Effects of Serotonin on Caudal Raphe Neurons: Inhibition of N- and P/Q-Type Calcium Channels and the Afterhyperpolarization

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Bayliss, Douglas A., Yu-Wen Li, and Edmund M. Talley. Effects of serotonin on caudal raphe neurons: inhibition of N- and P/Q-type calcium channels and the afterhyperpolarization. J. Neurophysiol. 77: 1362–1374, 1997. We characterized whole cell barium currents through calcium channels and investigated the effects of serotonin (5-HT) on calcium channel currents and firing behavior in visualized caudal raphe neurons of the neonatal rat in brain stem slices (n = 201). A subpopulation of recorded neurons was recovered after staining for tryptophan hydroxylase (TPH), the 5-HT synthesizing enzyme (n = 21); of those cells, 86% were TPH immunoreactive, suggesting that the majority of recorded neurons was serotonergic. Calcium channel currents began to activate near +40 mV in caudal raphe neurons and showed a peak amplitude of 952.2 ± 144.2 (SE) pA at −10 mV. A small low-voltage-activated current was also observed (~22 pA). Calcium channel currents were potentely inhibited by bath-applied 5-HT in most cells tested (~90%). The EC50 for inhibition of calcium current by 5-HT was 0.1 μM; a saturating concentration (1.0 μM) blocked ~49% of the current evoked at 0 mV from a holding potential of −70 mV (n = 101). Current inhibition was associated with a slowing of activation kinetics and a shift in the peak of the current–voltage relationship, and was partially relieved by strong depolarizations. Current inhibition by 5-HT was mimicked by 8-OH-DPAT, a specific 5-HT1A agonist, and blocked by the 5-HT1A antagonists NAN 190 and (+)-WAY 101,135, but was unaffected by ketanserin, a 5-HT2A/2C antagonist. ω-Conotoxin GVIA (ω-CgTx)-sensitive N-type and ω-agatoxin IVA (ω-AgaIVA)-sensitive P/Q-type channels together accounted for most of the calcium current (36 and 37%, respectively). Nimodipine had no effect on calcium current, indicating that caudal raphe neurons do not express dHPyR-sensitive L-type currents. A substantial residual current (27%) remained after application of ω-CgTx, ω-AgaIVA, and nimodipine. Most of the 5-HT-sensitive calcium current was blocked by ω-CgTx and ω-AgaIVA; 5-HT had little effect on the residual current. Inhibition of calcium current by 5-HT was irreversible when GTPγS, a nonhydrolyzable guanosine 5′-triphosphate (GTPγS) analogue, was substituted for GTP in the pipette. In addition, the effects of 5-HT were blocked by pretreating slices with pertussis toxin (PTX). Together these data indicate that inhibition of N- and P/Q-type calcium current in serotonergic caudal raphe neurons is mediated by a 5-HT1A receptor via PTX-sensitive G proteins. Under current clamp, calcium channel toxins (ω-CgTx and ω-AgaIVA) and 5-HT each caused a decrease in the spike afterhyperpolarization and enhanced the repetitive firing response to injected current. The similar effects of 5-HT and the calcium channel toxins on firing behavior suggest that those effects of 5-HT were secondary to inhibition of N- and P/Q-type calcium channels.

INTRODUCTION

Brain stem raphe neuronal cell groups provide a serotonergic innervation that is widespread throughout the CNS (Jacobs and Azmitia 1992). Neurons of the caudal medullary raphe nuclei, specifically in raphe obscurus (ROb) and raphe pallidus (RPa), project to motoneurons and spinal sympathetic preganglionic neurons (Jacobs and Azmitia 1992; Skagerberg and Bjorklund 1985) where serotonin (5-HT) is thought to provide a predominantly excitatory influence on motor and sympathetic outflow (Chalmers and Pilowsky 1991; Jacobs and Azmitia 1992). Thus information regarding the complement of channels that contribute to regulating behavior of caudal raphe neurons, and their modulation by neurotransmitters, is of substantial importance in understanding neural mechanisms of motor and sympathetic control.

Calcium channels are important in controlling neuronal behavior via the currents they generate, which contribute directly to electrical excitability, and via the influx of calcium they support, which influences numerous calcium-dependent cellular processes. Moreover, calcium currents in central neurons are subject to modulation by a number of neurotransmitters (Anwyl 1991; Hille 1994). Electrophysiological and pharmacological approaches have identified multiple subtypes of calcium currents that were initially classified as low- and high-voltage-activated (LVA and HVA) on the basis of differential activation and inactivation properties (Carbone and Swandulla 1993). Neuronal HVA current is now commonly separated into dihydropyridine-sensitive L-type; ω-conotoxin GVIA (ω-CgTx)-sensitive N-type; ω-agatoxin IVA (ω-AgaIVA)-sensitive P/Q-type channels; although occasionally further discrimination of P and Q types has been attempted (Randall and Tsien 1995); and a residual (R-type) current that is insensitive to the calcium channel antagonists (Dunlap et al. 1995). Although these types of currents are reasonably well described, their role in spontaneous firing behavior and the consequence on firing properties of calcium current inhibition by neurotransmitters is less well studied.

