Role of Calcium Conductances on Spike Afterpotentials in Rat Trigeminal Motoneurons

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Kobayashi, Masayuki, Tomio Inoue, Ryuji Matsuou, Yuji Masuda, Osamu Hidaka, Youngnam Kang, and Toshifumi Morimoto. Role of calcium conductances on spike afterpotentials in rat trigeminal motoneurons. J. Neurophysiol. 77: 3273–3283, 1997. Intracellular recordings were obtained from rat trigeminal motoneurons in slice preparations to investigate the role of calcium conductances in the depolarizing and hyperpolarizing spike after-potential (ADP and mAHP, respectively). The mAHP was suppressed by bath application of 1 µM apamin, 2 mM Mn2+, and 2 mM Co2+, and also by intracellular injection of ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetracetic acid (EGTA), suggesting that the potassium conductance generating the mAHP is activated by Ca2+ influx. Mn2+ (2 mM) or Cd2+ (500 µM) reduced the ADP, whereas the ADP amplitude was increased by raising extracellular Ca2+ concentration from 2 to 8 mM by bath application of Ba2+ (0.5–5 mM) and by intracellular injection of EGTA. This would suggest that Ca2+ itself is likely to be the charge carrier generating the ADP. Focal application of ω-conotoxin GVIA (10–30 µM) suppressed the mAHP and enhanced the ADP, whereas focal application of ω-agatoxin IVA (10–100 µM) reduced the ADP amplitude without apparent effects on the mAHP. We conclude that Ca2+ influx through ω-agatoxin IVA–sensitive calcium channels is at least in part responsible for the generation of the ADP and that Ca2+ influx through ω-conotoxin GVIA–sensitive calcium channels contribute to the generation of the mAHP. Because of the selective suppression of the ADP and mAHP by ω-agatoxin IVA and ω-conotoxin GVIA, respectively, it is suggested that both calcium channels are separated geometrically in rat trigeminal motoneurons.

INTRODUCTION

The action potential is followed by afterdepolarization (ADP) and medium-duration afterhyperpolarization (mAHP) in spinal (Granit et al. 1963; Harada and Takahashi 1983; Walton and Fulton 1986), hypoglossal (Viana et al. 1993b), and guinea pig trigeminal (Chandler et al. 1994) motoneurons. Additional spikes can be triggered from the ADP when it is enhanced (Viana et al. 1993a), whereas the mAHP is an important factor for slowing the rate of firing (Hille 1992). Thus the potential balance between the ADP and mAHP seems to determine the firing pattern of trigeminal motoneurons (TMNs) and affects the quantitative relationship between the input to TMNs and the final motor output of the muscles.

Voltage-gated calcium channels have been classified into at least five types referred to as the L, N, P, Q, and T types (Bean 1989; Hess 1990; Linas et al. 1989; Mintz et al. 1992; Randall and Tsien 1995), and multiple types of calcium channels coexist in a single neuron (Bean 1989; Miller 1987). It may be possible that different types of calcium channels participate in distinct physiological functions (Christie et al. 1995). Contribution of calcium currents to generating the ADP has been reported in spinal (Harada and Takahashi 1983; Walton and Fulton 1986) and hypoglossal (Umemiya and Berger 1994; Viana et al. 1993a) motoneurons. Furthermore, calcium-activated potassium current has been demonstrated to be involved in generating the mAHP in guinea pig TMNs (Chandler et al. 1994) as well as other motoneurons (Barrett and Barrett 1976; Nishimura et al. 1989; Sah and McLachlan 1992; Viana et al. 1993b; Walton and Fulton 1986; Zhang and Krnjevic 1987). Therefore Ca2+ influx may cause both the ADP and mAHP simultaneously. However, it is not clear whether the generation of the ADP and the mAHP were differentially regulated by distinct types of calcium channels in single neurons.

In the present study, differential regulations of the ADP and mAHP were investigated in rat TMN in vitro slice preparations by the use of the intracellular recording method. We demonstrate that Ca2+ influx through ω-agatoxin IVA (ω-Aga-IVA)–sensitive channels is responsible for the generation of the ADP, whereas Ca2+ influx through ω-conotoxin GVIA (ω-CTx-GVIA)–sensitive calcium channels activates calcium-activated potassium currents responsible for the mAHP. These results suggest that each specific type of calcium conductance may participate in distinct physiological functions in TMNs. Preliminary results of this study were reported previously in abstract form (Kobayashi et al. 1995).

