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GABAergic and serotonergic modulation of calcium currents in rat trigeminal motoneurons

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Abstract

We investigated the effects of a $GABA_B$ agonist baclofen, and serotonin, on the high voltage-activated Ca channel (HVACC) currents in trigeminal motoneurons. Immunohistochemical and reverse transcription-polymerization chain reaction (RT-PCR) studies demonstrated the expression of α_{1C}, α_{1B}, α_{1A}, and α_{1E} subunits in the trigeminal motoneurons, which form L-, N-, P/Q-, and R-type Ca channels, respectively. By use of specific Ca blockers, it was found that N-type (38%), P/Q-type (27%), L-type (16 %), and R-type Ca currents (19%) contribute to HVACC I_{Ba}. Baclofen inhibited HVACC I_{Ba} in the majority of trigeminal motoneurons tested ($n = 15$ out of 16), whereas serotonin only did in a small population ($n = 5$ out of 18). The I_{Ba} inhibition by baclofen and serotonin was associated with slowing of activation kinetics, relieved by strong prepulse, and prevented by *N*-ethylmaleimide (NEM), indicative of mediation of Gi/Go. These data provide evidence that GABAergic and serotonergic inputs to trigeminal motoneurons regulate neuronal activities through the inhibition of HVACC currents.

Keywords: Trigeminal motoneurons; High voltage-activated calcium channels; Reverse transcription-polymerization chain reaction; Serotonin; GABA

Calcium channels are of great importance in a wide variety of physiological functions including neurotransmitter release and neuronal excitability in the nervous system [1,2]. Neuronal Ca^{2+} channels have been classified on the basis of their electrophysiological properties into high voltage-activated Ca channels (HVACC), a class that includes L, N, P/Q-, and R-type, and low voltage-activated Ca channels (LVACC) or T-type Ca channels [3]. Recent advances in molecular biological approaches have elucidated that the subtypes of Ca channels result from expression of different pore-forming α_{1A} subunits. From these works, it was found that α_{1A} forms P/Q-type channel, α_{1A} N-type channel, and both α_{1C} and α_{1D} L-type, α_{1G}, α_{1H}, and α_{1I} T-type Ca channels, respectively [3]. However, it is still controversial if α_{1E} forms R-type channels in neurons [4,5].

The trigeminal motor nucleus contains motoneurons, the final output neurons of the brainstem responsible for orofacial motor function such as chewing, feeding, sucking, and speech. To date, most studies on these neurons were focused on characterization of their spontaneous discharge pattern or changes of discharge pattern in response to pharmacological or electrical stimulation onto other brain area or sensory receptors using intracellular or extracellular recording technique [6,7]. However, the ionic mechanisms controlling the motor behavior of trigeminal motoneurons are not understood completely [8–12]. In motor neurons, Ca channels are clearly involved in the release of transmitter from the axon at the neuromuscular junction (NMJ) [13,14], and Ca channels expressed in the soma and dendrites are involved in the control of firing properties

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[15]. In addition, it has been demonstrated that Ca influx through Ca channels, especially L-type, is critical for the rhythmic motor behavior through the mediation of *N*-methyl-D-aspartate (NMDA) oscillation in spinal motoneurons [16]. However, very little is known about modulation of Ca channels by neurotransmitters in the trigeminal motoneurons [8,10], although a number of studies investigated Ca channel present in motoneurons in other regions of the brainstem and in the spinal cord [17–20]. γ-Aminobutyric acid (GABA), one of the major inhibitory neurotransmitters in the central nervous system, is thought to act as a transmitter of the last order neurons projecting to the trigeminal motoneurons [21,22]. It has been also shown that trigeminal motoneurons receive serotonergic input [23] so that serotonin alters the excitability of trigeminal motoneurons [11,12,24].

In the present study, we therefore investigated the expression patterns of HVACC and if the activation of GABAergic and serotonergic neurons would modulate HVACC currents in trigeminal motoneurons.

Materials and methods

All procedures for animal were reviewed and approved by the Animal Care and Use Committee of the Seoul National University prior to the experiments.

Identification of trigeminal motor nucleus. The trigeminal motor nucleus was localized by retrograde labeling with a fluorescent dye, Evans blue. Sprague–Dawley rats (150–200 g) were lightly anesthetized with ether and 10% (w/v) Evans blue (10 μl) was injected into masseter muscle. After 48 h, the brainstem area was frozen-sectioned (14 μm thick) and trigeminal motor nucleus was identified under a fluorescent microscope (Fig. 1).

