Serotonergic Modulation of the Hyperpolarizing Spike Afterpotential in Rat Jaw-Closing Motoneurons by PKA and PKC

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Inoue, Tomio, Satuki Itoh, Masayuki Kobayashi, Youngnam Kang, Ryuji Matsu, Satoshi Wakisaka, and Toshifumi Morimoto. Serotonergic modulation of the hyperpolarizing spike afterpotential in rat jaw-closing motoneurons by PKA and PKC. J. Neurophysiol. 82: 626–637, 1999. Intracellular recordings were obtained from rat jaw-closing motoneurons (JCMNs) in slice preparations to investigate the effects of serotonin (5-HT) on the postspike medium-duration afterhyperpolarization (mAHP) and an involvement of protein kinases in the effects. Application of 50 μM 5-HT caused membrane depolarization and increased input resistance in the most cells without affecting the mAHP, whereas not only membrane depolarization and an increase in input resistance, but also the suppression of the mAHP amplitude was induced by higher dose of 5-HT (100 or 200 μM). On the other hand, when the mAHP amplitude was increased by raising [Ca$^{2+}$]o from 2 to 6 mM, 5-HT-induced attenuation of the mAHP amplitude was enhanced, and even 50 μM 5-HT reduced the mAHP amplitude. This 5-HT-induced suppression of the mAHP could be mimicked by application of membrane-permeable cAMP analogue 8-Bromo-cAMP, potentiated by the cAMP-specific phosphodiesterase inhibitor Ro 20-1724 and antagonized by protein kinase A (PKA) inhibitor H89. The enhancement of the mAHP attenuation induced by 50 μM 5-HT under raised [Ca$^{2+}$]o was blocked by a protein kinase C (PKC) inhibitor chelerythrine, suggesting an involvement of PKC in this enhancement. On the other hand, the attenuation of the mAHP induced by PKC activator phorbol 12-myristate 13-acetate was blocked almost completely by H89, suggesting that the PKC action on the mAHP requires PKC activation. Neither 5-HT$_{1A}$ antagonist NAN-190 or 5-HT$_{3}$ antagonist SB 203186 blocked 5-HT-induced attenuation of the mAHP. We conclude that 5-HT induces dose-dependent attenuation of the mAHP amplitude through cAMP-dependent activation of PKA and that PKC-dependent PKA activation is also likely to be involved in the enhancement of 5-HT-induced attenuation of the mAHP under raised [Ca$^{2+}$]o. Because the slope of the linear relationship between firing frequency and injected current was increased only when the mAHP amplitude was decreased by 5-HT, it is suggested that the relation between incoming synaptic inputs and firing output in JCMNs varies according to serotonergic effects on JCMNs and calcium-dependent modulation of its effects.

INTRODUCTION

The trigeminal motor nucleus receives a dense serotonergic input (Kolta et al. 1993; Nagase et al. 1997; Saha et al. 1991; Takeuchi et al. 1983) and contains serotonergic receptors (Kolta et al. 1993). In the brain stem slice preparations, 5-HT depolarizes trigeminal motoneurons and increases neuronal excitability (Hsiao et al. 1997; Inoue et al. 1995; Trueblood et al. 1996), as previously shown in facial (Aghajanian and Rasmussen 1989; Larkman et al. 1989), hypoglossal (Berger et al. 1992), and spinal (Elliott and Wallis 1992; Lindsay and Feldman 1993; Takahashi and Berger 1990; Wang and Dun 1990; White and Fung 1989) motoneurons. Trigeminal motoneurons innervate the jaw-closing or opening muscles. Iontophoretically applied serotonin (5-HT) facilitates spike discharge induced by glutamate in both jaw-closing motoneurons (JCMNs) and jaw-opening motoneurons (JOMNs) and also facilitates spike discharge of both motoneurons during cortically induced rhythmic jaw movements (Katakura and Chandler 1990; Kurasawa et al. 1990). However, an immunohistochemical study showed that JCMNs are contacted by a larger number of 5-HT-immunoreactive boutons than JOMNs, suggesting that JCMNs receive a more dense 5-HT innervation (Nagase et al. 1997). Furthermore the incidence of the facilitation by iontophoretically applied 5-HT is higher in JCMNs than JOMNs (Kurasawa et al. 1990). Thus 5-HT may modulate the excitabilities of JCMNs more strongly than those of JOMNs.

In addition to the depolarization, 5-HT decreases the postspike medium-duration afterhyperpolarization (mAHP) in juvenile guinea pig motoneurons (Hsiao et al. 1997), spinal motoneurons of adult cats (White and Fung 1989) and 2- to 3-wk-old rats (Wu et al. 1991), and hypoglossal motoneurons of neonatal rats (Berger et al. 1992). Because AHP is an important factor of controlling neuronal discharge (Hille 1992), the attenuation of AHP by 5-HT likely results in an enhancement of motoneuronal excitability by increasing input-output gain of motoneurons (Berger et al. 1992; Hsiao et al. 1997). However, 5-HT has little effect on the AHP in juvenile rat hypoglossal (Talley et al. 1997) and adult rat facial (Larkman and Kelly 1992) motoneurons. Thus the effects of 5-HT on the AHP are still controversial.

A calcium-activated potassium current is involved in generating the mAHP in trigeminal motoneurons (Chandler et al. 1994; Kobayashi et al. 1997) as well as other cranial motoneurons (Nishimura et al. 1989; Sah and McLachlan 1992; Viana et al. 1993). Bayliss et al. (1995) proposed that 5-HT suppresses high-voltage-activated calcium channels with a resultant decrease in the calcium entry necessary for activation of the potassium currents responsible for the AHP. In contrast, in hippocampal neurons, 5-HT suppressed the AHP without prominent reduction of the amplitude, time course, or threshold voltage of the calcium spike, suggesting that 5-HT suppressed the calcium-activated potassium...
channels directly (Andrade and Nicoll 1987). It has been reported that 5-HT suppresses the AHP by activation of protein kinase A (PKA) through an increase in intracellular cAMP in hippocampal neurons (Pedarzani and Storm 1993; Torres et al. 1995). Protein kinase C (PKC) also was reported to be involved in inhibition of AHP in hippocampal neurons (Baraban et al. 1985) and enteric neurons (Pan et al. 1997). However, it is not clear in trigeminal motoneurons whether cAMP/PKA or PKC cascades lead to direct suppression of calcium-activated potassium channels responsible for the mAHP or that suppression of calcium entry through voltage-activated calcium channels mediates the attenuation of the AHP.