METHODS

Subjects

Seventy-nine Sprague-Dawley rats (3–6 wk old) were used for slice preparations. To identify the recorded cell as a TMN histologically, we employed the fluorescence double-labeling technique (cf. Viana et al. 1990) in the first series of the experiments (20 of the 79 animals). Those 20 animals were anesthetized with ketamine HCl (150 mg/kg im) and chlorpromazine HCl (12.5 mg/kg im), and both sides of the masseter nerve were dissected. A small amount of dextran-tetramethylodamine-lysale (DRL; molecular weight 10,000, Molecular Probes) in crystal was applied to the central cut ends of the nerve for 30 min, and then the cut ends were rinsed with saline. After 2–5 days the 20 animals were reanesthetized for slice preparation. Biocytin (Sigma), with which the recording microelectrode had been filled, was injected into the recorded neurons (n = 40 neurons), and biocytin-injected neurons...
were visualized with fluorescein isothiocyanate (FITC). Of the 40 neurons, 15 were also labeled by DRL (Fig. 1), indicating that these neurons were masseter motoneurons. The remaining 25 neurons were also visualized with horseradish peroxidase (HRP), and all of the 25 neurons were located within the trigeminal motor nucleus. It is most likely that the neurons within the trigeminal motor nucleus are TMNs, because interneurons are very few in the trigeminal motor nucleus (Sessle 1977). Therefore no histological efforts were made to identify the recorded neurons in the second series of experiments (n = 108 neurons).

Slice preparation

Animals were anesthetized with ketamine HCl (150 mg/kg im) after an injection of chlorpromazine HCl (12.5 mg/kg im). After decapitation, the skull was removed and the brain was excised rapidly. The brain was placed in cold modified artificial cerebrospinal fluid (M-ACSF; see below for composition) and sectioned at the intercollicular level and at the obex. The rostral side of the brain stem was glued onto the stage of a microslicer (DTK-1500, Datsaka) with cyanoacrylate. Transverse slices (450 μm) were cut in cold M-ACSF. The slices were transferred to a holding chamber containing normal artificial cerebrospinal fluid (N-ACSF; see below for composition) maintained at room temperature.

Solutions

The composition of the N-ACSF was (in mM) 130 NaCl, 3 KCl, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, and 10 d-glucose. The M-ACSF was made from N-ACSF by replacing 130 mM NaCl with 260 mM sucrose. The N-ACSF and the M-ACSF were bubbled with a mixture of 95% O₂-5% CO₂, and the pH of these solutions was 7.35-7.40. Viable motoneurons were obtained by the use of M-ACSF during slice preparation (Aghajanian and Rasmussen 1989; Chandler et al. 1994). In some experiments the Ca²⁺ concentration was raised to 6-8 mM. When Mn²⁺, Co²⁺, Ba²⁺, or Cd²⁺ was added to the ACSF, 2 mM CaCl₂ was replaced with 2 mM MnCl₂, CoCl₂, or BaCl₂, or 500 μM CdCl₂, respectively, and NaH₂PO₄ was omitted to avoid precipitation. For anion substitution experiments, Cl⁻ was replaced with equimolar amounts of isethionate and the bath was grounded through an agar-KCl bridge. The following drugs were added directly to the perfusate: tetrodotoxin (1 μM) (Wako), tetraethylammonium chloride (TEA, 1-10 mM replacing equimolar NaCl), 4-amino-pyridine (4-AP, 0.5 mM), charybdotoxin (ChTX, 10-30 nM) (Peptide), NiCl₂ (0.5 mM), and nifedipine (10-20 μM dissolved in absolute ethanol). Apamin (10-20 μM) (Peptide), ω-CTX-GVIA (10-50 μM) (Peptide), and ω-Aga-IVA (10-100 μM) (Peptide) were each dissolved in N-ACSF and applied to the surface of a slice by pressure as microdroplets from a micropipette. All drugs were obtained from Nakarai tesque (Kyoto, Japan) unless otherwise specified.

Recording

After 2-12 h of incubation in the holding chamber, slices were transferred to an interface-type chamber. The recording chamber was continuously perfused with N-ACSF at a rate of 1-1.5 ml/min, and humidified 95% O₂-5% CO₂ flowed over the slice. All experiments were performed at 32 ± 1°C. Intracellular recordings were obtained with glass microelectrodes (1.2-1.5 mm OD) (Sutter Instruments). In the first series of experiments, the microelectrode was filled with 1% biocytin in 1 M KCl and 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.6) for intracellular labeling. In the second series of experiments, the microelectrode was filled with 1 M KCl and 0.05 M Tris buffer (pH 7.6). For intracellular injection of ethylene glycol-bis(β-aminoethylether)-N,N',N'′,N''-tetraacetic acid (EGTA) (Sigma), a calcium chelator, the microelectrode was filled with 0.25 M EGTA and 1 M KCl. The DC resistance of the microelectrodes ranged from 30 to 130 MΩ. The amplifier, an Axoclamp 2B (Axon Instruments), was used in either bridge or discontinuous current-clamp mode. During discontinuous current-clamp recordings, a 2- to 5-kHz sampling rate was employed at a 30% duty cycle. The head stage output was monitored on a separate oscilloscope to ensure proper capacitance adjustment and adequate settling of the microelectrode. Membrane potential and current were digitized and stored on a computer hard disk with the use of software (Clampex, Axon Instruments) through an A-D converter.

Data are presented as means ± SE. Comparisons of data before and after the drug application were based on Student’s paired t-tests. The Mann-Whitney U test was used when the difference of the variances was great. The Friedman test was performed for comparison of data on the amplitude of ADPs during repetitive firing and after the drug application. The level of P < 0.05 was assumed as significant.

