Properties and Function of Low- and High-Voltage-Activated Ca^{2+} Channels in Hypoglossal Motoneurons

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Calcium influx through voltage-gated Ca^{2+} channels plays an important role in neuronal function. In a thin-slice preparation of neonatal rat hypoglossal motoneurons (HMs) we recorded Ba^{2+} currents through voltage-gated Ca^{2+} channels using the whole-cell configuration of the patch-clamp technique. We found that HMs have low-voltage-activated (LVA) and at least three types of high-voltage-activated (HVA) Ca^{2+} channels (ω -Aga-IVA sensitive, ω -CgTx sensitive, and dihydropyridine sensitive), based on pharmacological and voltage-dependent properties. Of the Ca^{2+} current activated at 0 mV from a holding potential of -70 mV, approximately one-half was ω -Aga-IVA (200 nM) sensitive, one-third was ω -CgTx (3 μM) sensitive, whereas only 6% was DHP (nimodipine; 10 μM) sensitive. The residual current, after applying these three antagonists, had characteristics of LVA Ca^{2+} currents.

Based on this pharmacology we found that Ca^{2+} entry during a single action potential (AP) through LVA Ca^{2+} channels has a different role from Ca^{2+} entry through HVA Ca^{2+} channels. Ca^{2+} influx through ω -Aga-IVA-sensitive and ω -CgTx-sensitive HVA Ca^{2+} channels activates Ca^{2+} -activated K^{+} channels responsible for the AP afterhyperpolarization. On the other hand, Ca^{2+} entry through LVA Ca^{2+} channels is responsible for spike afterdepolarization and provides Ca^{2+} for the Ca^{2+} -activated K^{+} channels that contribute to AP repolarization.

[Key words: ω -agatoxin-IVA, ω -conotoxin-GVIA, calcium-activated potassium channel, action potential, afterhyperpolarization, ADP, development]

The presence of both low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca^{2+} currents has been demonstrated in a variety of motoneurons (McCobb et al., 1989; Berger and Takahashi, 1990; Mynlieff and Beam, 1992a; Viana et al., 1993a). Recent evidence suggests that in neurons there is considerable diversity in the Ca^{2+} channel subtypes that are responsible for these currents (Bean, 1989; Catterall, 1991; Tsien et al., 1991). Little information is known about the distribution and function of these subtypes in motoneurons. The primary purpose of the

present study was to determine the contribution of the various Ca^{2+} channel subtypes to the total Ca^{2+} current in neonatal hypoglossal motoneurons (HMs) and to investigate their function in neuronal behavior.

In some central neurons LVA Ca^{2+} channels are believed to be inactivated at resting membrane potential and deinactivated by hyperpolarization (Llinas, 1988; White et al., 1989; von Krosigk et al., 1993). For this reason, a role for LVA Ca^{2+} channels in neuronal function, such as burst firing, involves excursions of membrane potential negative to resting potential in order to remove inactivation (Crunelli et al., 1989; White et al., 1989; Viana et al., 1993a). In contrast, it has been reported that LVA Ca^{2+} channels can contribute significantly to the amount of Ca^{2+} entry during a short-duration depolarization, as during the action potential (AP) (McCobb and Beam, 1991; Scroggs and Fox, 1992). Thus, Ca^{2+} entry during the AP through LVA channels may contribute to the AP and spike afterpotential trajectories, either directly or through Ca^{2+} -activated channels.

Two types of Ca^{2+} -activated K^{+} channels, one with fast kinetics (e.g., BK) and one with slower kinetics (SK), contribute to AP repolarization and afterhyperpolarization (AHP) (Blatz and Magleby, 1987; Lancaster and Nicoll, 1987; Storm, 1987; Morita and Barrett, 1990; Takahashi, 1990). The question of whether Ca^{2+} influx through different types of Ca^{2+} channels contribute differentially to the activation of various types of Ca^{2+} -activated K^{+} channels in motoneurons is not known. In the present study, this question was investigated in HMs.

Selective blocking of LVA Ca^{2+} channels in central neurons to assess their function appears not to be feasible because there are no specific and potent antagonists of these channels. Amiloride, known as a specific antagonist of LVA Ca^{2+} channels in peripheral neurons (Tang et al., 1988; Scroggs and Fox, 1992), is not as effective in central neurons (Mogul and Fox, 1991; Viana et al., 1993a). Alternatively, a strategy to investigate the function of LVA Ca^{2+} channels is first to block that portion of the total Ca^{2+} current arising from HVA Ca^{2+} channels. There are specific and potent antagonists to various HVA Ca^{2+} channel subtypes: dihydropyridines (DHP) (L-channel), ω -agatoxin-IVA (ω -Aga-IVA) (P-channel), and ω -conotoxin-GVIA (ω -CgTx) (N-channel). Using these three antagonists, most HVA Ca^{2+} channels have been shown to be blocked (Hess, 1990; Mintz et al., 1992). During application of all three antagonists, the dominant Ca^{2+} influx by depolarization may be through LVA Ca^{2+} channels, although an HVA Ca^{2+} channel resistant to these three antagonists is present in some neurons (Ellinor et al., 1993).