In the present study, the effects of 5-HT on the mAHP evoked in JCMNs were investigated in juvenile rat slice preparation containing the trigeminal motor nucleus by the use of the intracellular recording method. We demonstrate that 5-HT induces a dose-dependent attenuation of the mAHP amplitude through an increase in intracellular cAMP and activation of PKA. When the mAHP amplitude was increased by raising [Ca\textsuperscript{2+}], from 2 to 6 mM, 5-HT-induced attenuation of the mAHP amplitude was enhanced. PKC-dependent activation of PKA is likely to be involved in the enhancement under raised [Ca\textsuperscript{2+}]. Preliminary results of this study were reported previously in abstract forms (Inoue et al. 1997, 1998).

**METHODS**

**Animal preparation**

Transverse brain stem slices (450 \mu m) including the trigeminal motor nucleus were prepared from 87 juvenile Sprague-Dawley rats (3 to 6-wk old) as previously described (Kobayashi et al. 1997). To identify the recorded cell as a jaw-closing motoneuron histologically, we employed the fluorescence double-labeling technique (cf. Viana et al. 1990) in the first series of the experiments (37 of the 87 animals). The 37 animals were anesthetized with ketamine HCl (150 mg/kg im) and chlorpromazine HCl (12.5 mg/kg im), and both sides of the animal preparation were performed at 32 \pm 1 °C. Intracellular recordings were obtained from 2 to 6 M KCl and 0.05 M Tris buffer (pH 7.6) for intracellular staining in the slice preparation. The microelectrode was filled with 1% biocytin in 1 M NaCl, 3 KCl, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 2 CaCl\textsubscript{2}, and 10 D-glucose. The M-ACSF was made of N-ACSF by replacing 130 mM NaCl with 260 mM sucrose. In some experiments, the Ca\textsuperscript{2+} concentration was raised to 6 mM. Tetrodotoxin (TTX, 1 \mu M; Wako), methysergide (20 \mu M; Novartis), 5-hydroxytryptamine creatinine sulfate (5-HT, 20–200 \mu M; Sigma), 8-Bromo-cAMP (1 mM; RBI), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20–1724, 15–25 \mu M; RBI), N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide (H-89, 10–20 \mu M; Seikagaku), phosphol 12-myristate 13-acetate (PMA, 2–5 \mu M; Sigma), 1,2-dimethoxy-12-methyl-[1,3]benzodioxolo[5,6-c]phenanthridinium chloride (chelerythrine, 10 \mu M; RBI), (+)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydropyridine (8-OH-DPAT, 25–50 \mu M; RBI), 1-[2-methoxyphenyl]-4-[4-(2-phtalimidobutyl)piperazin (NAN-190, 10 \mu M; Sigma) and 1-piperidinylethyl 1H-indole-3-carboxylate (SB 203186, 10 \mu M; Tocris) were added directly to the perfusate. In some experiments, microdrops of 5-HT (10–20 mM dissolved in N-ACSF) were applied to the surface of a slice by pressure-ejection using a Picospritzer II from a broken micropipette positioned upstream of the recording electrode.

**Recording**

After 2–10 h incubation in the holding chamber, slices were transferred to an interface-type chamber. The recording chamber was perfused continuously with N-ACSF at a rate of 1–1.5 ml/min and humidified 95% O\textsubscript{2}-5% CO\textsubscript{2} 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, 20–50 M\Omega) (Sutter Instruments). The microelectrode was filled with 1% biocytin in 1 M KCl and 0.05 M Tris buffer (pH 7.6) for intracellular staining in the first series of the experiments or filled with 2 M KCl and 0.05 M Tris...
buffer (pH 7.6) in the second series of experiments. Membrane potentials were recorded using an Axoclamp 2B amplifier (Axon Instruments) in either bridge or discontinuous current-clamp (DCC) mode. During DCC 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. When membrane potential changed during or after application of a drug, the membrane potential was clamped manually to the resting potential level in control by injecting constant hyperpolarizing or depolarizing current to evaluate the effects of the drug, except for measuring a change in the membrane potential after the drug application and evaluating the effects of microdroplet application of 5-HT. Membrane potential and current were digitized and stored on a computer hard disk using software (Clampex, Axon Instruments) through A-D converter, and analyzed with the use of Clampfit (Axon Instruments) and Excel (Microsoft) software.

Data obtained from each neuron except for resting membrane potential, rheobase and firing frequency are the mean of 5–8 trials. Data are presented as means ± SE. Comparisons of data before and during the drug application within groups were based on Student’s paired t-tests. Differences of data between groups were analyzed by Student’s t-tests, one-way or two-way ANOVA. ANOVA was followed by post hoc Newman-Keuls multiple comparison tests when justified. Probabilities <0.05 were considered significant.

**RESULTS**

**General properties**

The present study was based on the recordings from 111 JCMNs (20 MAMNs, 22 CLMNs, and 69 DLMNs), which had stable resting potentials more negative than −55 mV (−67.3 ± 0.6 mV; n = 111) and displayed action potentials (measured from resting membrane potential to the spike peak) >70 mV (98.3 ± 0.7 mV; n = 111). Input resistance was 9.6 ± 0.3 MΩ (n = 111), which was calculated from the relationship between injected current intensity (−0.2 to −0.5 nA) and the voltage response. Such values were similar to those previously reported (Kobayashi et al. 1997) and were not significantly different among MAMNs, CLMNs, and DLMNs. Furthermore, the effects of 50 μM 5-HT applied in normal perfusate (2 mM Ca²⁺) on resting membrane potential, input resistance, rheobase, and characteristics of spike afterpotentials were not significantly different between MAMNs (n = 8) and CLMNs (n = 8), although the effects on DLMNs were not examined under this condition. Thus MAMNs, CLMNs, and DLMNs were not distinguished for further analysis.