Histology

After recordings, the slices obtained from the DRL-injected animals were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 12-24 h, and were then transferred to 25% sucrose in 0.02 M phosphate-buffered saline for 12-24 h. The slices were frozen, sectioned at 25-30 μm with a cryostat, and thaw-mounted onto poly-L-lysine-subbed glass slide. The sections were incubated with FITC-conjugated streptavidin (1:100) (Amersham) for 10
min, examined with a fluorescein microscope equipped with an appropriate excitation filter (B2 filter for FITC; G filter for DRL), and photographed. To examine whether bicuculline-injected neurons were localized in the trigeminal motor nucleus, sections were re- cubated with HRP-conjugated streptavidin (1:500) (Dako) for 60 min at room temperature, and HRP was developed with 3,3'-diaminobenzidine and 0.003% H2O2 in 0.5 M Tris-HCl buffer (pH 7.4) with nickel ammonium sulfate intensification. Sections were counterstained with neutral red.

RESULTS

General properties

Through the stereomicroscope, we could see the trigeminal motor nucleus as a pale gray area medial to the trigeminal sensory nucleus. We placed the recording electrode in this area. The present study is based on recordings from 148 neurons that had stable resting potentials more negative than −55 mV (−67.2 ± 0.5 mV; n = 148) and displayed spikes >70 mV (measured from resting membrane potential to the spike peak). Input resistance, calculated from the relation- ship between injected current intensity (−0.2 to −0.5 nA) and the steady-state (>200 ms) voltage response, was 13.4 ± 0.7 MΩ (n = 141). Rheobase, measured as the mini- mum current (duration 300 ms) necessary to evoke a single action potential 100% of the time in response to at least five consecutive stimuli, was 1.5 ± 0.1 nA (n = 94). No neurons fired spontaneously. Single action potentials were elicited by an injection of brief (2- to 3-ms) depolarizing current pulses. Action potential amplitude was 94.8 ± 0.8 mV (n = 148), and duration was 0.7 ± 0.01 ms (n = 148; measured 10 mV positive to threshold).

Action potentials were followed by a biphasic afterhyper- polarization (AHP), consisting of the fast AHP (fAHP) and mAHP as shown in Fig. 2A. The mAHP amplitude, mea- sured from resting potential to the most negative peak (Fig. 2A, open arrowhead), was 4.7 ± 0.2 mV (n = 148). The half-decay time of the mAHP was 25.4 ± 0.7 ms (n = 148). In 132 (89%) of the 148 neurons, these two AHPs were separated by a depolarizing waveform, the ADP. The ampli- tude of the ADP, measured from the resting potential to its peak (Fig. 2A, filled arrowhead), was 10.6 ± 0.5 mV (n = 132). In response to injection of subthreshold depolarizing current pulses, the ADP was rarely observed without genera- tion of action potentials. The remaining 16 neurons did not display a prominent ADP between the fAHP and the mAHP, and were not included in the analysis of the ADP. As shown in Fig. 2B, there is a significant negative correlation between the amplitude of the mAHP and that of the ADP (r = −0.56, P < 0.001).

Ionic basis for the action potential

Bath application of 1 and 10 mM TEA invariably in- creased the spike duration by 118 ± 19% (n = 6) and 497 ± 66% (n = 5), respectively, without affecting rising phase of the action potential (Fig. 2C). By contrast, 1 mM TEA increased the amplitude and the half-decay time of the mAHP by 66 ± 17% (n = 6) and 23 ± 6% (n = 6), respectively, whereas 10 mM TEA reduced the mAHP ampli- tude by 34 ± 5% in four of five neurons tested and pro- longed the half-decay time of the mAHP by 32 ±12% (n = 5). These effects of TEA were reversible. Similarly to the effect of 1 mM TEA, bath application of 500 μM 4-AP increased the spike duration in all neurons tested by 141 ± 10% (n = 4) without affecting rising phase of the action potential (Fig. 2D). However, we could not analyze the effect of 4-AP on the ADP and mAHP because of a substan- tial increase in spontaneous synaptic potentials. No consist- tent effects on the resting membrane potential were observed after 4-AP application. These results suggest that TEA-sensi- tive, voltage-dependent, delayed-rectifier-type potassium currents and 4-AP-sensitive currents are involved in generat- ing spike repolarization in rat TMNs as has been reported in TMNs of the guinea pig (Chandler et al. 1994), facial motoneurons (Nishimura et al. 1989), and hypoglossal moto- neurons (Viana et al. 1993b).

