments with the P4 leak subtraction protocol, TEA- and 4AP-resistant currents showed outward deactivating tail cur-
rent on membrane repolarization to \(-60\) mV (data not shown). Thus TN-GnRH cells may not have such nonselective cation current. Another possibility that may not be completely excluded is that some, if not all, TEA- and 4AP-resistant currents can be regarded as residual K\(^+\) outward currents that failed to be blocked by 20 mM or higher concentrations of TEA. This is because of the fact that the activation threshold of TEA- and 4AP-resistant current evoked from a holding potential of \(-100\) mV is similar to the activation threshold of TEA-sensitive current. However, this current should be negligible because higher concentration of TEA (\(\leq 60\) mM) did not cause further blockade of TEA- and 4AP-resistant currents (Fig. 4A).

**Functional significance of K\(^+\) currents**

We previously proposed a hypothesis that may be relevant to the peptidergic neuromodulatory system of vertebrate brains in general; the modulator neurons have endogenous rhythmic activities that vary according to the animal’s physiological (hormonal or environmental) conditions, and they regulate the excitability of target neurons in a wide variety of brain regions simultaneously (Oka 1992a, 1997; Oka and Matsushima 1993). TN-GnRH cells mainly exhibit regular beating discharge activities (pacemaker activities), and a TTX-resistant persistent Na\(^+\) current, \(I_{\text{Na(slow)}}\) supplies the persistent depolarizing drive. Therefore \(I_{\text{Na(slow)}}\) contributes to the depolarizing phase of the pacemaker potentials (Oka 1995, 1996). We assumed that an interplay between the persistent inward current and counteracting outward current should generate basic rhythmic pacemaker activities. Therefore we studied the outward K\(^+\) currents and examined which type of K\(^+\) currents are involved in the generation of pacemaker potentials.

We identified four types of voltage-dependent K\(^+\) currents and concluded that the TEA-sensitive K\(^+\) current is the most likely candidate that contributes to the repolarizing phase of pacemaker potentials, based on the voltage- and current-clamp studies. It is suggested that \(I_{\text{Na(slow)}}\) and TEA-sensitive K\(^+\) current interact in the following manner to generate the subthreshold pacemaker potentials. When the TN-GnRH cells are at a negative potential the \(I_{\text{Na(slow)}}\) is deinactivated and supplies the persistent depolarizing drive, and the membrane potentials gradually depolarizes. When the membrane potential reaches the activation threshold for the TEA-sensitive K\(^+\) current, outward current gradually develops and the net flux of current reverses. Then the membrane potential becomes hyperpolarized and deactivates the K\(^+\) current, and the next cycle begins. Thus the pacemaker mechanism in TN-GnRH cells is different from those reported in other pacemaker cells where interaction between the Ca\(^{2+}\) current and Ca\(^{2+}\)-dependent K\(^+\) current was suggested (see review by Connor 1985).

In our hypothetical model on the peptidergic and monoaminergic modulatory neurons, it was suggested that the frequency of intrinsic pacemaker activities or the firing mode may change according to the physiological or environmental conditions, and the change may be the neural basis for long-lasting changes in animal behavior (Oka 1992a,b, 1997; Oka and Matsushima 1993). Here it was further suggested that ionic channels underlying the pacemaker activities may be the target for modulation by hormones or transmitters. In fact, we recently found that certain transmitters or hormones modify the frequency of pacemaker activities in TN-GnRH cells (unpublished observations). For example, in the bag-cell neurons of *Aplysia*, a set of command neurons that trigger egg laying, an elevation of cyclic AMP by exogenous application of an analog induces a period of high-frequency burst discharges lasting up to one-half hour in normally quiescent cells of the intact ganglion (Kaczmarek et al. 1978). Voltage-clamp studies on the bag-cell neurons in culture demonstrated a modulation (significant diminution) by cAMP analogs of TEA-sensitive “delayed rectifier” K\(^+\) currents (Connor 1985; Strong and Kaczmarek 1985). Thus it would be an interesting future problem to study possible modulation of TEA-sensitive K\(^+\) currents (or other current) by various hormones or transmitters.

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