**Subthreshold membrane properties**

The effects of bath (20–200 μM) or microdroplet (10–20 mM) application of 5-HT in normal perfusate (2 mM Ca²⁺) were tested in 47 JCMNs (16 MAMNs, 21 CLMNs, and 10 DLMNs). As has been reported in cranial and spinal motoneurons (reviewed by White et al. 1996), bath (50 μM) or microdroplet (20 mM) application of 5-HT produced a slow depolarization (4.1 ± 0.6 mV, P < 0.001, n = 31) and increased input resistance by 31% (13.1 ± 1.0 MΩ from 10.2 ± 0.8 MΩ, P < 0.001, n = 31) (Fig. 2A, left). These effects persisted in the presence of tetrodotoxin (n = 5). The nonselective 5-HT antagonist methysergide (20 μM) antagonized the effects of 5-HT on input resistance and resting membrane potential (Fig. 2A, right; n = 3). Those results suggest that 5-HT produced those changes through direct activation of 5-HT receptors in JCMNs.

**Effects of 5-HT on spike afterpotentials**

**NORMAL PERFUSATE.** The effects of 5-HT on spike afterpotentials were examined in 31 JCMNs (11 MAMNs, 11 CLMNs, and 9 DLMNs) in normal perfusate. As we previously reported (Kobayashi et al. 1997), an afterdepolarization (ADP) and a medium-duration afterhyperpolarization (mAHP) followed single action potentials which were elicited by an injection of brief (2–3 ms) depolarizing current pulses. In 5 of the 31 neurons a prominent ADP was not observed, and the ADP was not analyzed in the five neurons. As described in the preceding text, 50 μM 5-HT induced depolarization in 16 JCMNs tested (8 MAMNs and 8 CLMNs; 3.5 ± 0.7 mV, n = 16, P < 0.001) and increased input resistance by 28.7 ± 7.1% (9.4 ± 0.7 to 12.0 ± 1.0 MΩ, n = 16, P < 0.005). However, this dose of 5-HT had relatively minor effects on the afterpotentials. An exemplary response to 50 μM 5-HT obtained from a CLMN is shown in Fig. 2B. The traces before and after application of 50 μM 5-HT were superimposed, and each trace was obtained by averaging five records before or after 5-HT application by triggering with the peak of the action potential. Application of 50 μM 5-HT did not change the mAHP (5.0 ± 0.4 to 5.2 ± 0.6 mV, n = 16, P > 0.2) and ADP (9.0 ± 1.2 to 8.4 ± 1.4 mV, n = 12, P > 0.1) amplitude in the 16 JCMNs (Fig. 2B).

Then the effects of higher dose of 5-HT (100 or 200 μM) were examined in 18 JCMNs (5 MAMNs, 4 CLMNs, and 9 DLMNs). Compared with the effect of 50 μM 5-HT, 200 μM 5-HT invariably decreased the mAHP amplitude by 31.2 ± 6.6% (3.6 ± 0.2 to 2.5 ± 0.3 mV, n = 10, P < 0.001) and increased the ADP amplitude by 23.3 ± 6.0% (9.1 ± 0.9 to 11.1 ± 1.1 mV, n = 10, P < 0.05; Fig. 2C). Application of 100 μM 5-HT also significantly reduced mAHP by 13.2 ± 5.4% (5.2 ± 0.6 to 4.5 ± 0.6 mV, n = 8, P < 0.05) although the effect was less than those of 200 μM 5-HT. This dose of 5-HT was not effective on the ADP amplitude (6.2 ± 0.3 to 6.1 ± 0.4 mV, n = 6, P > 0.4). In contrast to the alteration of spike afterpotentials, no significant change was observed in spike height or spike half-amplitude duration during application of 5-HT.

**RAISED [Ca²⁺], ENHANCED 5-HT-INDUCED ATTENUATION OF THE mAHP.** Because both mAHP and ADP are calcium-dependent in rat trigeminal motoneurons (Kobayashi et al. 1997), raising [Ca²⁺], results in enhancements of the mAHP and ADP probably due to an increase in Ca²⁺ influx. To make the effects of 5-HT on the mAHP and ADP more evident, we examined the effects of 5-HT under raised [Ca²⁺], from 2 to 6 mM in 22 JCMNs (4 MAMNs, 1 CLMN, and 17 DLMNs). Slices were held under raised [Ca²⁺],[i] for 1–2 h and then intracellular recordings were performed. Before 5-HT application, those neurons under raised [Ca²⁺],[i] showed significantly larger amplitude of both ADP and mAHP compared with neurons incubated in normal perfusate (Table 1). The effects of 5-HT under raised [Ca²⁺],[i] were different from those under normal [Ca²⁺],[i]. Under raised [Ca²⁺],[i], 50 μM 5-HT invariably decreased the mAHP amplitude by 19.4 ± 3.4% (6.0 ± 0.8 to 5.0 ± 0.8 mV, P < 0.001, n = 9) and increased the ADP amplitude by 15.5 ± 8.0% (11.8 ± 1.8 to 13.1 ± 1.7 mV, n = 6, P < 0.05; Fig. 3A). Effects of 200 μM 5-HT under raised [Ca²⁺],[i] were examined in 13 JCMNs. In 12 of 13 neurons, 200 μM 5-HT under this condition further decreased the mAHP.
amplitude by 40.4 ± 6.8% (5.0 ± 0.6 to 3.2 ± 0.6 mV, P < 0.001, n = 12) and increased the ADP amplitude by 10.1 ± 3.0% (14.5 ± 1.4 to 16.0 ± 1.5 mV, P < 0.01, n = 10; Fig. 3B).