Calcium-activated potassium channels have been sug- gested to be involved in the generation of spike repolarization in rat spinal (Takahashi 1990), hypoglossal (Umemiya and Berger 1994), and vagal (Sah and McLachlan 1992) motoneurons. To examine the extent to which calcium-acti- vated potassium currents are involved in spike repolariza- tion, effects of apamin, a selective blocker for small-conduc- tance calcium-activated potassium channels (SK channels) (Blatz and Magleby 1986; Pennefather et al. 1985), or ChTX, a selective blocker for large-conductance calcium-activated potassium channels (Blatz and Magleby 1987; La- torre et al. 1989), were examined. Microdroplet application of 10–20 μM apamin to the surface of the slice or bath application of 10–30 nM ChTX had almost no effects on the spike duration (Fig. 3, insets in A and B), suggesting that calcium-activated potassium channels are not essential in spike repolarization of TMNs in young adult rats. A similar conclusion has been reached in TMNs (Chandler et al. 1994; Kim and Chandler 1995) and facial motoneurons (Nishimura et al. 1989) of the guinea pig.

Ionic basis for the mAHP and ADP

To determine the calcium dependence of the mAHP, the following experiments were performed. First, replacement of extracellular Ca2+ concentration ([Ca2+]o; 2 mM) with the inorganic calcium channel blocker Mn2+ (2 mM) and Ca2+ (2 mM) reduced the mAHP amplitude by 88 ± 8% (n = 5) and 92 ± 4% (n = 5), respectively, 30–60 min after substitution (Fig. 4A). Second, an intracellular injection of EGTA also depressed the mAHP. In five neurons tested, the mAHP amplitude was invariably and completely suppressed 10–40 min after impalement of the microelectrode con- taining 0.25 M EGTA into the neuron (Fig. 4B). Third, application of apamin to the surface of the slice (10–20 μM, n = 15) or to the perfusate (0.5 or 1 μM, n = 2) almost completely depressed the mAHP in all the neurons tested. As shown in Fig. 3A, the mAHP amplitude was reduced by 98 ± 2% (n = 17) without any appreciable changes in the height and duration of action potential and input resistance. On the other hand, bath application of ChTX (10–30 nM) had little effect on the ADP and mAHP (n = 8; Fig. 3B). These results indicate that the mAHP is calcium dependent and that SK channels are likely responsible for generating the mAHP.

Calcium dependence of the ADP was also examined.
To quantitatively evaluate the amplitude of the ADP that is generated in association with action potentials, the membrane potential response evoked by a short pulse with an intensity just below the threshold to elicit the action potentials was digitally subtracted from the threshold response with an action potential (Fig. 5, A and B). The effect of apamin on the ADP was evaluated by the use of this digitally calculated waveform in the 15 neurons in which apamin decreased the mAHP amplitude by >90%. Apamin application did not change the peak potential level and the peak time of the fAHP in the 15 neurons examined, but delayed the peak time of the ADP by 0.6 ± 0.1 ms (n = 15; Fig. 5B). Therefore the ADP amplitude was measured at the respective peak times of ADPs in control conditions...
FIG. 4. Calcium-dependent generation of mAHP and ADP. A: superimposed traces of action potentials before and after bath application of 2 mM Mn²⁺ (replacing extracellular Ca²⁺ with Mn²⁺). mAHP is blocked by application of Mn²⁺. Inset: ADP is also suppressed by application of Mn²⁺. Dotted line: resting membrane potential (−64 mV). B: progressive suppression of mAHP and progressive enhancement of ADP are observed with ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)-containing electrode. Resting membrane potential: −68 mV (dotted line). Ca: superimposed traces of action potentials before and after bath application of 1 and 2 mM Ba²⁺ (0 Ca²⁺). Suppression of mAHP and enhancement of ADP are observed. After application of 2 mM Ba²⁺, an additional spike was triggered from ADP. Resting membrane potential: −75 mV (dotted line). Cb: injection of short (2-ms) depolarizing current pulse induces burst firing emerging from an enhanced ADP in same neuron as in Ca after bath application of 5 mM Ba²⁺ (0 Ca²⁺). Resting membrane potential: −75 mV (dotted line). With increasing extracellular Ba²⁺ concentration ([Ba²⁺]₀), ADP progressively augments, leading to a generation of a burst firing.