Immunohistochemistry. Rats anesthetized with pentobarbital were perfused transcardially with cold pre-fixative solution (4 °C) and 4% paraformaldehyde solution. Brainstem including trigeminal motor nucleus was excised, post-fixed in 4% paraformaldehyde for 1 h, then placed in 10%, 30%, and 50% sucrose for 1 day at each concentration, and then frozen-sectioned transversely (30 μm thick). LSAB (labeled streptavidin–biotin) Kit (K680, Dako, USA) was used to perform immunohistochemistry according to the manufacturer's instruction.

Isolation of trigeminal motoneurons. Trigeminal motoneurons were acutely isolated with modification of methods described previously [25]. Briefly, 5–10-days-old Sprague–Dawley rats were anesthetized with ether and the brainstem was rapidly removed. Transverse slices (300 μm thickness) were prepared using Vibratome (Technical Products International, St. Louis, MO) in ice-cold oxygenated artificial cerebrospinal fluid (aCSF; NaCl 126, KCl 3, NaH_2PO_4 1, $NaHCO_3$ 26.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5, and glucose 10, pH 7.4, 310 mosm) gassed with 95% O_2 and 5% CO_2. Slices were then enzymatically treated with 15 U/ml papain at 35 °C for 30 min, kept in a holding chamber containing aCSF bubbled with 95% O_2, 5% CO_2 for at least 1 h. When needed, trigeminal motor nucleus regions were micropunched under the dissecting microscope. The cells were then mechanically dissociated into single cells using a series of fire-polished glass pipettes.

Reverse transcription-polymerization chain reaction. Total RNA was prepared from trigeminal motoneurons using Trizol reagent (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript Preamplification System (Life Technologies) according to the manufacturer's instruction. The primers for PCR were designed for each α_1 calcium channel subunit—α_{1A}, α_{1B}, α_{1C}, and α_{1E} based on GenBank rat cDNA sequences. The primer sequences are as follows: $\alpha_{1A\text{-}S}$, 5′-cgtggtgagaaaatacgccaaa-3′; $\alpha_{1A\text{-}AS}$, 5′-agctggcgactcaccctggatg-3′; $\alpha_{1B\text{-}S}$, 5′-acgtcg tccgcaaatacgcta ag-3′; $\alpha_{1B\text{-}AS}$, 5′-atcacactgacgagcaggggatcttt-3′; $\alpha_{1C\text{-}S}$, 5′-tggctggaggtgac atcgagggagaa-3′; $\alpha_{1C\text{-}AS}$, 5′-atcgaacgtgctcctacgggtctgc a-3′; $\alpha_{1E\text{-}S}$, 5′-catt gtcaggaaatacgccaagaagct-3′; and $\alpha_{1E\text{-}AS}$, 5′-ttgttcatgaagcatgctcgatgcaa c-3′. PCRs with both cDNA from rat whole brain and water were run in parallel as positive and negative controls, respectively.

Electrophysiology. Whole cell patch clamp technique [26] was performed to record barium currents (I_{Ba}) from trigeminal motoneurons labeled with Evans blue. Pipette solution was composed of (mM): CsCl 100, $MgCl_2$ 1, Hepes 10, BAPTA 10, Mg-ATP 3.6, phosphocreatine 14, GTP 0.1, and creatine phosphokinase 50 U/ml, adjusted to pH 7.4 with CsOH. Extracellular solution contained (mM): tetraethylammonium chloride (TEACl) 151, Hepes 10, $BaCl_2$ 5, $MgCl_2$ 1, and glucose 10, adjusted to pH 7.4 with TEAOH. The I_{Ba} were obtained by a test pulse to 0 mV from the holding potential (−80 mV). Double-pulse protocol was also employed, in which I_{Ba} was evoked by the application of 0 mV depolarization (5 ms) from a holding potential of −80 mV either without (−prepulse) or almost directly from a strong depolarizing prepulse (90 mV, +prepulse) every