The effects of 5-HT under normal and raised [Ca\(^{2+}\)]\(_o\) are summarized in Fig. 3C and D. The effects of 5-HT on the mAHP and ADP amplitude were different when [Ca\(^{2+}\)]\(_o\) and/or 5-HT concentration were altered. Two-way ANOVA showed that percent reduction in the mAHP amplitude from control after 200 μM 5-HT application was larger than those after 50 μM 5-HT (P < 0.001; Fig. 3C). Percent reduction in the mAHP amplitude under raised [Ca\(^{2+}\)]\(_o\) was also larger than that under normal [Ca\(^{2+}\)]\(_o\) (P < 0.05, 2-way ANOVA) and post hoc Newman-Keuls test revealed that the reduction after 50 μM 5-HT application under raised [Ca\(^{2+}\)]\(_o\) were larger than that under normal [Ca\(^{2+}\)]\(_o\) (P < 0.05; Fig. 3C). On the other hand, no significant differences were observed between the effects of 5-HT on the ADP amplitude under normal and raised [Ca\(^{2+}\)]\(_o\) (P > 0.1, 2-way ANOVA) and between those effects of 50 and 200 μM 5-HT (P > 0.5, 2-way ANOVA; Fig. 3D). However, a significant interaction was found between the factors of [Ca\(^{2+}\)]\(_o\) and concentration of 5-HT (P < 0.05, 2-way ANOVA), and post hoc Newman-Keuls test showed that the effect of 200 μM 5-HT on the ADP amplitude was significantly larger than that of 50 μM 5-HT under normal [Ca\(^{2+}\)]\(_o\) (P < 0.05). The effects of 5-HT on the resting membrane potential and input resistance under normal [Ca\(^{2+}\)]\(_o\) were not different from those under raised [Ca\(^{2+}\)]\(_o\) (P > 0.3, 2-way ANOVA).

### INVOLVEMENT OF cAMP/PKA AND PKC CASCADES

To examine whether activation of PKA is involved in 5-HT-induced attenuation of the mAHP in rat JCMNs, the following experiments were performed under normal [Ca\(^{2+}\)]\(_o\). First, effects of 8-Bromo-cAMP (1 mM), a membrane permeable cAMP analogue was examined in four DLMNs in the normal perfusate. Similar to the effects of 200 μM 5-HT, 8-Bromo-cAMP invariably decreased the mAHP amplitude by 39.3 ± 8.1% (3.1 ± 0.2 to 1.9 ± 0.3 mV, n = 4, P < 0.01) and increased the ADP amplitude by 9.8 ± 3.0% (14.1 ± 2.3 to 15.3 ± 2.2 mV, n = 4, P < 0.01; Fig. 4A).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>6 mM Ca(^{2+})</th>
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<tbody>
<tr>
<td>Resting potential, mV</td>
<td>-67.1 ± 1.0 (22)</td>
<td>-68.4 ± 1.1 (21)</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>9.3 ± 0.5 (22)</td>
<td>10.2 ± 0.7 (21)</td>
</tr>
<tr>
<td>Spike amplitude, mV</td>
<td>97.3 ± 1.6 (22)</td>
<td>99.6 ± 1.4 (21)</td>
</tr>
<tr>
<td>ADP amplitude, mV</td>
<td>9.1 ± 0.9 (17)</td>
<td>12.9 ± 1.1* (17)</td>
</tr>
<tr>
<td>mAHP amplitude, mV</td>
<td>4.7 ± 0.3 (22)</td>
<td>5.6 ± 0.5* (21)</td>
</tr>
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Number tested in parentheses. JCMN, jaw-closing motoneuron; ADP, afterdepolarization; mAHP, medium-duration afterhyperpolarization. * Statistically significant difference between Normal and 6 mM Ca\(^{2+}\) Ringer (P < 0.05).
Application of 8-Bromo-cAMP also increased the spike amplitude slightly (from 103.3 ± 1.3 to 104.4 ± 1.0 mV, n = 4, P < 0.05) and induce depolarization by 2.3 ± 0.5 mV (from −68.8 ± 1.9 mV, n = 4, P < 0.01), however, no significant change was observed in spike half-amplitude or input resistance. Second, the effects of Ro 20–1724, a cAMP-specific phosphodiesterase inhibitor were examined in five DLMNs. The mAHP amplitude was not altered during application of 15–25 μM Ro 20–1724 for 25–30 min (3.1 ± 0.6 to 3.3 ± 0.6 mV, n = 5, P > 0.05; Fig. 4Ba). However, when the same dose of Ro 20–1724 was coapplied with 25–50 μM 5-HT, which did not alter the mAHP amplitude by itself, the mAHP amplitude was reduced by 28.8 ± 5.7% (3.3 ± 0.6 to 2.4 ± 0.6 mV, n = 5, P < 0.005; Fig. 4Bb). In contrast, the ADP amplitude was not altered by this dose of 5-HT in the presence of Ro 20–1724 (14.1 ± 0.9 to 14.0 ± 1.2 mV, n = 4, P > 0.1). Third, we examined the effects of H89, a selective inhibitor of PKA in five DLMNs. Slices were preincubated with H89 (10–20 μM) for 1.5–2.5 h and then intracellular recordings were performed. In the presence of H89, 200 μM 5-HT reduced the mAHP amplitude to only a small degree (4.2 ± 0.5 to 3.9 ± 0.6 mV, n = 5, P < 0.05; Fig. 4C), and percent reduction in the mAHP amplitude after coapplication of 200 μM 5-HT and H89 was about one fourth of the percent reduction after 200 μM 5-HT application by itself (8.1 ± 3.3% vs. 31.2 ± 6.6%, P < 0.05; Fig. 4D). In the presence of H89, 200 μM 5-HT had no effect on the ADP amplitude, either (5.0 ± 2.2 to 5.2 ± 1.9 mV, n = 5, P > 0.3). These results suggest that activation of PKA through an increase in intracellular cAMP is involved in 5-HT-induced attenuation of the mAHP.