In the present study we determined the distribution and function of various HVA Ca^{2+} channel subtypes: L, P, and N. In addition, using the three antagonists to block Ca^{2+} entry through HVA Ca^{2+} channels, we compared the role of Ca^{2+} entry through

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Figure 1. Effect of holding potentials on Ca^{2+} currents. *A*, Peak currents plotted against test potential. Currents elicited by depolarizing steps (50 msec) from holding potentials of -80 mV (solid diamonds) and -50 mV (open diamonds). *B*, Activation and steady-state inactivation relationships. Voltage dependence of activation was determined from analysis of tail currents obtained after returning from each test potential (open diamonds). Data were fitted with a Boltzmann relationship (solid lines; see Materials and Methods). Steady-state inactivation at different holding potentials was determined at both -40 mV (solid circles) and 0 mV (open circles) test potentials. The solid line on the data from -40 mV test potential is a line fitted with a Boltzmann relationship. Data from a test potential of 0 mV are simply connected with a line. Values and error bars are means $\pm$ standard errors. *C* and *D*, Sample traces of currents from two holding potentials to 0 mV (*C*) and to -40 mV (*D*).

residual Ca^{2+} channels (presumably involving mostly LVA Ca^{2+} channels) with that in the absence of Ca^{2+} entry (zero Ca^{2+} in external solution) on HM function.

Materials and Methods

Preparation. Procedures for whole-cell recording were essentially identical to those described previously (Edwards et al., 1989; Takahashi, 1992; Viana et al., 1993a). Briefly, neonatal male and female Sprague-Dawley rat pups (3–6-d-old) were anesthetized by hypothermia and rapidly decapitated. The brainstem was isolated and cut in 120–130 μm transverse sections with a vibrating slicer (DSK-1000, Dosaka Co. Ltd., Kyoto, Japan). Slices were incubated at 37°C for at least 1 hr and then maintained at room temperature while being superfused with a bicarbonate buffer solution (see below).

Recording. Brainstem slices were viewed with Nomarski optics and HMs were visually identified by their location within the hypoglossal motor nucleus and by their size and shape. The surface of an identified HM was cleaned with a saline jet stream (Edwards et al., 1989). Patch electrodes were pulled from borosilicate glass capillaries (Clark, UK) to a DC resistance of $5\text{ M}\Omega$. Whole-cell recordings were made with an EPC-7 patch-clamp amplifier (List, Darmstadt, Germany) at room temperature (23 – 25°C). Currents elicited by depolarizing pulses, applied every 20 sec, were filtered at 2 kHz with a 4-pole, low-pass Bessel filter (Cornerstone, Dagan) and analyzed using pCLAMP software (Axon Instruments). Series resistance, usually less than $20\text{ M}\Omega$, was compensated 60–70%. Leak currents were subtracted on line using a $P/4$ protocol. Membrane capacitance was determined from the compensation circuit of the EPC-7 amplifier. Liquid junction potential, measured in symmetrical and recording solutions, was less than 1 mV.

To determine voltage dependence of Ca^{2+} current activation, Ca^{2+} currents were elicited using 20-msec-duration depolarizing pulses to different potentials from a holding potential of -70 mV. Ca^{2+} currents were measured 0.4 msec after the end of the test pulse (as a tail current) and normalized to the maximum tail current (usually at a test potential of 0 mV). To measure steady-state inactivation, the membrane potential was held at different potentials for 20 sec, and Ca^{2+} currents were elicited by a 20 msec duration depolarizing pulse (typically to 0 mV). The peak Ca^{2+} currents during the depolarizing pulse were normalized to the

maximum current. Activation and inactivation data were fitted to Boltzmann relationships, $I/I_{\text{max}} = 1/(1 + \exp((V_{1/2} - V)/k))$ for activation and $I/I_{\text{max}} = 1/(1 + \exp((V - V_{1/2})/k))$ for inactivation. Time constants of inactivation were calculated by fitting the Ca^{2+} current data with a single exponential function and assuming that at infinite time the current fully inactivated.

Solutions. The ionic composition of the solution used for preparation of slices was (mM) NaCl, 130; NaHCO_3 , 26; NaH_2PO_4 , 1.25; KCl, 3; glucose, 10; CaCl_2 , 1; and MgCl_2 , 5. The solution used to maintain slices was similar in composition but CaCl_2 was raised to 2 mM, MgCl_2 was reduced to 2 mM, and lactic acid (4 mM) (Takahashi, 1992) was added. For current-clamp recording the external solution contained (mM) NaCl, 140; HEPES, 10; CaCl_2 , 2; MgCl_2 , 2; and glucose, 10 (pH = 7.4 titrated using NaOH); and the internal solution contained KCl, 17.5; K-glucuronate, 122.5; HEPES, 10; EGTA, 0.2; NaCl, 9; and MgCl_2 , 1 (pH = 7.2 titrated using KOH). In Ca^{2+} -free conditions equimolar amounts of MgCl_2 were substituted for CaCl_2 . To measure Ba^{2+} current through Ca^{2+} channels, the external solution contained NaCl, 140; HEPES, 10; BaCl_2 , 2; MgCl_2 , 2; glucose, 10; and TTX, 0.001 (pH = 7.4 titrated using NaOH); and the internal solution contained CsMeSO_4 , 100; TEA-Cl, 30; MgCl_2 , 1; CaCl_2 , 0.5; NaCl, 5; HEPES, 10; EGTA, 10; ATP-Mg, 3; and GTP-Tris, 0.3 (pH = 7.2 titrated using TEA-OH). All inward currents elicited by depolarization were abolished by replacing BaCl_2 in external solution with MgCl_2 or by adding 0.1 mM CdCl_2 to the external solution.