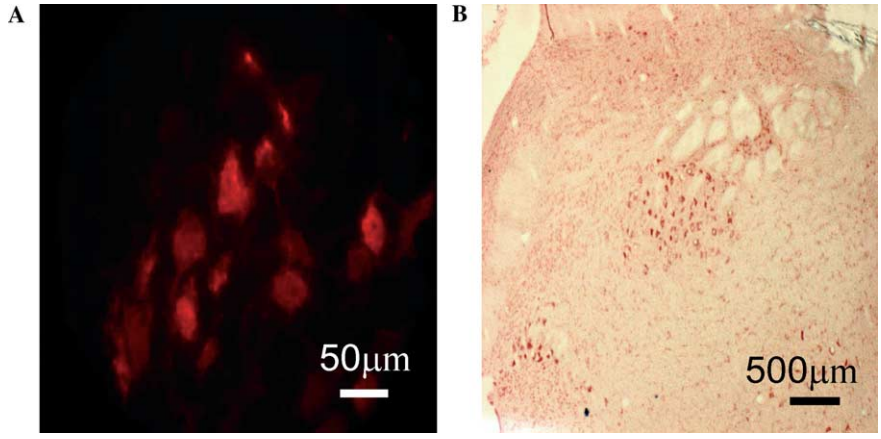

Fig. 1. Identification of trigeminal motoneurons. (A) The trigeminal motor nucleus was localized by retrograde labeling with a fluorescent dye, Evans blue (400×). Cells were visualized using a fluorescent filter which can detect Evans blue (emission wavelength 611 nm). (B) The trigeminal motor nucleus was counterstained with 1% neutral red (40×).

20 s. Whole-cell currents were recorded with Axopatch-1C amplifier (Axon Instruments, USA). All experiments where recovery was <75% after correcting the average rundown were disregarded. Partial series resistance compensation was employed and currents were low pass-filtered at 2 kHz and sampled at 10 kHz. The pClamp6 (Axon Instruments, USA) software was used during experiments and analysis. Statistics are given as means ± SEM.

Drugs. Nifedipine, baclofen, and serotonin were purchased from Sigma (St. Louis, MO). Nifedipine was dissolved in dimethyl sulfoxide (DMSO) at 10 mM to make stock solution and kept in a light-proof container at −20 °C. The final concentration of DMSO was less than 0.1% (v/v), which did not affect I_{Ba} ($n = 5$). Just before experimentation, nifedipine was diluted to their final concentration using the extracellular solution. ω-Conotoxin GVIA and ω-agatoxin-IVA (Alomone labs, Jerusalem, Israel) were dissolved in distilled water to make stock solution and stored at −20 °C. These toxins and drugs were applied directly to the bath by gravity using a continuous bath perfusion system at a flow rate of 1 ml/min.

Results

Expression of multiple Ca channels in trigeminal motoneurons

Immunohistochemical study was performed to examine which subtypes of HVACCs would be expressed in trigeminal motoneurons. As illustrated in Fig. 2, α_{1A},