Then we examined whether activation of PKC is also involved in 5-HT-induced attenuation of the mAHP or not. Bath application of PMA (2–5 μM), a PKC activator, invariably reduced the mAHP amplitude by 30.5 ± 4.7% (3.9 ± 0.3 to 2.7 ± 0.3 mV, P < 0.005; Fig. 5A) under normal [Ca²⁺]o in
four DLMNs tested, suggesting that PKC might reduce the mAHP. In contrast the ADP amplitude was not altered by PMA (8.6 ± 1.0 to 8.9 ± 1.2 mV, n = 4, P > 0.2; Fig. 5A). Because PKC is activated Ca2+ dependently (Hug and Sarre 1993; Nishizuka 1995), it might be possible that a transient increase in [Ca2+]i generated by an action potential activates more PKC under raised [Ca2+]o and results in an enhancement of 5-HT-induced attenuation of the mAHP. To examine this possibility, we investigated the effects of chelerythrine, a selective inhibitor of PKC, on the attenuation of the mAHP induced by 50 μM 5-HT under raised [Ca2+]o in five DLMNs. In slices preincubated with chelerythrine (10 μM) for 1.5–2.5 h under raised [Ca2+]o, 50 μM 5-HT had no significant effect on the mAHP amplitude under this condition (6.6 ± 0.8 to 6.3 ± 1.0 mV, n = 5, P > 0.1; Fig. 5C). The difference between percent decreases of the mAHP amplitude after 50 μM 5-HT application under raised [Ca2+]o in the presence and absence of chelerythrine was significant (6.6 ± 4.6% vs. 19.4 ± 3.4%, P < 0.05; Fig. 5D). These results suggest that activation of PKC might be involved in the enhancement of 5-HT-induced attenuation of the mAHP under raised [Ca2+]o.

Thus both PKA and PKC are likely to be involved in the 5-HT effects on the mAHP. Cross-talk between cAMP/PKA and PKC cascades might occur, as demonstrated in several cellular systems (Cooper et al. 1995; Pieroni et al. 1993). To examine whether PKA activation is required for the effects of PKC on the mAHP, the effects of PMA (5 μM) on the attenuation of the mAHP in the presence of H89 (10–20 μM) were investigated under normal [Ca2+]o in five DLMNs. In slices preincubated with H89 for 1.5–2.5 h, PMA did not induce any significant attenuation of the mAHP (3.6 ± 0.8 to 3.5 ± 0.8 mV, n = 5, P > 0.2; compare Fig. 5, A and B). There was a significant difference in percent reduction of the mAHP amplitude after PMA application between in the presence and absence of H89 (30.5 ± 4.7% vs. 2.1 ± 2.2%; P < 0.005; Fig. 5D). Because the K_i value of H89 for PKC that were obtained from purified enzyme assay was reported to be 31.7 μM (Chijiwa et al. 1990), it is possible that 10–20 μM H89 might block PMA-induced attenuation of the mAHP by inhibiting PKC activity. However, the K_i value of H89 for PKC in in vivo experiments is likely to be higher than that obtained from enzyme assay. In fact, 30 μM H89 had no effects on PKC

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**FIG. 4.** Involvement of a cAMP/protein kinase A (PKA) cascade in 5-HT-induced attenuation of mAHP. A: superimposed traces of a DLMN before and after bath application of 1 mM 8-Bromo-cAMP under normal [Ca2+]o. Marked suppression of mAHP and enhancement of ADP (inset) were observed. Ba: superimposed traces of a DLMN before and after application of 15 μM Ro 20–1724 under normal [Ca2+]o. Neither mAHP nor ADP (inset) was altered during Ro 20–1724 application. Bb: same neuron in Ba. Application of 50 μM 5-HT in addition to 15 μM Ro 20–1724 attenuated mAHP under normal [Ca2+]o. C: superimposed traces of a DLMN before and after bath application of 200 μM 5-HT in the presence of 10 μM H89 under normal [Ca2+]o. H89 antagonized 5-HT-induced attenuation of mAHP. D: summary data of the effects of 8-Bromo-cAMP (n = 4), Ro 20–1724 (n = 5), the addition of 50 μM 5-HT to Ro 20–1724 (n = 5), 200 μM 5-HT (n = 10, same data in Fig. 3C) and 200 μM 5-HT in the presence of H89 (n = 5) on mAHP. Error bars: SE associated with each group. † and ‡: P < 0.05 and P < 0.01 vs. control, respectively. * and **: P < 0.05 and P < 0.01 between groups, respectively. Each trace in A–C is the average of 5 records. ・・ resting membrane potential in control.
activity in PC12D pheochromocytoma cells (Chijiwa et al. 1990), and hypoxia-induced inhibition of whole cell Na⁺ current in dissociated hippocampal neurons was not altered by inclusion of 30 μM H89 in the patch electrode but was greatly attenuated by inclusion of PKC inhibitors such as calphostin C or PKCi in the electrode (O’Reilly et al. 1997). In the present study, percent reduction of the mAHP amplitude after PMA application in the presence of 10 μM H89 was 2.5% (n = 3).

Thus it is less likely that H89 inhibits PKC activity, leading to abolishing the effects of PMA on the mAHP. Therefore it can be assumed that PKC-induced attenuation of the mAHP is mediated by PKA activation.

RECEPTOR PHARMACOLOGY. Because 5-HT-induced attenuation of the AHP was reported to be mediated by 5-HT₁A receptors in neonatal rat hypoglossal motoneurons (Bayliss et al. 1995; Talley et al. 1997) or by 5-HT₄ receptors in hippocampal neurons (Andrade and Chaput 1991; Torres et al. 1994), we tested whether those receptors mediate 5-HT-induced attenuation of the mAHP in JCMNs. The effects of a 5-HT₁A agonist 8-OH-DPAT (25–50 μM) and a 5-HT₁A antagonist NAN-190 (10 μM) were examined under normal [Ca²⁺]₀ in six and four DLMNs, respectively. Although 8-OH-DPAT slightly reduced the mAHP amplitude by 6.9 ± 0.8% (P < 0.01; Fig. 6A), in slices preincubated with NAN-190 for 1.5–2.5 h, 200 μM 5-HT could reduce the mAHP amplitude by 41.1 ± 12.6% (P < 0.01; Fig. 6B). Then we examined the effects of SB 203186 (10 μM), a 5-HT₄ antagonist under normal [Ca²⁺]₀ in four DLMNs. In slices preincubated with SB 203186 for 1.5–2.5 h, 200 μM 5-HT could reduce the mAHP amplitude by 45.6 ± 9.7% (P < 0.01; Fig. 6B). The effects of 200 μM 5-HT co-applied with NAN-190 or SB 203186 were not significantly different from those of 200 μM 5-HT by itself (P > 0.01). Those results suggest an involvement of receptor subtype(s) other than 5-HT₁A and 5-HT₄ in 5-HT-induced attenuation of the mAHP.