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After apamin application, the ADP amplitude at the former and latter times increased by 1.0 ± 0.3 mV (n = 15; 12 ± 4% of control) and 2.3 ± 0.3 mV (n = 15; 32 ± 6% of control), respectively. These results suggest that apamin-sensitive potassium conductances responsible for the generation of the mAHP become active slightly before the ADP peak and become maximum around the peak of the mAHP. Therefore the ADP is likely to be curtailed by the early phase of the mAHP. In later analysis, the ADP amplitude was measured at the time of the ADP peak in control by the use of such digitally calculated waveform. Then effects of Mn²⁺, Cd²⁺, or high-Ca²⁺ Ringer solution on the ADP were investigated after suppression of the mAHP by apamin. These inorganic calcium channel blockers invariably reduced the ADP amplitude by 42 ± 9% (n = 6) in the presence of apamin, without any appreciable changes in the peak time of the ADP (Fig. 5C). Raising [Ca²⁺]₀ from 2 to 6–8 mM invariably increased the ADP amplitude by 25 ± 10% in the presence of apamin (n = 3; Fig. 5D). These results indicate that the currents underlying the ADP are at least in part calcium dependent. An intracellular injection of EGTA abolished the mAHP and also increased the ADP amplitude by 149 ± 54% (n = 5; Fig. 4B). In contrast to effects of apamin on the ADP, the peak potential of the fAHP was depolarized by EGTA. Furthermore, bath application of 0.5–5 mM Ba²⁺ with no added Ca²⁺ enhanced the ADP dose dependently (Fig. 4, Ca and Cb). Because Ba²⁺ blocks K⁺ channels (Hille 1992), bath application of Ba²⁺ might cause a plateau-type potential, as shown in Fig. 4Cb. However, 10 mM TEA and 500 μM 4-AP, which block many K⁺ channels, did not produce a plateau-type potential but prolonged and enhanced the mAHP in the present study. Furthermore, apamin did not produce any plateau-type potentials. Thus effects of Ba²⁺ may be different from those of K⁺ channel blockers but somewhat similar to those of intracellular injection of EGTA, in which the peak level of fAHP was commonly depolarized. Therefore it can be assumed that removal of Ca²⁺-dependent inactivation of Ca²⁺ currents by Ba²⁺ or EGTA might have caused enhancements of
ADP. These observations suggest that Ca\(^{2+}\) itself is likely to be the charge carrier generating the ADP, rather than to trigger a calcium-dependent process.

**Effects of selective calcium channel blockers on the ADP and mAHP**

We further examined the effects of selective blockers of high-voltage-activated (HVA) calcium currents on the ADP and mAHP. Microdroplet application of \(\omega\)-CTx-GVIA (10–30 \(\mu\)M), an N-type calcium channel blocker, strongly decreased the mAHP amplitude by 87 ± 9% \((n = 5)\), whereas \(\omega\)-CTx-GVIA increased the amplitude of both the fAHP and ADP by 21 ± 9% and 33 ± 5%, respectively \((n = 5; \text{Fig. 6A})\). Such an increment of the ADP by \(\omega\)-CTx-GVIA is significantly larger than that by apamin \((P < 0.01)\). On the other hand, \(\omega\)-Aga-IVA \((10–100 \mu\)M\), a potent blocker of P-type calcium channels, decreased the ADP amplitude by 37 ± 7% \((n = 5)\), but did not alter apparently the peak mAHP amplitude \((\text{Fig. 6B})\). We also applied \(\omega\)-Aga-IVA in combination with apamin. \(\omega\)-Aga-IVA \((100 \mu\)M\) reduced the ADP amplitude by 37 ± 11% \((n = 6)\) in the presence of apamin \((\text{Fig. 6C})\). Neither the ADP nor mAHP was apparently affected by nifedipine \((10–20 \mu\)M\), a selective blocker of L-type calcium channels \((n = 3; \text{data not shown})\).

We also examined the effect of blockers of low-voltage-activated calcium currents on the ADP and mAHP. Ni\(^{2+}\) has been reported to block low-voltage-activated calcium currents in spinal (McCobb et al. 1989) and hypoglossal (Viana et al. 1993a) motoneurons, although it has been shown to reduce HVA calcium currents as well (Randall and Tsien 1995; Zhang et al. 1993). Addition of 500 \(\mu\)M Ni\(^{2+}\) to perfusate containing 2 mM Ca\(^{2+}\) decreased the ADP amplitude by 37 ± 12%, whereas Ni\(^{2+}\) did not significantly affect the amplitude of the mAHP \((n = 4)\) \((\text{Fig. 6D})\). Next we examined the selective effects of Ni\(^{2+}\) on the ADP during blockade of the mAHP by apamin in five neurons. Ni\(^{2+}\) \((500 \mu\)M\) reduced the ADP amplitude in all of the five neurons by 33 ± 3% in the presence of apamin \((\text{Fig. 6E})\). Both \(\omega\)-Aga-IVA–sensitive and Ni\(^{2+}\)-sensitive currents were likely involved in the generation of the ADP, because effects of \(\omega\)-Aga-IVA and Ni\(^{2+}\) on the ADP were additive in the presence of apamin \((\text{Fig. 6F})\). However, there was a difference between the effects of Ni\(^{2+}\) and \(\omega\)-Aga-IVA on the ADP when applied during repetitive firing, as described in the next section.
Differential effects of calcium channel blockers on the spike afterpotentials during repetitive firing