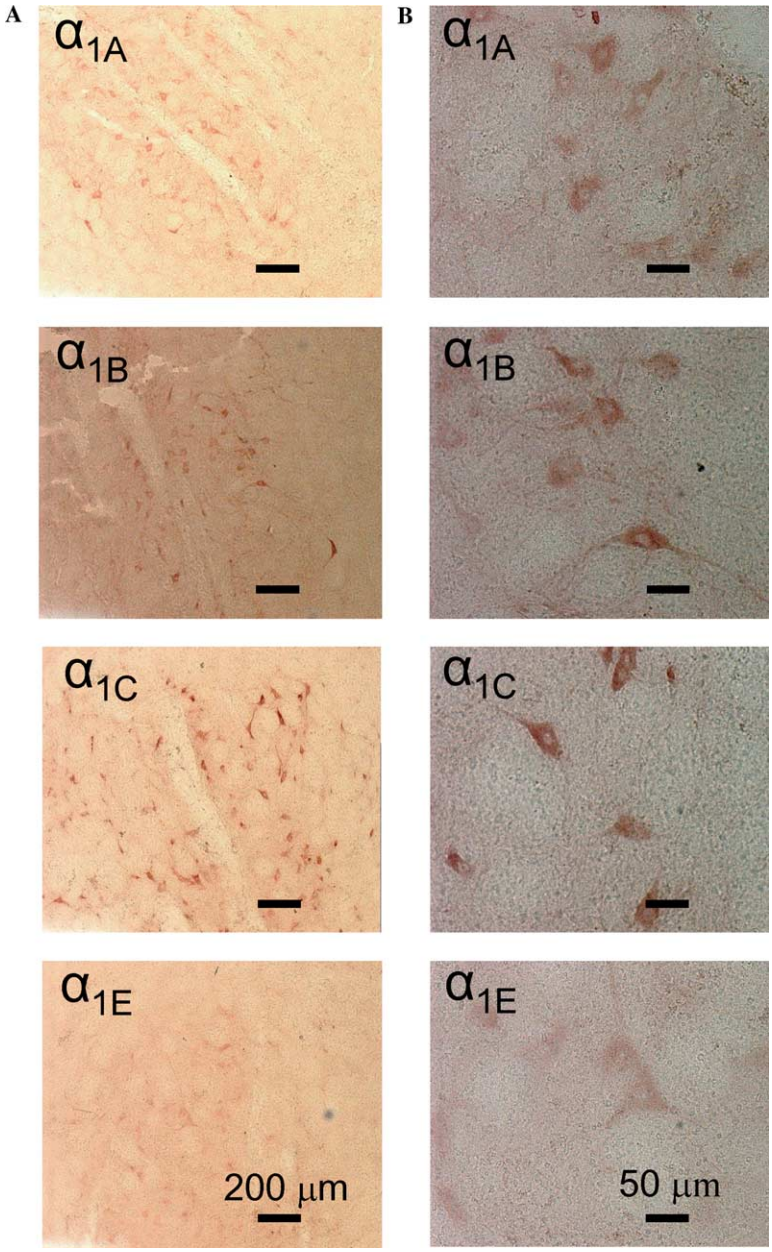

Fig. 2. Immunohistochemical identification of Ca channels in trigeminal motoneurons. Trigeminal motoneurons were densely labeled when stained with polyclonal rabbit anti-rat antibodies: anti-α_{1A}, anti-α_{1B}, anti-α_{1C}, and anti-α_{1E}. Left panel (100×), Right panel (400×).

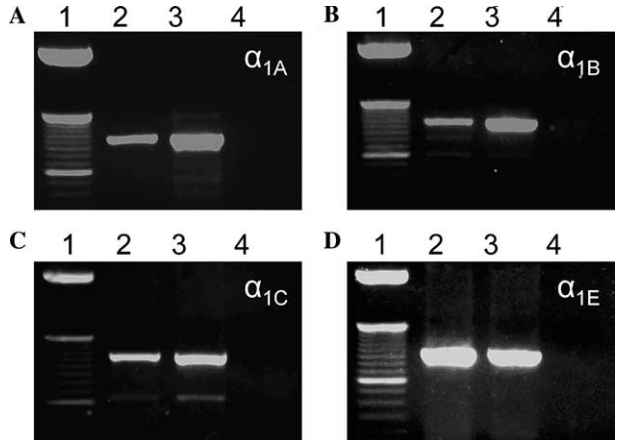

Fig. 3. RT-PCR analysis of calcium channel expression on trigeminal motoneurons. The results demonstrate the presence of mRNA of α_{1A} (A), α_{1B} (B), α_{1C} (C), and α_{1E} (D) Ca channel subunit in trigeminal motoneurons. Lanes 2 and 3 in each panel show PCR products obtained from amplification by primers selected specifically detect each Ca subunit (lane 2, Vmot and lane 3, rat whole brain). Lane 1 contains 50 bp ladder. Lane 4 indicates no amplification products with H_2O.

α_{1B}, α_{1C}, and α_{1E} are homogeneously expressed on the trigeminal motoneurons, indicating the presence of P/Q-, N-, L-, and R-type Ca channels in the individual trigeminal neurons. The expression of P/Q-, N-, L-, and R-type Ca channels in trigeminal motoneurons was further confirmed by RT-PCR. The mRNAs of α_{1A}, α_{1B}, α_{1C}, and α_{1E} Ca channels were clearly detected in trigeminal motoneurons (Fig. 3), consistent with immunohistochemical study (Fig. 2).