Drugs. Stock solutions of ω -CgTx, 48 μM (Calbiochem), and ω -Agar-IVA, 3.2 μM (Peptide Institute, Inc., Osaka, Japan), were prepared in recording solutions containing 0.4% cytochrome *c* and stored at -20°C . Toxins were applied to the bath after stopping perfusion and the concentration was calculated based on the volume of the chamber (0.4 ml). *S*(-)-BayK-8644 and nimodipine (Research Biochemicals Inc.) were dissolved in DMSO or ethanol and stored at -20°C . The final concentration of vehicle in the perfusing solutions was less than 0.01%, and the same concentration of vehicle alone had no effect on Ca^{2+} currents.

Results

Whole-cell Ba^{2+} currents through Ca^{2+} channels were recorded from HMs using the patch-clamp technique. Ba^{2+} was used as

Figure 2. Effect of Ca^{2+} current antagonists. **A**, Effect of amiloride (1 mM) on the LVA Ca^{2+} current. Currents were elicited by depolarizing pulses to -40 mV from a holding potential of -70 mV (20 msec duration, applied every 20 sec). Peak current amplitude was plotted against time. **B**, Effect of nimodipine (10 μM). Currents were elicited by depolarizing pulses to 0 mV from a holding potential of -70 mV (20 msec duration, applied every 20 sec) and mean current amplitude between 6 and 8 msec after the start of the depolarization was plotted against time (**B–D**). **C** and **D**, The effect of increasing doses of $\omega\text{-CgTx}$ (**C**) and $\omega\text{-Aga-IVA}$ (**D**). Antagonists were applied to the bath after stopping perfusion. *Insets* show sample traces of currents in control condition and in the peak response of each concentration of the antagonist. Results were from different neurons. Calibration: **A**, 50 pA, 5 msec; **B–D**, 500 pA, 5 msec.

a charge carrier to minimize Ca^{2+} current rundown. In this condition, Ba^{2+} currents were stable up to 30 min at a holding potential of -70 mV. Figure 1*A* shows the current–voltage (I – V) relationship from two holding potentials. From the more negative holding potential (-80 mV), the evoked currents were larger at all potentials. The threshold for current activation and the potential at which the peak current was measured were more hyperpolarized when activated from -80 mV than from -50 mV. In addition, the Ca^{2+} current elicited at 0 mV from a holding potential of -80 mV showed somewhat greater inactivation than did that from -50 mV. For example, as shown in Figure 1*C*, from a holding potential of -80 mV, the time constant of inactivation was 0.4 sec compared to 1.3 sec from a holding potential of -50 mV. Thus, the Ca^{2+} current elicited at 0 mV has at least two components. One component is relatively insensitive to holding potential and is less inactivated during the test pulse, whereas the other is sensitive to holding potential and shows greater inactivation.

To characterize further Ca^{2+} currents in HMs, we determined the voltage dependence of activation and steady-state inactivation (Fig. 1*B*). The activation curve was determined from the tail currents at -70 mV following depolarizing steps to various potentials from a holding potential of -70 mV. The Ca^{2+} current began to activate at -40 mV. The half-activation voltage of the Ca^{2+} current was -16.8 ± 1.6 mV with a slope factor of 4.5 ± 0.5 mV (mean $\pm$ SEM; Fig. 1*B*, diamonds; $n = 4$). To determine the voltage dependence of steady-state inactivation, Ca^{2+} currents were activated from different holding potentials. Holding potentials positive to -30 mV could not be tested because of rapid rundown of Ca^{2+} current at depolarized potentials. Figure 1*B* shows the holding potential-dependent in-

activation of Ca^{2+} current at a test potential of 0 mV (open circles), and this inactivation is discussed below.

LVA Ca^{2+} current was isolated based on the voltage dependency of activation and inactivation. Ca^{2+} currents elicited at -40 mV from two holding potentials are shown in Figure 1*D*. From a holding potential of -80 mV, the activation of Ca^{2+} current was relatively slow and it inactivated during the depolarization. At -40 mV from a holding potential of -70 mV, the mean time to peak was 19.3 ± 4.6 msec and time constant of the inactivation was 30.2 ± 2.5 msec ($n = 5$), while at 0 mV these were 6.7 ± 0.7 msec and 730 ± 203 msec ($n = 5$), respectively. This current was almost completely inactivated at a holding potential of -50 mV. Currents activated at -40 mV from different holding potentials were measured to determine steady-state inactivation. The half-inactivation potential was -61.6 ± 1.8 mV with slope factor of 7.5 mV (Fig. 1*B*, solid circles; $n = 4$). The half-inactivation voltage measured at a -30 mV test potential was similar (-63.5 ± 2.3 mV, $n = 4$) to that at -40 mV test potential. It was clear that Ca^{2+} currents elicited at -30 mV and -40 mV test potentials had an inactivation that was much more sensitive to holding potential than the currents activated at 0 mV (compare open and solid circles in Fig. 1*B*). Thus, kinetics, activation potential, and sensitivity to holding potential of Ca^{2+} current activated at -40 mV are similar to LVA Ca^{2+} currents measured in various neurons (Berger and Takahashi, 1990; Chen and Hess, 1990; Swandulla et al., 1991; Viana et al., 1993a).