FIG. 5. Contribution of protein kinase C (PKC) to 5-HT-induced attenuation of mAHP. A: superimposed traces of a DLMN before and after bath application of 5 μM phorbol 12-myristate 13-acetate (PMA) under normal [Ca²⁺]₀. PMA reduced mAHP amplitude, whereas PMA did not affect ADP (inset). B: superimposed traces of a DLMN before and after application of 5 μM PMA in the presence of 10 μM H89 under normal [Ca²⁺]₀. H89 almost completely blocked PMA-induced attenuation of mAHP. C: superimposed traces of a DLMN before and after application of 50 μM 5-HT in the presence of 10 μM chelerythrine under raised [Ca²⁺]₀. Chelerythrine blocked mAHP attenuation induced by 50 μM 5-HT under raised [Ca²⁺]₀. D: summary data of the effects of PMA (n = 4), PMA in the presence of H89 (n = 5), 50 μM 5-HT under raised [Ca²⁺]₀ (n = 9, same data shown in Fig. 3C) and 50 μM 5-HT under raised [Ca²⁺]₀ in the presence of chelerythrine (n = 5) on mAHP. Error bars: SE associated with each group. † and ‡: P < 0.05 and P < 0.01 vs. control, respectively. * and **: P < 0.05 and P < 0.01 between groups, respectively. Each trace in A–C is the average of 5 records. zz, resting membrane potential in control.
Effects of 5-HT on repetitive firing properties

The effects of 5-HT on repetitive firing discharge in response to intracellular injection of long (1–2 s) depolarizing current pulses were examined in 19 JCMNs (3 MAMNs, 4 CLMNs, and 12 DLMNs). After application of 50 μM 5-HT under normal [Ca\(^{2+}\)]\(_{o}\), rheobase was decreased significantly by 0.4 ± 0.1 nA (1.7 ± 0.3 to 1.3 ± 0.3 nA, n = 9, P < 0.001), and a higher frequency spike train was elicited compared with control (Fig. 7A, compare left and right). The relationship between firing frequency (f) and injected current intensity (I) was plotted in Fig. 7D. Both f–I relationships for the first interspike interval (1st ISI; Fig. 7D, left) and steady-state firing (average firing discharge over the last half of the injected current pulse; Fig. 7D, right) were shifted to the left. However, neither f–I slope of linear region of 1st ISI or steady-state relationship was significantly altered after 50 μM 5-HT under this condition (1st ISI: 19.0 ± 3.5 to 18.8 ± 2.4 Hz/nA, n = 7, Fig. 7D, left; steady-state: 13.5 ± 2.5 to 12.8 ± 1.5 Hz/nA, n = 7, Fig. 7D, right). Similar results have been reported in neonatal rat phrenic (Lindsay and Feldman 1993) and adult rat hypoglossal (Talley et al. 1997) motoneurons. On the other hand, 200 μM 5-HT under raised [Ca\(^{2+}\)]\(_{o}\) from 2 to 6 mM affected the slope of the linear region of the f–I relationship. Before application of 5-HT the f–I slopes were not very steep for both the 1st ISI and the steady-state firing under raised [Ca\(^{2+}\)]\(_{o}\) (1st ISI: 12.7 ± 2.5 Hz/nA, n = 6; steady-state: 6.3 ± 0.6 Hz/nA, n = 6; Fig. 7F, left and right, ○). As mentioned above, mAHP amplitude of those neurons was large under this condition. Such large mAHP most likely slowed the neuronal firing. After 5-HT application, the mAHP amplitude was remarkably reduced (Fig. 7E) and the f–I relationships were shifted to the left and their slopes became significantly steeper (1st ISI: 20.2 ± 4.7 Hz/nA, P < 0.05; steady-state: 8.0 ± 0.5 Hz/nA, P < 0.05; Fig. 7F, left and right, ●). Slopes of the linear region of f–I relationships for both 1st ISI and steady-state also were increased by 50 μM 5-HT under raised [Ca\(^{2+}\)]\(_{o}\) (n = 3) and 200 μM 5-HT under normal [Ca\(^{2+}\)]\(_{o}\) (n = 3). In all neurons that were examined on the f–I relationships, percent increases in the f–I slope for 1st ISI and steady-state after 5-HT application were correlated significantly with percent decrease in the mAHP amplitude (1st ISI: R\(^2\) = 0.53, n = 19, P < 0.05; steady-state: R\(^2\) = 0.30, n = 19, P < 0.05). These results suggest that the 5-HT-induced attenuation of the mAHP amplitude likely leads to an increase in the f–I slope in rat JCMNs as reported in other motoneurons (Berger et al. 1992; Hsiao et al. 1997; Talley et al. 1997).

DISCUSSION

Mechanisms for 5-HT-induced change in the afterpotentials

It has been proposed that the 5-HT-induced suppression of the mAHP may be a property of neonatal but not juvenile or adult motoneurons (Talley et al. 1997). Consistent with this previous observation, 50 μM 5-HT under normal [Ca\(^{2+}\)]\(_{o}\) (2 mM) caused membrane depolarization and an increase in input resistance but did not reduce the mAHP amplitude in juvenile rat JCMNs. However, we demonstrated that even in juvenile motoneurons 5-HT suppresses the mAHP when it is applied either under raised [Ca\(^{2+}\)]\(_{o}\) from 2 to 6 mM or at a high concentration (100 or 200 μM).