Trains of spikes were elicited by intracellular injections of long (1-s) depolarizing current pulses (Fig. 7). The ADP was observed following individual action potentials. In 67 of 71 neurons examined, the amplitude of the first ADP (arrowhead) was larger than those of the remaining ADPs in spike trains (Fig. 7, A, left and B, left). An application of $\omega$-Aga-IVA hyperpolarized the peak potentials of the first and the remaining ADPs (Fig. 7A, compare left and right). On the other hand, $\text{Ni}^{2+}$ hyperpolarized the peak potentials of the first ADP but did not hyperpolarize that of the remaining ADPs markedly (Fig. 7B, compare left and right). The amplitude of ADPs measured from the baseline potential became smaller successively and reached a steady level by the 6th–10th ADPs. Because the peak level of the ADPs varied from spike train to spike train depending on the intensity of injected current pulses, the amplitude of respective ADPs was normalized to the mean amplitude of the 6th–10th ADPs and was plotted against the order of ADPs in respective spike trains obtained by injections of current pulses with varying intensities (Fig. 8A). The normalized amplitude of ADPs did not significantly differ in terms of the current intensity (Friedman test, $P < 0.001$). Thus, regardless of the current intensity, the profile of successive changes in the normalized amplitude of ADPs was almost stereotyped. In terms of the profile of successive changes in the normalized amplitude of the ADP, effects of $\omega$-Aga-IVA or $\text{Ni}^{2+}$ on individual ADPs in spike trains were further analyzed. Figure 8, B and C, shows the effects of $\omega$-Aga-IVA and $\text{Ni}^{2+}$, respectively. Open and filled circles indicate the mean of the normalized values before and after application of $\omega$-Aga-IVA, respectively. Open and filled triangles indicate those values before and after application of $\text{Ni}^{2+}$, respectively. The normalized amplitude of neither the first nor remaining ADPs changed after application of $\omega$-Aga-IVA, respectively. Open and filled triangles indicate those values before and after application of $\text{Ni}^{2+}$, respectively. The normalized amplitude of neither the first nor remaining ADPs changed after application of $\omega$-Aga-IVA (Fig. 8B). However, $\text{Ni}^{2+}$ significantly decreased the normalized amplitude of the first ADP (Mann-Whitney U-test, $P < 0.01$) without altering the normalized amplitude of remaining ADPs. These findings suggest that $\omega$-Aga-IVA attenuates all ADPs whereas $\text{Ni}^{2+}$ mainly decreases the first ADP.

**DISCUSSION**

**Calcium conductances underlying the mAHP and ADP**

Umemiya and Berger (1994) reported that the mAHP was attenuated by either $\omega$-CTx-GVIA or $\omega$-Aga-IVA but not by

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**Fig. 6.** Effects of selective calcium channel blockers on afterpotentials. A: superimposed traces of action potentials (truncated) before (Control) and after ($\omega$-CTx-GVIA) microdroplet application of $\omega$-conotoxin GVIA ($\omega$-CTx-GVIA) (20 $\mu$M) on surface of slice. Dotted line: resting membrane potential (−68 mV). B: superimposed traces of action potentials before (Control) and after ($\omega$-Aga-IVA) microdroplet application of 100 $\mu$M $\omega$-agatoxin IVA ($\omega$-Aga-IVA) (resting membrane potential: −65 mV). C: superimposed traces of action potentials before (Apamin) and after (Apamin + $\omega$-Aga-IVA) microdroplet application of 100 $\mu$M $\omega$-Aga-IVA in presence of apamin. Resting membrane potential: −65 mV. D: superimposed traces of action potentials before (Control) and after ($\text{Ni}^{2+}$/ bath application of 500 $\mu$M $\text{Ni}^{2+}$ (resting membrane potential: −68 mV). E: superimposed traces of action potentials before (Apamin) and after (Apamin + $\text{Ni}^{2+}$) bath application of 500 $\mu$M $\text{Ni}^{2+}$ in presence of apamin. Subthreshold membrane potential was subtracted digitally from action potentials.

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**Calcium conductances underlying the mAHP and ADP**

Umemiya and Berger (1994) reported that the mAHP was attenuated by either $\omega$-CTx-GVIA or $\omega$-Aga-IVA but not by
FIG. 7. Effects of ω-Aga-IVA and Ni\textsuperscript{2+} on individual ADPs during spike trains. A: trains of spikes before (left) and after (right) application of ω-Aga-IVA, in response to injections of 1-s depolarizing current pulses of 1.8 nA. After ω-Aga-IVA application, a depolarizing DC was injected to keep original membrane potential (bottom dotted line; −68 mV). B: trains of spikes before (left) and after (right) application of Ni\textsuperscript{2+}, in response to injections of 1-s depolarizing current pulses of 3.0 and 2.2 nA, respectively. After Ni\textsuperscript{2+} application, a hyperpolarizing DC was injected to keep original membrane potential (bottom dotted line; −69 mV). Arrowheads: 1st ADPs in spike trains. Top and middle dotted lines in A and B: peak level of 1st ADP and level of mean amplitude of 6th–10th ADPs in each spike trains, respectively. Note that both 1st and remaining ADPs were suppressed after ω-Aga-IVA application, whereas Ni\textsuperscript{2+} suppressed 1st ADP but had little effect on remaining ADPs.