To characterize Ca^{2+} currents further, we tested the effects of various Ca^{2+} channel antagonists. Amiloride inhibits LVA Ca^{2+} current in peripheral neurons (Tang et al., 1988; Scroggs and Fox, 1992). However, it was not very effective in mammalian

Figure 3. Effect of sequential application of three antagonists on HVA current. The antagonists were applied in the order shown in *A*. ω -Aga-IVA (as a final concentration of 200 nM) was applied directly to the chamber after perfusion was stopped (indicated by the lower bar). Nimodipine (10 μ M) was applied in the bath after perfusion was started again, and ω -CgTx (3 μ M) was applied to the chamber when perfusion was again stopped. *B* shows sample traces of currents. Letters correspond to the time points in *A*.

CNS neurons (Mogul and Fox, 1991; Viana et al., 1993a). We tested the effect of amiloride (1 mM) on LVA Ca^{2+} current in HMs using Ba^{2+} as a charge carrier. We used the higher concentration of amiloride than the saturating concentration in peripheral neurons (500 μ M; Tang et al., 1988). Currents elicited at -40 mV from a holding potential of -70 mV were inhibited reversibly by amiloride and the percentage of amiloride-sensitive current was $27.6 \pm 12.8\%$ ($n = 4$; Fig. 2*A*). While T-type Ca^{2+} channels in peripheral neurons are sensitive to amiloride, it is possible that multiple subtypes of Ca^{2+} channels are responsible for LVA Ca^{2+} current in HMs (Huguenard and Prince, 1992; Soong et al., 1993). As the effect of amiloride was only 27.6%, we conclude that amiloride did not block LVA Ca^{2+} current effectively in HMs.

To test the contribution of L-type channels to total Ca^{2+} current, we performed experiments using DHP antagonists and agonists. HMs were held at -70 mV and Ca^{2+} currents were elicited by depolarizing pulses to 0 mV (Fig. 2*B*). Nimodipine (10 μ M) blocked only $6.6 \pm 1.7\%$ ($n = 19$) of total Ca^{2+} current. We also tested for a voltage-dependent block of nimodipine from a -50 mV holding potential (data not shown). At this potential, the rundown of Ca^{2+} current was faster than from a holding potential of -70 mV (at -50 mV the rundown time constant = 38 ± 3 min, $n = 3$). The effect of nimodipine increased somewhat at this more negative holding potential ($10.9 \pm 3.7\%$, $n = 3$), but this was not significantly different. It should be noted that DHP-sensitive channels have been reported to be

rather insensitive to holding potential (Hess, 1990). In our case, the amplitude of Ca^{2+} current elicited from a holding potential of -50 mV is about 80% of that from a holding potential of -70 mV. Therefore, the difference in the effect of nimodipine at the different holding potentials may be due to a preferential inactivation of the DHP-resistant component of Ca^{2+} current. In any case, the DHP-sensitive component of the total current at 0 mV is relatively minor. This conclusion is further supported by the finding that the DHP agonist *s*(-)-BayK-8644 (2 μ M), potentiated the current by only $3.8 \pm 3.8\%$ (data not shown, $n = 5$). As the nimodipine-sensitive current was small, we could not readily determine a saturating dose of nimodipine. In the following experiments we used a concentration of 10 μ M nimodipine, since this appears to be a saturating dose in other neural systems (McCarthy and TanPiengco, 1992; Sayer et al., 1993).

Next we tested the effect of the P-type and N-type Ca^{2+} channel antagonists ω -Aga-IVA and ω -CgTx, respectively. To determine the saturating concentrations of these peptide antagonists, increasing concentrations of each were applied to HMs (Fig. 2*C,D*). The effect of either antagonist was irreversible. A substantial decrease in Ca^{2+} current was found with 40 nM ω -Aga-IVA; increasing the concentration to 80 nM caused a further but smaller decrease in the Ca^{2+} current. Further increase in concentration to 120 nM caused no additional reduction in the current. Thus, 80 nM ω -Aga-IVA appeared to be a saturating concentration. Following a similar protocol, we found that the

Figure 4. Voltage dependency and I - V relationship of the effect of three antagonists. *A*, Percentage of antagonist-sensitive currents at both -40 mV and 0 mV test potentials are shown. Holding potential was -70 mV. Error bars indicate SE. *B*, I - V relationship of the currents during sequential application of the antagonists in one HM. The holding potential was -70 mV and peak current amplitude was plotted against test potential.

Figure 5. Voltage-dependent activation and steady-state inactivation of current resistant to three HVA Ca^{2+} channel antagonists (ω -Aga-IVA, 200 nM; ω -CgTx, 3 μM ; nimodipine, 10 μM). *Aa*, Currents were elicited at test potentials indicated from a holding potential of -70 mV. *Ab*, Steady-state inactivation of residual current. Currents were elicited at 0 mV test potential from the indicated holding potentials. *B*, Activation curve (diamonds) was obtained from tail currents, and steady-state inactivation curves (-30 mV, solid circles; 0 mV, open circles) were obtained from peak current amplitudes. Current amplitude was normalized to peak current. Lines represent the Boltzmann relationships that were fitted to these data. Values and error bars are means $\pm$ SEs.

effect of ω -CgTx saturated at 0.3 μM (Fig. 2C). Increasing concentration up to 3 μM caused no additional reduction in the Ca^{2+} current.