Classification of HVACC currents in trigeminal motoneurons

Whole-cell recording of Ca^{2+} currents was performed using 5 mM Ba^{2+} as the charge carrier under experimental conditions that suppress other voltage-dependent currents, such as Na^+ and K^+ currents. To isolate various subtypes of HVACCs in the same cells, 10 μM nifedipine, 1 μM ω-conotoxin, and 200 nM ω-agatoxin which were known to block L-, N-, and P/Q-type currents, respectively, were sequentially applied to the bath (Figs. 4A and B). The residual currents insensitive to nifedipine, ω-conotoxin, and ω-agatoxin were designated as R-type currents, which can be blocked by the nonspecific calcium channel blocker $CaCl_2$ (data not shown). L-, N-, P/Q-, and R-type currents were separated by subtracting the current after the application of respective blocker from that before application, and the percentages of each Ca current out of the total currents were then calculated. We found that L-type Ca currents contributed to the total HVACC currents by $16 \pm 1\%$, N-type by $38 \pm 3\%$, and P/Q-type by $27 \pm 2\%$ ($n = 5$) (Fig. 4C). R-type currents also substantially contributed to the HVACC currents by $19 \pm 2\%$ ($n = 5$) (Fig. 4C).

Inhibition of I_{Ba} via Gi/Go protein by baclofen and serotonin in trigeminal motoneurons

In the majority of the cells tested ($n = 15$ out of 16), baclofen (25 μM) inhibited HVACC I_{Ba} by $48 \pm 3\%$ in a reversible manner (Figs. 5A and B). Serotonin (10 μM) also reversibly depressed HVACC I_{Ba} by $47 \pm 6\%$ (Figs. 5C and D), although serotonin did only in subpopulations of trigeminal motoneurons ($n = 5$ out of 18). We then tested if baclofen and serotonin would inhibit I_{Ba} in the same cell. In all neurons tested ($n = 8$), while baclofen produced clear HVACC I_{Ba} inhibition, serotonin did not (Fig. 5E), indicating that $GABA_B$ and serotonin receptors are rarely expressed together on the same cell. To determine if the effect of baclofen was mediated by Gi/Go protein, we next applied *N*-ethylmaleimide (NEM), a sulfhydryl alkylating agent that has been shown to selectively inhibit Gi/Go function [27]. As shown in Fig. 5F, the inhibition of I_{Ba} by baclofen was completely blocked by NEM pretreatment, suggesting the mediation of membrane-delimited pathway for the I_{Ba} inhibition by baclofen.

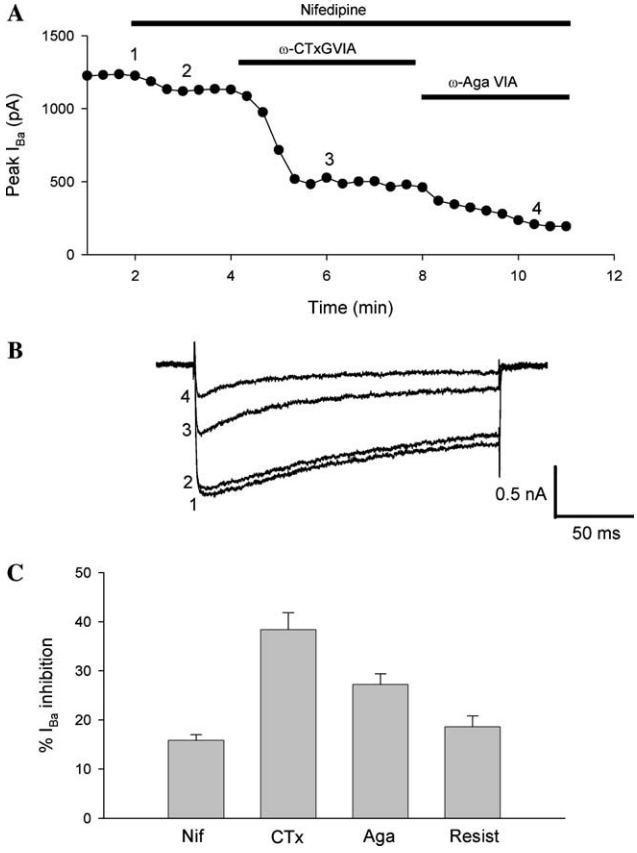

Fig. 4. Inhibition of I_{Ba} by drugs and toxins in trigeminal motoneurons. (A) Plot of peak I_{Ba} versus time. Sequential application of nifedipine, ω-conotoxin, and ω-agatoxin blocked L-, N-, and P/Q-type currents, respectively, in trigeminal motoneurons. R-type currents still remained. (B) Current profile at the points indicated at A. (C) Average inhibition of I_{Ba} by specific drugs and toxin used ($n = 5$).