We previously reported that N-type Ca\(^{2+}\) currents activate apamin-sensitive calcium-activated potassium channels (SK channels) responsible for generating the mAHP in trigeminal motoneurons (Kobayashi et al. 1997). Thus it might be possible that 5-HT decreases Ca\(^{2+}\) influx through N-type Ca\(^{2+}\) channels. High-voltage-activated Ca\(^{2+}\) currents including N-type Ca\(^{2+}\) currents activate apamin-sensitive calcium-activated potassium channels (SK channels) responsible for generating the mAHP in trigeminal motoneurons (Kobayashi et al. 1997). Thus it might be possible that 5-HT decreases Ca\(^{2+}\) influx through N-type Ca\(^{2+}\) channels. High-voltage-activated Ca\(^{2+}\) currents including N-type Ca\(^{2+}\) currents have been reported to be inhibited by 5-HT through activation of 5-HT\(_{1A}\) receptors in pyramidal neurons (Foehring 1996), hypothalamic neurons (Koike et al. 1994; Rhee et al. 1996), dorsal (Penington and Kelly 1990), and caudal raphe neurons (Bayliss et al. 1997). In addition to those reports Bayliss et al. (1995) have suggested that 5-HT decreased N- and P-type Ca\(^{2+}\) currents responsible for the mAHP through activation of 5-HT\(_{1A}\) receptors in neonatal hypoglossal mo-
toneurons. However, in the present study, inhibition of 5-HT$_{1A}$ receptors by NAN-190 did not alter 5-HT-induced inhibition of the mAHP. Talley et al. (1997) have shown that expression of 5-HT$_{1A}$ receptor greatly decreases in rat hypoglossal motoneurons with maturation and 5-HT did not inhibit the mAHP in juvenile animals. It is also possible that 5-HT$_{1A}$ receptor might be expressed to a small extent in juvenile JCMNs, as was the case with juvenile rat hypoglossal motoneurons. Furthermore a
membrane-delimited G-protein pathway has been shown repeatedly to mediate the inhibition of N-type Ca\(^{2+}\) channels by 5-HT in various vertebrate neurons (Anwyl 1991; Foehring 1996; Penington et al. 1991) as well as hypoglossal motoneurons (Bayliss et al. 1995). Suppression of N-type Ca\(^{2+}\) channels by neurotransmitter through the membrane-delimited pathway was shown to be independent of soluble intracellular messengers (Hille 1994). On the other hand, we found that 5-HT-induced attenuation of the mAHP could be mimicked by 8-Bromo-cAMP, potentiated by Ro 20–1724, and blocked by H89, suggesting that a cAMP/PKA cascade is involved in 5-HT-induced attenuation of the mAHP. Therefore it is likely that 5-HT-induced suppression of the mAHP in JCMNs is not mediated mainly by the inhibition of N-type Ca\(^{2+}\) channels through a membrane-delimited G-protein pathway. However, we cannot exclude the possibility that PKA might inhibit N-type Ca\(^{2+}\) channels because PKA was reported to reduce N-type Ca\(^{2+}\) channels indirectly in rat neostriatal neurons (Surmeier et al. 1995).

As calcium-dependent potassium channels are known to be important targets for modulation by protein phosphorylation (Levitan 1994), 5-HT might have reduced the mAHP amplitude through PKA-dependent phosphorylation of SK channels. In CA1 hippocampal pyramidal neurons, activation of 5-HT\(_4\) receptors elicits reduction in the slow AHP through a cAMP/PKA cascade (Torres et al. 1995). However, it was suggested in these cells that 5-HT reduces the slow AHP by inhibiting calcium-induced calcium release (CICR) because Ca\(^{2+}\) increase due to CICR triggered by Ca\(^{2+}\) influx may activate K\(^+\) channels responsible for the slow AHP (Torres et al. 1996). The mAHP in rat trigeminal motoneurons peaks rapidly in amplitude (<30 ms), lasts 50–100 ms after even a burst of action potentials (Fig. 7, A and B) and is apamin-sensitive (Kobayashi et al. 1997), whereas the slow AHP in CA1 pyramidal neurons has a slow rising phase, lasts several seconds, and is apamin-insensitive (Sah 1996). Furthermore blockade of 5-HT\(_4\) receptors by SB 203186 did not alter 5-HT-induced attenuation of the mAHP. Thus the mAHP in JCMNs appears to be generated in a different way from the slow AHP in CA1 pyramidal neurons. It is most likely that inflowing Ca\(^{2+}\) directly gates SK channels leading to the generation of the mAHP (Sah 1996; Schwindt et al. 1992). 5-HT receptor(s) other than 5-HT\(_4\) receptors might be involved in the effects on the mAHP, although we could not determine which subtype of 5-HT receptors mediates an increase in cAMP. 5-HT\(_6\) and 5-HT\(_7\) receptors were reported to activate adenylyl cyclase (Hoyer et al. 1994), and activation of both or either receptor(s) might increase intracellular cAMP and activate PKA, leading to phosphorylation of SK channels.

It has been shown that 5-HT enhances the inward rectifier current activated by membrane hyperpolarization (I\(_h\)) in motoneurons (Hsiao et al. 1997; Larkman and Kelly 1992; Takahashi and Berger 1990), and an increase in I\(_h\) is suggested to decrease the mAHP amplitude (Schwindt et al. 1988; Spain 1994; Spain et al. 1987). Thus it is possible that an enhancement of I\(_h\) by 5-HT might result in a decrease in the mAHP amplitude. However, even in the presence of 5-HT the activation of I\(_h\) by the mAHP could be small because the membrane potential of the mAHP peak was only 3–7 mV lower than resting membrane potential (~67 mV). I\(_h\) was reported to be activated around ~70 mV with a potential for half-maximal activation of ~86 to ~88 mV in the presence of 5-HT in guinea pig trigeminal motoneurons (Hsiao et al. 1997) and rat facial motoneurons (Larkman and Kelly 1992). Therefore an enhancement of I\(_h\) by 5-HT is less likely to be involved in 5-HT-induced suppression of the mAHP under both normal and raised [Ca\(^{2+}\)]\(_o\).