To rule out possible nonspecific blocking by the two peptide antagonists at concentrations of 400 nM ω -Aga-IVA and 3 μM ω -CgTx, we compared the fraction of total current sensitive to each by alternating the order of antagonist application. When applied first, the percentage of ω -Aga-IVA-sensitive current was $45.6 \pm 4.4\%$ ($n = 10$). After ω -CgTx, the percentage of ω -Aga-IVA-sensitive current was $50.4 \pm 7.0\%$ ($n = 4$). These values were not significantly different. Similarly, the percentage of ω -CgTx-sensitive current was not different whether applied before ω -Aga-IVA ($29.3 \pm 6.4\%$, $n = 4$) or after ω -Aga-IVA ($29.1 \pm 4.3\%$, $n = 10$). Therefore, we conclude there was no significant cross-reaction between these two antagonists, and that each antagonist targets a distinct type of Ca^{2+} channel in HMs. In the following experiments, ω -CgTx and ω -Aga-IVA were applied at 3 μM and 200 nM, respectively.

Figure 3 shows the effect of sequential application of ω -Aga-IVA, nimodipine, and ω -CgTx on the Ca^{2+} current elicited at 0 mV. In this HM, 38% of Ca^{2+} current was sensitive to ω -Aga-IVA, 37% to ω -CgTx, 3.8% to nimodipine, and the residual

Ca^{2+} current following application of all three antagonists was 21.5%. Note that the residual current showed inactivation during the depolarizing test pulse (Fig. 3B, trace d).

Figure 4 shows the average effect of the antagonists on the current from pooled data. Figure 4A (open bars) shows that for a test potential of 0 mV from a holding potential of -70 mV, the percentage of Ca^{2+} current sensitive to each antagonist was, for ω -Aga-IVA, $49.4 \pm 4.5\%$ ($n = 13$); ω -CgTx, $29.4 \pm 2.9\%$ ($n = 19$); and nimodipine, $6.6 \pm 1.7\%$ ($n = 19$). We found that $24.1 \pm 4.2\%$ ($n = 8$) of the Ca^{2+} current was resistant to these antagonists. Figure 4A (solid bars) also shows the fraction of antagonist-sensitive current at a test potential of -40 mV (i.e., mostly LVA). Here, the ω -Aga-IVA-sensitive current was $5.5 \pm 4.7\%$ ($n = 6$), the ω -CgTx-sensitive current was $4.4 \pm 2.9\%$ ($n = 7$), and the nimodipine-sensitive current was $13.1 \pm 4.8\%$ ($n = 8$). Therefore, most of the Ca^{2+} current at -40 mV ($85.5 \pm 6.3\%$, $n = 5$) was resistant to these three antagonists.

Figure 4B shows that the effect of the antagonists began at -40 mV. The percentage of the Ca^{2+} current resistant to the HVA Ca^{2+} channel antagonists decreased with the activation of HVA Ca^{2+} current and became constant at potentials positive to the maximum activation potential of the total Ca^{2+} current. The effect of nimodipine (as a percentage) was constant at different potentials, while the effect of ω -CgTx and ω -Aga-IVA increased with the activation of HVA Ca^{2+} current. In some neurons the LVA Ca^{2+} current has been reported to be sensitive to DHP and ω -CgTx (McCleskey et al., 1987; Akaike et al., 1989). If nimodipine blocked HVA Ca^{2+} current and had no effect on the LVA Ca^{2+} current, the percentage of the sensitive current would increase with activation of HVA Ca^{2+} current. The constant effect of nimodipine through different potentials suggests that some LVA Ca^{2+} channels in HMs may be sensitive to nimodipine (Fig. 4). In contrast, the effect of ω -CgTx and ω -Aga-IVA began at -40 mV and increased in parallel with the activation of HVA Ca^{2+} current. Thus, it is unlikely that LVA Ca^{2+} channels were sensitive to ω -CgTx and ω -Aga-IVA.

In HMs, 24.1% of Ca^{2+} current at a test potential of 0 mV was resistant to application of the three HVA Ca^{2+} channel antagonists. We believe that a major portion of this residual current is LVA Ca^{2+} current. To investigate this, we characterized the residual current by its kinetics and voltage dependency. Characteristics of this residual current were similar to those of LVA Ca^{2+} currents observed in other cells (Akaike et al., 1989; Chen and Hess, 1990). The kinetics of activation were voltage dependent, becoming faster at more positive potentials. From a holding potential of -70 mV, the time to peak was 13.9 ± 3.2 msec at -40 mV, while it was 8.5 ± 1.1 msec at 0 mV test potential ($n = 5$). The residual currents inactivated during depolarization and the inactivation was faster than that of total Ca^{2+} current (LVA plus HVA) observed in the absence of the antagonists (Fig. 5A). From a holding potential of -70 mV, the time constant of inactivation at 0 mV was 730 ± 203 msec ($n = 5$) in control condition and was 36.1 ± 10.1 msec in the presence of the antagonists ($p < 0.005$, $n = 5$). Figure 5B shows voltage dependence of activation and steady-state inactivation of this current. The half-activation voltage of the residual current was -28.8 ± 0.3 mV (Fig. 5B, diamonds; $n = 3$), which is significantly more hyperpolarized (by 12 mV) than that of the control Ca^{2+} current (control, -16.8 ± 1.6 mV; $p < 0.01$, $n = 4$). The half-inactivation voltage at a -30 mV test potential was -70.1 ± 1.4 mV (Fig. 5B, solid circles; $n = 4$), and this was significantly more negative than that observed for a test