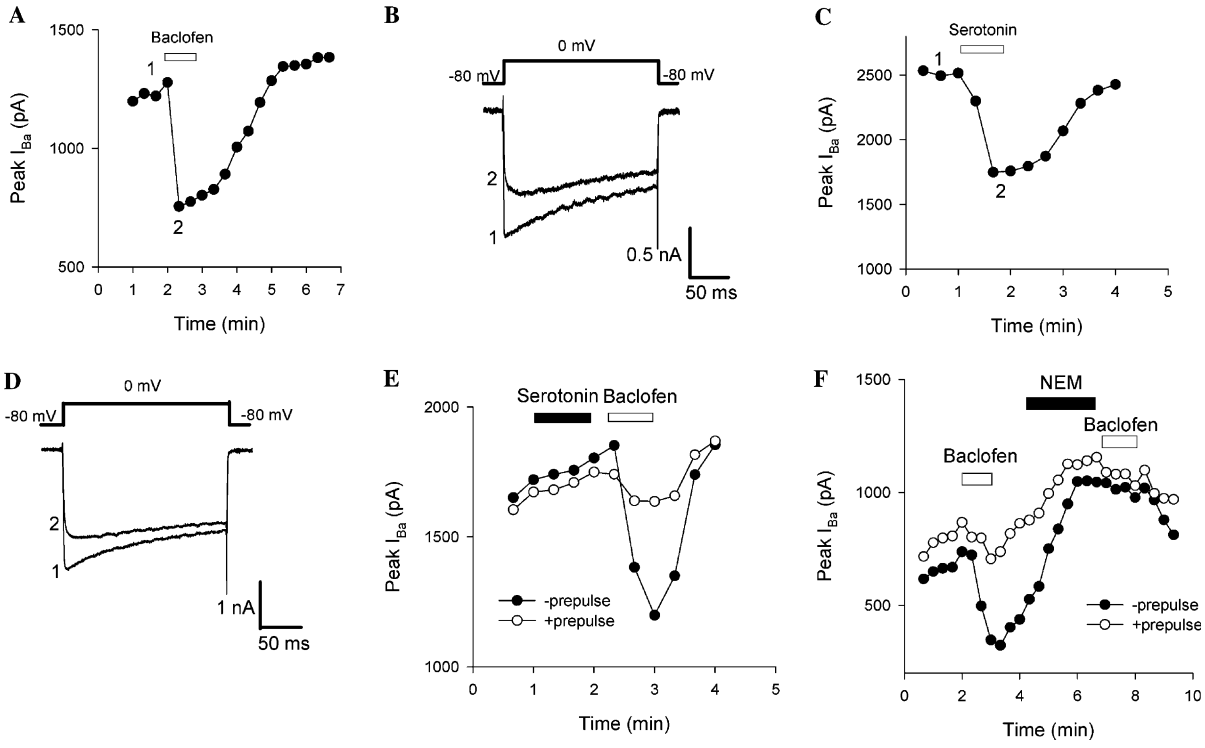

Fig. 5. Effects of baclofen and serotonin on the I_{Ba} in trigeminal motoneurons. (A,C) Plot of peak barium currents versus time. The application of baclofen (25 μM) or serotonin (10 μM) inhibited I_{Ba} in trigeminal motoneurons. (B,D) Current profiles at the points indicated at A and C, respectively. (E) The illustrated is a representative example of neuron that is responsive to baclofen, but not to serotonin. (F) NEM (50 μM) pretreatment prevented the I_{Ba} inhibition by baclofen. The illustrated is time course of I_{Ba} inhibition by baclofen.

Voltage-dependent I_{Ba} inhibition by baclofen and serotonin

Membrane-delimited modulation of Ca channels often exhibits a voltage dependency, being relieved by strong depolarizing prepulse. To determine the voltage-dependency of I_{Ba} inhibition, we used a double-pulse protocol [28,29] (Fig. 6A). Baclofen (25 μM) inhibited the I_{Ba} by $49 \pm 3\%$ (Fig. 6B) and the I_{Ba} inhibition was reduced to $20 \pm 3\%$ by a depolarizing prepulse ($n = 9$) (Fig. 6C). Serotonin (10 μM) also inhibited the I_{Ba} by $47 \pm 5\%$ (Fig. 6D) and a depolarizing prepulse relieved the I_{Ba} inhibition by $24 \pm 6\%$ ($n = 5$) (Fig. 6E).