FIG. 8. Effects of ω-Aga-IVA and Ni\textsuperscript{2+} on normalized amplitude of individual ADPs during repetitive firing. A: plots of normalized amplitude of ADP vs. order of ADPs in respective spike trains obtained by injections of current pulses with varying intensities (2.6–3.6 nA). Amplitude of individual ADPs was normalized to mean amplitude of 6th–10th ADPs. Note that profile of successive changes in normalized amplitude of ADPs was almost stereotyped regardless of current intensity. B and C: plots of mean normalized amplitude of ADP vs. order of ADPs before and after application of ω-Aga-IVA (B) or Ni\textsuperscript{2+} (C). Open and filled circles: mean normalized amplitude before and after application of ω-Aga-IVA, respectively. Open and filled triangles: values before and after application of Ni\textsuperscript{2+}, respectively. Each symbol and error bar indicate mean and SE values calculated from normalized amplitude obtained by current pulses with 6 different intensities of 1.5–2.0 nA (before ω-Aga-IVA application), 1.3–1.8 nA (after ω-Aga-IVA application), 2.4–3.4 nA (before Ni\textsuperscript{2+} application), and 2.0–3.0 nA (after Ni\textsuperscript{2+} application). Note that ω-Aga-IVA had little effect on profile of successive changes in normalized amplitude of ADPs, whereas Ni\textsuperscript{2+} altered that profile by reducing normalized amplitude of 1st ADP.
nemodipine in neonatal rat hypoglossal motoneurons. In the present study, \( \omega \)-CTx-GVIA almost abolished the mAHP and neither \( \omega \)-Aga-IVA (microdroplet application as high as 100 \( \mu \)M) nor nifedipine apparently affected the mAHP. Therefore N-type calcium channels are most likely responsible for activating the calcium-activated potassium conductance underlying the mAHP conductance in rat TMsNs. Because \( \omega \)-Aga-IVA reduced the ADP almost selectively without marked effects on the mAHP, the discrepancy of the effects of \( \omega \)-Aga-IVA on the mAHP between the two studies might not be due to the difference in the doses of \( \omega \)-Aga-IVA but might be related to age or origin of the motoneurons.

In the presence of apamin application, inorganic calcium channel blockers reduced the ADP amplitude and high-Ca\(^{2+}\) Ringer increased the ADP amplitude in the rat TMsNs. These results indicate that the currents underlying the ADP are at least in part calcium dependent. Such calcium dependence of the ADP is in accord with the results from motoneurons (Harada and Takahashi 1983; Viana et al. 1993a; Walton and Fulton 1986), cortical neurons (Connors et al. 1982; Higashi et al. 1993), thalamic neurons (Bal and McCormick 1993), and sympathetic preganglionic neurons (Yoshimura et al. 1987).

It has been also suggested that calcium-dependent nonsensitive cation currents (Bal and McCormick 1993; Swandulla and Lux 1985) or calcium-dependent chloride currents (Higashi et al. 1993; Sanchez-Vives and Gallego 1994) might be responsible for the ADP. However, Cl\(^-\) is less likely to be involved in generating the ADP, because reducing [Cl\(^-\)], from 140 to 15–75 mM had little effect on the ADP (n = 4, data not shown). Because chelation of intracellular free calcium by intracellular injection of EGTA and substitution of extracellular Ca\(^{2+}\) with Ba\(^{2+}\) did not decrease the ADP amplitude but increased it, it is likely that at least some fraction of the ADP is directly generated by calcium currents. The increment of the ADP by EGTA and Ba\(^{2+}\) is probably due to removal of the calcium-dependent inactivation of calcium current. However, it is possible that calcium-independent inward currents such as nonsensitive cation current and sodium current are also involved in producing the ADP (Alzheimer 1994; Azouz et al. 1996), because inorganic calcium channel blockers failed to abolish the ADP.

We tried to determine which type of specific calcium conductance contributes to the generation of the ADP. During repetitive firing in response to injection of long (1-s) depolarizing current pulses, the first ADP was larger than the remaining ADPs in the spike train, implying that some components of the ADP might be inactivating. Ni\(^{2+}\) largely suppressed the first ADP but had little effect on the remaining ADPs. These results suggest that Ni\(^{2+}\) -sensitive rapidly inactivating currents might be involved in the first ADP, but did not contribute to the generation of the remaining ADPs. In contrast to the effect of Ni\(^{2+}\), application of 10–100 \( \mu \)M \( \omega \)-Aga-IVA reduced not only the first ADP but also the remaining ADPs to the same extent during repetitive firing. Furthermore, \( \omega \)-Aga-IVA decreased the ADP even after application of apamin and Ni\(^{2+}\). Although this dose (10–100 \( \mu \)M) was relatively high, the effects of \( \omega \)-Aga-IVA did not seem to be nonspecific, because the mAHP appeared to be insensitive to \( \omega \)-Aga-IVA. Therefore it is likely that \( \omega \)-Aga-IVA–sensitive HVA calcium currents mainly contribute to the generation of calcium-dependent component of the ADP. Nevertheless, the mean peak value of the ADP was around –56 mV, lower than the threshold of the HVA calcium currents in other neurons (Hille 1992). There are two possible explanations for this finding. First, the calcium currents responsible for the generation of the ADP might occur in dendritic site and the potential might be attenuated during electrotonic spread from the dendritic site to the soma. Second, the intermediate threshold calcium currents, as has been observed in neurons that display pacemaker activity (Alonso and Llinas 1992; Kang and Kitai 1993; Onimaru et al. 1996), might be involved in generation of the ADP. In contrast to those studies, subthreshold depolarizing currents rarely evoked the ADP in the present study. Thus it is more likely that \( \omega \)-Aga-IVA–sensitive HVA calcium currents generated at the dendritic site are responsible for the ADP.