Figure 6. Developmental changes in membrane capacitance (C_m , open circles), total Ca^{2+} current density (solid circles), and percentage of resistant Ca^{2+} current (diamonds). Resistant current was measured after application of all three HVA Ca^{2+} channel antagonists. Numbers in parentheses are the number of HMs at each postnatal age. Stars indicate significant differences in the percentage of resistant current between 3- and 4-d-old rats. Currents were measured by stepping to a 0 mV test potential from a holding potential of -70 mV. Values and error bars are means $\pm$ SEs.

potential of -40 mV in control conditions (-61.6 ± 1.8 mV, $p < 0.01$). This difference in the half-inactivation voltages suggests some contamination of LVA current by HVA Ca^{2+} current in control conditions at the -40 mV test potential. Half-inactivation voltage of the residual current to 0 mV test potential was -62.0 ± 1.9 mV (Fig. 5B, open circles; $n = 5$), and was significantly more depolarized than that at a -30 mV test potential ($p < 0.05$). This may indicate that HMs have HVA Ca^{2+} current components that are insensitive to the three antagonists we used. Nevertheless, since the residual current activates at more hyperpolarized potentials, and because inactivation is more steeply dependent on holding potential, we conclude that most of this residual current is the LVA Ca^{2+} current.

In chick and mouse motoneurons it has been reported that the density of LVA Ca^{2+} channels decreases with development, while the density of HVA Ca^{2+} channels increases (McCobb et al., 1989; Mynlieff and Beam, 1992b). If this were also true for rat HMs, and the dominant component of the residual current is LVA Ca^{2+} current, then the residual current density should also decrease with postnatal development. To test this possibility, we compared the fraction of the total Ca^{2+} current that is residual Ca^{2+} current in 3- to 6-d-old rats (Fig. 6). We found no significant change in the membrane capacitance during this period. As membrane capacitance is proportional to the surface area of a neuron, this result shows that HMs exhibit little change in size during this period (Fig. 6, open circles), which is consistent with other studies of the postnatal maturation of HMs (He et al., 1992; Viana et al., 1994). During this same period we observed that although the density of total Ca^{2+} current did not change (Fig. 6, filled circles), the fraction of residual current decreased. The fraction of residual Ca^{2+} current at 3 d old was significantly higher ($35.6 \pm 1.4\%$, $n = 4$) than that at 4 d old

Figure 7. Effects of HVA channel antagonists and 0 Ca^{2+} on AP, ADP, and AHP recorded in current clamp. Traces in A and B were determined in control external solution (see Materials and Methods), after application of all three HVA Ca^{2+} channel antagonists (ω -Aga-IVA, 200 nM; ω -CgTx, 3 μM ; nimodipine, 10 μM) and then with zero Ca^{2+} in the external solution. A and B have different voltage and time scales, and all traces begin at -60 mV. In A, single sweeps are illustrated, while in B, each trace is average of 15 sweeps.

(16.5 ± 3.2 , $n = 5$, $p < 0.01$) and 6 d old (9.5 ± 2.5 , $n = 4$, $p < 0.001$). This developmental change in residual current supports the idea that this current is mostly Ca^{2+} LVA current. Further, the fraction of LVA Ca^{2+} current appears to decline during this period (3- to 6-d-old), and since the total current density did not change, the density of HVA Ca^{2+} current must have increased.

To evaluate the contribution to the AP and spike afterpotentials of the various Ca^{2+} current components, we studied HMs in current-clamp conditions (Takahashi, 1990). The membrane potential was held at -60 mV and APs were elicited by brief injected current pulses (5 msec duration) (Fig. 7). The AP is followed by an after depolarization (ADP) and an AHP. As previously described, this AHP has also been called the medium-duration AHP (Viana et al., 1993b). The AHP was attenuated by either ω -CgTx (3 μM , $n = 4$) or ω -Aga-IVA (200 nM, $n = 3$), but not by nimodipine (10 μM , $n = 3$), when each was applied independently (data not shown). Since Ca^{2+} -activated K^{+} channels contribute to the AHP, and this K^{+} channel is dependent on intracellular Ca^{2+} , the absence of an effect of nimodipine on the AHP may reflect the minor contribution that L-type channels make to Ca^{2+} influx during the AP. Figure 7B shows that simultaneous application of nimodipine, ω -CgTx and ω -Aga-IVA almost completely abolished the AHP ($n = 6$). Figure 7A shows that the duration of AP was unchanged after application of these three antagonists ($n = 5$). Action potential duration increased after eliminating Ca^{2+} from the external solution, indicating that Ca^{2+} -activated K^{+} currents are involved in spike repolarization ($n = 4$ of 5 HMs) (Fig. 7A). Note that the ADP, which increased in amplitude following application of the three antagonists (perhaps due to attenuation of the AHP, $n = 4$) (Fig. 7B), disappeared in Ca^{2+} -free solution ($n = 4$), indicating that the ADP is clearly dependent on Ca^{2+} influx. These results indicate that the Ca^{2+} -activated K^{+} currents that contribute to AP repolarization and to the AHP depend on Ca^{2+} entry through different types of Ca^{2+} channels. The AHP is dependent on Ca^{2+} influx through HVA Ca^{2+} channels sensitive to ω -CgTx and ω -Aga-IVA. On the other hand, the conductance responsible for the ADP and Ca^{2+} -activated K^{+} channels underlying spike repolarization is dependent primarily on Ca^{2+} influx through Ca^{2+} channels resistant to the three HVA Ca^{2+} channel antagonists.