Discussion

Calcium channels, widely expressed in the nervous system, are critical for the release of neurotransmitters and in the control of neuronal excitability [2,3], and biological functions of many neurotransmitters are mediated by the modulation of HVACC [1,2].

Our immunohistochemical and RT-PCR studies clearly demonstrate that multiple components of HVACCs are widely expressed in the trigeminal motoneurons. These results are consistent with our electrophysiological study, which indicates that a single neuron expresses multiple subtypes of HVACC currents—i.e., L-, N-, P/Q-, and R-type. The major component of the HVACC currents was N-type (~38%) and its contribution was comparable to that in facial motor neurons (30–50%) [19,30]. L-type Ca current was a minor component of HVACC currents in most motoneurons including facial (5%) [19] and hypogossal motoneurons (6%) [31], but the contribution of L-type channels to HVACC currents (16%) was relatively larger in trigeminal motoneurons. P/Q-type Ca currents contribute to HVACC Ca currents by 27% in trigeminal motoneurons. Although this finding is consistent with the previous reports studied in other motoneurons [32], this results contrasts with that in facial motoneurons [19], in which P-type channels were absent in the cell body. However, it should not be neglected that since we used dissociated neurons with few dendrites, our determination could be less than those observed in slice preparation, where most dendrites are preserved at least within the slice.

It is of interest that baclofen inhibits I_{Ba} in the majority of trigeminal motoneurons, but serotonin did only in subpopulations. In addition, it is likely that $GABA_B$ receptor and serotonin receptor are rarely expressed together on the same cells, at least on the cell bodies. Morphological studies have demonstrated that trigeminal motoneurons have both GABAergic and serotonergic axonal contacts through the cell body to