Differential contribution of calcium currents to the ADP and mAHP

\( \omega \)-CTx-GVIA reduced the mAHP and enhanced the ADP, whereas \( \omega \)-Aga-IVA reduced the ADP without marked effects on the mAHP. Thus calcium currents contributing to the ADP and mAHP could be different. However, two important questions now arise. Why didn’t \( \omega \)-Aga-IVA reduce the mAHP? Why didn’t \( \omega \)-CTx-GVIA reduce the ADP but enhance it?

It has been reported that distinct types of calcium channels are differentially distributed in neurons (Christie et al. 1995; Elliott et al. 1995), and that some types of calcium channels and calcium-activated potassium channels are located close to each other (Robitaille and Charlon 1992; Westenbrook et al. 1992). Because \( \omega \)-CTx-GVIA reduced the mAHP whereas \( \omega \)-Aga-IVA apparently did not reduce the mAHP in the present study, it is likely that SK channels are located closer to \( \omega \)-CTx-GVIA–sensitive calcium channels than to \( \omega \)-Aga-IVA–sensitive ones. Such colocalization of SK channels and \( \omega \)-CTx-GVIA–sensitive calcium channels was also suggested in hypoglossal (Viana et al. 1993a) and vagal (Sah 1995) motoneurons. If these channels are closely located, Ca\(^{2+}\) influx through \( \omega \)-CTx-GVIA–sensitive calcium channels during an action potential may activate the considerable number of SK channels. Because EGTA and Ba\(^{2+}\) enhanced the ADP markedly, \( \omega \)-Aga-IVA–sensitive calcium channels might inactivate calcium dependently. If this is the case, the amount of Ca\(^{2+}\) influx through \( \omega \)-Aga-IVA–sensitive channels in dendrites might be relatively small because of the inactivation. And the amount of intracellular Ca\(^{2+}\) of \( \omega \)-Aga-IVA–sensitive channels origin might become smaller in association with its diffusion through dendrites to somatic SK channels, or it might be buffered (Lancaster and Pennefather 1987) before activation of SK channels. Thus Ca\(^{2+}\) influx through \( \omega \)-Aga-IVA–sensitive channels might activate only a small number of SK channels. This may explain why \( \omega \)-Aga-IVA did not reduce the mAHP. And it is likely that both \( \omega \)-CTx-GVIA–sensitive and \( \omega \)-Aga-IVA–sensitive calcium channels may be geometrically segregated to some extent.

Because \( \omega \)-CTx-GVIA abolished the mAHP and enhanced the ADP, it is likely that \( \omega \)-CTx-GVIA indirectly increased the ADP amplitude by suppressing the mAHP, as was the
case with apamin. However, in contrast to the case of apamin, the peak level of the fAHP where mAHP is not involved was depolarized by $\omega$-CTX-GVIA (Fig. 6A) and the enhancement of the ADP by $\omega$-CTX-GVIA was larger than that of apamin. These observations indicate the presence of an additional cause. If Ca$^{2+}$ influx through $\omega$-CTX-GVIA–sensitive calcium channels contributes to the inactivation of $\omega$-Aga-IVA–sensitive calcium channels, application of $\omega$-CTX-GVIA may lead to direct enhancement of the ADP.

**Role of afterpotentials for firing patterns**

The suppression of the mAHP by calcium channel blockers or apamin greatly increased the firing frequency of repetitive firing (data not shown), as reported in other cranial motoneurons (Chandler et al. 1994; Nishimura et al. 1989; Sah and McLachlan 1992; Viana et al. 1993b), spinal motoneurons (Walton and Fulton 1986), and cortical neurons (Schwindt et al. 1988). When the ADP was enhanced by Ba$^{2+}$ application or by attenuation of the mAHP, additional action potentials could be triggered from the several ADPs in the initial phase of a train of firing in response to long depolarizing current pulses and the interval between the first and second spikes could be greatly decreased (data not shown). Thus repetitive firing patterns were greatly affected by alterations of the ADP and mAHP. Burke et al. (1970) demonstrated that the insertion of a single extra action potential in a low-frequency spike train of a spinal motoneuron can cause marked, long-lasting tension enhancement produced by the muscle fibers that are innervated by the motoneuron. Therefore not only the average firing rate of a motoneuron but also the pattern of firing seems to alter the amount of force produced by the muscle fibers. It has been also reported that some neuromodulators, such as serotonin and noradrenaline, affect the mAHP and/or the ADP in cranial and spinal motoneurons (Berger et al. 1992; Parkis et al. 1995; Takahashi and Berger 1990). It is likely that those neuromodulators may regulate changes in muscle tension by affecting these spike afterpotentials.

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