Discussion

Percentage of antagonist-sensitive Ca^{2+} current

Neurons in the CNS have multiple types of Ca^{2+} channels. Previously, our laboratory has shown that neonatal HMs have both LVA and HVA Ca^{2+} channels, and that Ca^{2+} influx through Ca^{2+} channels plays an important role in the behavior of this neuron (Viana et al., 1993a,b). In the present study, we have identified at least four Ca^{2+} channel types in HMs. Based on their voltage dependence of activation and inactivation, the total Ca^{2+} current can be separated into LVA and HVA Ca^{2+} components. Of the total Ca^{2+} current, about half is sensitive to ω -Aga-IVA, 29% is sensitive to ω -CgTx, and only about 6% is DHP sensitive. This pharmacological distribution is similar to that found in dissociated rat spinal cord interneurons (Mintz et al., 1992), which has a somewhat higher fraction of DHP-sensitive channels and lower fraction of antagonist-resistant current.

We have good evidence that much of the residual (antagonist-resistant) current was carried by LVA Ca^{2+} channels, and we found that expression of LVA Ca^{2+} channels in HMs decreases with postnatal development. Consistent with these latter results, our laboratory has reported that the incidence of burst firing was highest among HMs just at or after birth (P0) and disappeared by P10 and the LVA current was shown to contribute to the burst firing (Viana et al., 1993a). A similar developmentally related reduction in LVA Ca^{2+} current has also been observed in rat hippocampal neurons and chick and mouse spinal motoneurons (McCobb et al., 1989; Thompson and Wong, 1991; Mynlieff and Beam, 1992b).

Dominance of LVA Ca^{2+} current in the resistant current

The half-inactivation voltage (around -62 mV) of the LVA Ca^{2+} current in control conditions was more positive than that observed in Purkinje cells (Regan, 1991), hippocampal neurons (Mogul and Fox, 1991), and thalamic neurons (Huguenard and Prince, 1992). In those neurons, the half-inactivation voltage is more negative than -80 mV. On the other hand, in mouse motoneurons and rat DRG neurons the half-inactivation voltages (-46 and -68 mV, respectively) are more positive (Mynlieff and Beam, 1992a; Scroggs and Fox, 1992). In our case, the half-inactivation voltage of LVA Ca^{2+} current in control conditions was around -60 mV at both -40 mV and -30 mV test potentials. One possible explanation for these differences among various neurons is a molecular diversity in LVA Ca^{2+} channels. Distinct isoforms of LVA Ca^{2+} channels can be expressed in various tissues and neurons. For example, in thalamic neurons, two LVA Ca^{2+} currents have been isolated (Huguenard and Prince, 1992). A recently discovered rat brain Ca^{2+} channel α_1 subunit has characteristics similar to a subset of LVA Ca^{2+} channels, but it was resistant to amiloride (Soong et al., 1993). The weak effect of amiloride on LVA Ca^{2+} current in central neurons compared with peripheral neurons (Tang et al., 1988; Mogul and Fox, 1991; Scroggs and Fox, 1992; Viana et al., 1993a) further suggests the presence of distinct isoforms of these channels.

It is possible that the activation of HVA Ca^{2+} channels shifts the voltage dependency of steady-state inactivation in a positive direction. In HMs, 14.5% of the Ca^{2+} current activated at -40 mV was sensitive to HVA Ca^{2+} channel antagonists. Following blockade of HVA Ca^{2+} current by antagonists, the half-inactivation voltage at a test potential of -30 mV was -70 mV. This

difference in half-inactivation voltage supports the positive shift of steady-state inactivation by the activation of HVA Ca^{2+} channels. We used Ba^{2+} as a charge carrier, which may shift the activation of HVA Ca^{2+} channels to a more negative potential than when Ca^{2+} is used (Mynlieff and Beam, 1992a). It is also possible that the charge carrier itself changes the voltage dependency of LVA Ca^{2+} channels and causes shifts in the activation and inactivation relationships. In any case, a half-inactivation voltage of -70 mV during pharmacological blockade of HVA Ca^{2+} currents is most likely the half-inactivation voltage of the LVA Ca^{2+} current in HMs.