In accordance with suppression of the mAHP, the ADP amplitude was enhanced when 5-HT was applied under raised [Ca\(^{2+}\)]\(_o\), or applied at a high concentration. However, the ADP and the mAHP were differentially affected by 5-HT when [Ca\(^{2+}\)]\(_o\) was raised. Under raised [Ca\(^{2+}\)]\(_o\) the mAHP was suppressed by 5-HT in a dose-dependent manner, whereas the enhancement of the ADP by 5-HT was not dose-dependent. Furthermore the mAHP was similarly suppressed by 8-Bromo-cAMP and PMA, whereas the ADP was enhanced only by 8-Bromo-cAMP but not by PMA (compare Figs. 4A and 5A). Therefore the increase in the ADP amplitude might not be simply explained by 5-HT-induced suppression of the mAHP alone. 5-HT is most likely to increase Ca\(^{2+}\) currents responsible for the generation of the ADP; however, further studies will be necessary to clarify the mechanisms for this enhancement.

**Involvement of PKA and PKC in the enhancement of 5-HT-induced attenuation of the mAHP under raised [Ca\(^{2+}\)]\(_o\)**

Because PKC activation induced by PMA suppressed the mAHP and inhibition of PKC by chelerythrine application blocked the attenuation of the mAHP induced by 50 μM 5-HT under normal [Ca\(^{2+}\)]\(_o\). PKC activation is likely to be involved in the enhancement of 5-HT-induced attenuation of the mAHP under raised [Ca\(^{2+}\)]\(_o\). On the other hand, intracellular Ca\(^{2+}\) was shown to stimulate some isoforms of adenylyl cyclase through calmodulin (Cooper et al. 1995). Thus a transient increase in [Ca\(^{2+}\)]\(_i\) generated by an action potential might have stimulated adenylyl cyclase and also resulted in an enhancement of 5-HT-induced attenuation of the mAHP. However, the contribution of this pathway to the enhancement of the mAHP attenuation should be minor because the percent decrease in the AHP amplitude after application of 50 μM 5-HT under raised [Ca\(^{2+}\)]\(_o\) was small in the presence of chelerythrine.

As mentioned in the preceding text, a cAMP/PKA cascade is involved in 5-HT-induced attenuation of the mAHP. Because H89 almost completely blocked PMA-induced attenuation of the mAHP, PKA activation is likely to be required for PKC action on the mAHP. Furthermore H89 greatly reduced the attenuation of the mAHP induced by 200 μM 5-HT, suggesting that PKA activation appears to be the key component in 5-HT-induced attenuation of the mAHP. Therefore it can be assumed that through activation of PKA, PKC might inhibit Ca\(^{2+}\)-activated K\(^+\) channels responsible for the mAHP, as suggested in enteric neurons (Pan et al. 1997). PKC has been shown to increase the activity of adenylyl cyclases and the level of cAMP (reviewed by Cooper et al. 1995; Pieroni et al. 1993). If this is the case, a transient increase in [Ca\(^{2+}\)]\(_i\), generated by an action potential under raised [Ca\(^{2+}\)]\(_o\), might stimulate PKC, leading to an increase in PKA activity through elevation of intracellular cAMP when 5-HT was applied. This increased activity of PKA might phosphorylate more Ca\(^{2+}\)-activated K\(^+\) channels and result in more suppression of the mAHP. Therefore it is likely that PKC-dependent PKA activation might be
involved in the enhancement of 5-HT-induced attenuation in the mAHP under raised [Ca2+]o.

Role of 5-HT-induced modulation of afterpotentials for firing patterns

Application of 50 μM 5-HT to normal perfusate shifted the f-I relationships for both 1st ISI and steady-state to the left, however, it did not change the f-I slope of the linear relationships. Such constancy of the slope after 50 μM 5-HT application is probably due to the unchanged mAHP amplitude because the percent increases in the f-I slopes of the linear relationship for 1st ISI and steady-state after 5-HT application were correlated significantly with the percent decrease in the mAHP amplitude. On the other hand, 200 μM 5-HT added to normal perfusate or 5-HT in combination with high Ca2+ perfusate reduced the mAHP amplitude and increased the f-I slope of the linear relationship as well as shifting the relationship to the left. Similar changes in the mAHP and the f-I slope induced by lower dose (10 μM) of 5-HT were reported in juvenile guinea pig trigeminal motoneurons (Hsiao et al. 1997). The discrepancy of the dose of 5-HT necessary to induce those changes between rat and guinea pig trigeminal motoneurons might be related to species of the motoneuron or the activities of 5-HT reuptake mechanism in the slice (Wang and Dun 1990). The increase in the f-I slope indicates that such serotonergic inputs do not only lower the threshold to induce spike firing but also increase input-output gain of JCMNs, suggesting that small changes in incoming synaptic inputs would produce large alterations in spike frequency output of JCMNs. Under physiological condition, such increase in input-output gain of JCMNs might occur only when JCMNs receive serotonergic inputs under raised [Ca2+]o because a low dose (50 μM) of 5-HT by itself could not attenuate the mAHP amplitude. Elevation of [Ca2+]o could be induced via Ca2+ influx through N-methyl-D-aspartate (NMDA) channels (MacDermott et al. 1986), by release of Ca2+ from intracellular stores that are, for example, caused by activation of some types of metabotropic glutamate receptors (Pin and Duvoisin 1995), or by repetitive spike firing at high-frequency. In fact, activation of NMDA receptors was reported to elicit a Ca2+-dependent increase in cAMP in hippocampal neurons (Chetkovich et al. 1991). Thus it is possible that the relation of incoming synaptic inputs and firing output in JCMNs is modulated by combination of serotonergic and other inputs inducing an increase in [Ca2+]o. Activities of both jaw-closing and -opening muscles are influenced by physical properties of food (Wejs and Dantuma 1981); however, the jaw-closing muscles are likely to be facilitated more readily by peripheral sensory inputs during chewing than the jaw-opening muscles (Hidaka et al. 1997; Inoue et al. 1989). Such serotonergic modulation of activities of JCMNs could contribute to adequate regulation of masticatory force for the properties of food.

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References


SEROTONERGIC MODULATION OF JAW-CLOSING MOTONEURONS