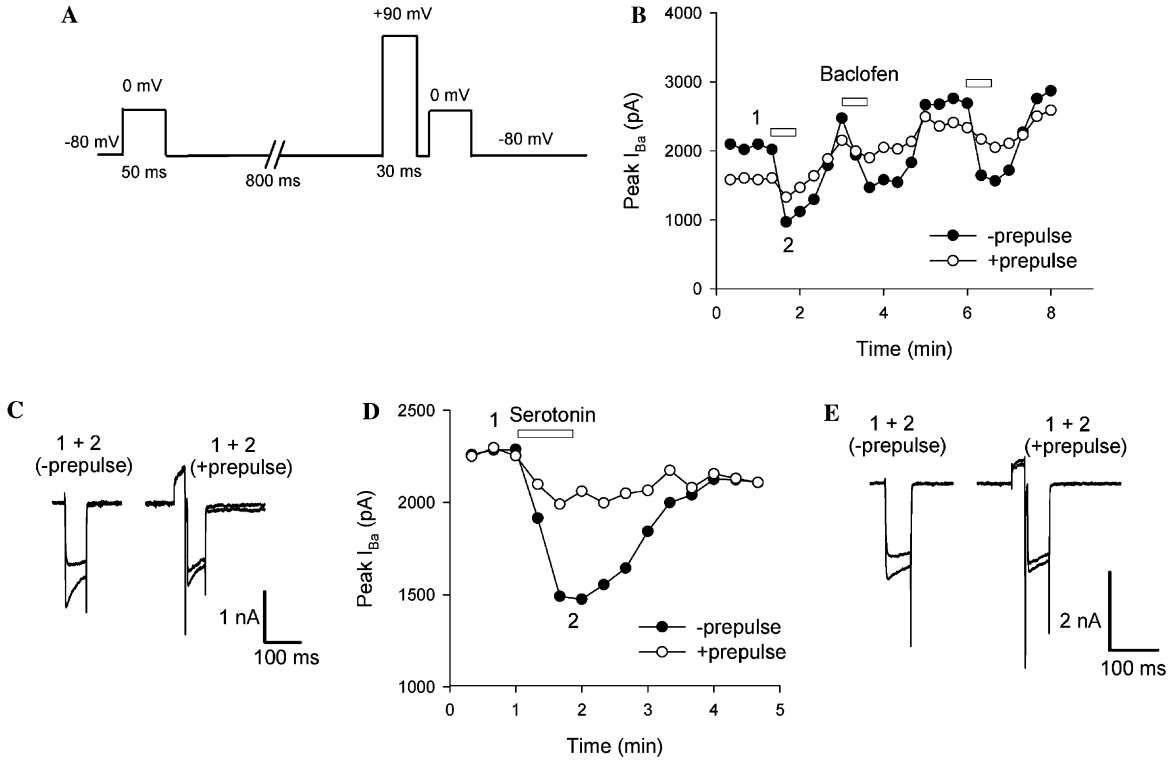

Fig. 6. Effects of baclofen and serotonin on the I_{Ba} in trigeminal motoneurons. (A) Illustration of the double-pulse protocol employed in our experiments. The current was first recorded with a 50-ms test pulse to +10 mV (−prepulse); then, after 800 ms, the second test pulse following a 30-ms conditioning prepulse to +90 mV was applied (+prepulse). (B) Time course of the effect of baclofen (25 μM) on I_{Ba} in trigeminal motoneurons. (C) Superimposed I_{Ba} evoked by test pulse with (+prepulse) and without prepulse (−prepulse) at the points indicated in B. The I_{Ba} inhibition exhibited prepulse facilitation. (D) Time course of the effect of serotonin (10 μM) on I_{BA} in a trageminal motoneuron. (E) Superimposed I_{Ba} evoked by test pulse with (+prepulse) and without prepulse (−prepulse) at the points indicated in D. The current profiles illustrate the relief of serotonin-induced I_{Ba} inhibition with a depolarizing prepulse.

dendrites [22,23]. Our findings clearly show that whereas $GABA_B$ receptors are widely expressed on the cell bodies of trigeminal motoneurons, serotonin receptors are more preferentially expressed intermediate or tertiary dendrites rather than cell bodies of most trigeminal neurons or expressed on the cell bodies in limited subpopulations of trigeminal motoneurons.

GABAergic and serotonergic synaptic inputs to trigeminal motoneurons seem to play distinct physiological roles in trigeminal motoneurons. It has been demonstrated that there are two kinds of trigeminal motoneurons [33,34]. One is jaw-closing motoneurons which are composed of rhythmic alterations of excitation and inhibition coincident with the jaw-closing and jaw-opening phases, respectively. The other is jaw-opening motoneurons which consist of only a rhythmic excitation in the jaw-opening phase. Our results suggest that although glycine predominately mediates the inhibitory phase of jaw-closing motoneurons during the excitatory phase of jaw-opening motoneurons [35], the activation of GABAergic receptors widely expressed on the jaw-closing motoneurons also contributes, at least in part, to this phase through the inhibition of neurotransmitter release from NMJ via blocking Ca channels. The iontophoretically applied serotonin in the masseter motoneurons in the guinea pig did not produce any changes in discharge pattern by itself, but enhanced glutamate-induced discharge [12], and induced NMDA bursting, suggesting 'enabling' role for serotonin [8,10]. In addition, many serotonergic inputs (for example, from medullary raphe nucleus) to trigeminal motoneurons exhibited excitatory effects rather than inhibitory effects in trigeminal motoneurons [23]. These reports may imply why we rarely observed I_{Ba} inhibition by serotonin in trigeminal motoneurons. However, there should be potential roles of the I_{Ba} inhibition by serotonin. One possibility is that the reduction of Ca^{2+} influx through voltage-dependent Ca channels might inhibit the Ca^{2+}-dependent K^+ currents responsible for post-spike hyperpolarization (AHPslow), thereby increasing neuronal excitability [9]. Or the activation of serotonin receptor could result in different effects on trigeminal motor activity according to the subtypes of serotonin receptors involved, as suggested by Mori et al. [24].

We also have demonstrated that the I_{Ba} inhibition produced by baclofen and serotonin was relieved by a depolarizing prepulse. This indicates that Ca channel inhibition is mediated by a "membrane delimited"

pathway, probably involving the interaction of G-protein βγ subunits with the Ca channel α_1 subunit. The I_{Ba} inhibition produced by baclofen was blocked by NEM, an agent which blocks G-protein-effector interactions by alkylating the α-subunits of PTX-sensitive G-proteins. This result indicates that the I_{Ba} inhibition is mediated by Gi/Go-proteins [36].

In summary, trigeminal motoneurons express diverse subtypes of HVACC. Both GABAergic and serotonergic neurons regulate neuronal activities of trigeminal motoneurons through the inhibition of HVACC Ca currents.

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