In the presence of HVA Ca^{2+} channel antagonists, the half-inactivation voltage is more positive at a 0 mV than that at a -30 mV test potential. One possibility for this voltage dependency of inactivation is that HMs possess HVA Ca^{2+} channels that are resistant to the three antagonists used. In cerebellar granular cells, there is a type of HVA Ca^{2+} channel that is insensitive to these three antagonists (Ellinor et al., 1993). Another possibility is the facilitation of LVA Ca^{2+} channels by large depolarizations. For example, DHP-sensitive and ω -CgTx-sensitive Ca^{2+} currents can be facilitated by depolarization (Artalejo et al., 1991; Ikeda, 1991). The same mechanism might take place for the LVA Ca^{2+} current. If LVA Ca^{2+} channels can open directly from the inactivated state, acceleration of activation may be greater at more positive potentials; as a consequence, more Ca^{2+} current would be activated at 0 mV than at -30 mV test potential (Chen and Hess, 1990). As a result, the steady-state inactivation curve could shift to more depolarized potentials.

Differential contribution of HVA and LVA Ca^{2+} currents to Ca^{2+} -activated K^+ channels

It is highly probable that HMs have two distinct types of Ca^{2+} -activated K^+ channels (Viana et al., 1993a,b); one is TEA sensitive and similar to the so-called BK channel, and the other is apamin sensitive and similar to the so-called SK channel (Blatz and Magleby, 1987; Rudy, 1988). Our data show that Ca^{2+} entry through LVA Ca^{2+} channels probably activates different Ca^{2+} -activated K^+ channels from the Ca^{2+} entering via HVA channels. Ca^{2+} -activated K^+ channels responsible for the AHP depend on Ca^{2+} entry through HVA Ca^{2+} channels that are sensitive to ω -CgTx or ω -Aga-IVA. The Ca^{2+} -activated K^+ channel involved in spike repolarization depends mostly on Ca^{2+} entry through LVA Ca^{2+} channels. One possible explanation of this difference is a differential sensitivity of the relevant K^+ channels to internal Ca^{2+} concentration. In this case, the rise in Ca^{2+} concentration by influx through LVA Ca^{2+} channels is high enough to activate BK channels but not SK channels. However, this is unlikely because BK channels are actually less sensitive to internal Ca^{2+} concentration than SK channels (Rudy, 1988). Another possibility is that HVA Ca^{2+} channels are distributed in close proximity to SK channels, and LVA Ca^{2+} channels are distributed close to BK channels. There is evidence for a differential distribution of Ca^{2+} channels in neurons and a close spatial association between Ca^{2+} channels and Ca^{2+} -activated K^+ channels (Robitaille and Charlton, 1992; Westenbroek et al., 1992).

It is also possible that the voltage dependency of Ca^{2+} channel activation can also explain this difference. Since HVA Ca^{2+} channels activate mostly at positive potentials, Ca^{2+} enters through HVA channels during the repolarizing phase and after repolarization (Llinas et al., 1981). The activation of LVA chan-

nels begins earlier than HVA channels because LVA channels have a lower threshold of activation. During the rising phase of the AP, the membrane potential is more negative than the Ca^{2+} reversal potential and Ca^{2+} enters through LVA channels. This Ca^{2+} activates BK channels, which in turn contribute to AP repolarization. The activation of BK channels depends on both intracellular Ca^{2+} concentration and membrane potential. Thus, BK channels could open in lower intracellular Ca^{2+} at depolarized potentials than at hyperpolarized potentials (Barrett et al., 1982), and this voltage-dependent activation is appropriate for BK channels to contribute to AP repolarization.

The conductance responsible for the ADP in HMs could not be determined in these experiments. In rat dorsal root ganglion neurons, the conductance responsible for the ADP is LVA Ca^{2+} current (White et al., 1989). In neonatal HMs, Viana showed that the ADP amplitude is strongly dependent on holding potential and suggested that this indicated that an LVA Ca^{2+} current may be involved in its generation (Viana et al., 1993a). Also, that study showed that the ADP depends on Ca^{2+} influx and was not blocked by substituting external Ca^{2+} by Ba^{2+} . Since Ba^{2+} passes through Ca^{2+} channels but does not activate intracellular Ca^{2+} -dependent processes, it is unlikely that a calcium-dependent process is responsible for the ADP. Thus, it is possible that divalent ion movement itself is responsible for the ADP in HMs.

In summary, our results show that there are at least four types of Ca^{2+} channels in HMs (three types of HVA Ca^{2+} channels— ω -Aga-IVA-sensitive, ω -CgTx-sensitive, and DHP-sensitive channels—and an LVA Ca^{2+} channel). In the absence of these three HVA Ca^{2+} channels during pharmacological blockade, the LVA Ca^{2+} channel is the dominant channel. While Ca^{2+} -activated K^{+} channels responsible for the AHP depend on Ca^{2+} influx through HVA Ca^{2+} channels, the conductance responsible for the ADP and the Ca^{2+} -activated K^{+} channels contributing to spike repolarization depends, for the most part, on Ca^{2+} entry through LVA Ca^{2+} channels. The half-inactivation voltage at a 0 mV test potential for this LVA Ca^{2+} current was between -60 and -70 mV, and this potential is close to the resting membrane potential of HMs (Viana et al., 1993a,b). A small change in membrane potential around rest can result in large changes in steady-state inactivation of the LVA Ca^{2+} current, since the steady-state inactivation exhibits a steep voltage dependence in this region. Therefore, LVA Ca^{2+} current can have a significant role in Ca^{2+} influx during the AP, and its function is different from that of the HVA Ca^{2+} channels.

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