Currently nothing is known about the types of calcium currents in caudal raphe neurons, their neuromodulation, or their role in firing behavior. Therefore we characterized whole cell calcium channel currents in caudal raphe neurons of neonatal rats. In addition, we also determined the effects of 5-HT on calcium currents and firing behavior in those cells. We found that caudal raphe neurons displayed little LVA current or dihydropyridine-sensitive L-type current, but did express prominent N- and P/Q-type currents, as well as a substantial residual current. The N- and P/Q-type current components were inhibited by 5-HT1A receptor activation through a pertussis toxin (PTX)-sensitive G protein. Inhibition of N- and P/Q-type calcium channels with calcium channel toxins, or treatment with 5-HT, inhibited the afterhyperpolarization (AHP) and increased the firing response to current input. These data suggest that the inhibition of N- and P/Q-type calcium channels by 5-HT may mediate the reduction in AHP amplitude in caudal raphe neurons. Thus inhibition of N- and P/Q-type channels by 5-HT may
serve to enhance the raphe neuronal response to suprathreshold inputs. A preliminary account of these results has been presented (Bayliss 1995).

**METHODS**

**General preparation**

Whole cell recordings were obtained from caudal raphe neurons using thin brain stem slices obtained from neonatal rats (postnatal day 8 or younger), as described in the accompanying paper (Bayliss et al. 1997). Briefly, thin transverse brain stem slices (150 μm) were prepared from neonatal rats and submerged in a chamber mounted on a fixed-stage microscope (Axioskop FS, Zeiss) equipped with Nomarski optics and a ×40 water immersion objective. Caudal raphe neurons were identified visually by their position along the midline in the slice and by their morphology (Jacobs and Azmitia 1992; Steinbusch and Nieuwenhuys 1983). In some cases we filled cells with Lucifer yellow for post hoc identification and immunostaining with antibodies to tryptophan hydroxylase (TPH), the synthesizing enzyme for 5-HT, according to methods detailed in the accompanying paper (Bayliss et al. 1997).

**Recording**

Patch-clamp recordings were obtained at room temperature with pipettes pulled to a DC resistance of 2–7 MΩ using an Axopatch 200A patch-clamp amplifier (Axon Instruments) and the pCLAMP suite of programs (Axon). Series resistance was typically <20 MΩ and was compensated by ~70%.

To generate calcium channel current-voltage (I-V) curves under whole cell voltage clamp, currents were elicited by applying voltage step commands (50 ms) to varying potentials from a holding potential of −70 mV. Difference currents obtained by digital subtraction of currents elicited during voltage steps to −40 mV from −70 and −100 mV were used to define LVA currents. To test effects of 5-HT, HVA currents were elicited during 20-msec voltage steps to 0 mV at 12- to 20-s intervals. The peak current during the voltage step was measured under control conditions and then isochronally throughout the experiment (Penington et al. 1992). To test whether current inhibition by 5-HT was relieved by strong depolarizations, we used a protocol in which a test pulse to 0 mV was generated before and then after a step to +70 mV (Elmslie et al. 1990). In current clamp, action potentials were recorded during spontaneous firing or during firing induced by depolarizing intracellular current injection. AHP amplitude was taken as the difference between the peak of the AHP and the spike threshold. The relationship between firing frequency and injected current (I-V curve) was determined by injecting long (2 s) depolarizing current pulses of varying intensity. When necessary, the membrane potential was restored to control levels by DC injection before action potential or repetitive firing characteristics were recorded.

Data are presented as means ± SE. Concentration response data were fitted with a logistic equation of the form y = (a − c)/(1 + (5-HT/EC50)0.5) + c, where a and c are the theoretical maximum and minimum, respectively, and b is a slope function (SigmaPlot, Jandel). Data were analyzed statistically with the use of paired t-tests or one-factor analysis of variance (ANOVA) as indicated in the text; to evaluate prior hypothesized differences among group means, ANOVAs were followed by a Bonferroni modification of the t-test (Wallenstein et al. 1980). In all cases significance was accepted if P < 0.05.

**Solutions and drugs**

Solutions used for preparation and maintenance of slices were as described in the accompanying paper (Bayliss et al. 1997). For current-clamp recordings we used an external N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-based Ringer solution that contained (in mM) 140 NaCl, 3 KCl, 10 HEPES, 2 CaCl2, 2 MgCl2, and 10 glucose, pH adjusted to 7.3 with NaOH. The internal solution contained (in mM) 17.5 KCl, 122.5 potassium gluconate, 10 HEPES, 0.2 ethylene glycol-bis-(β-aminomethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 9 NaCl, 1 MgCl2, 3 MgATP, and 0.3 guanosine 5’-triphosphate (GTP)–tris(hydroxymethyl)aminomethane (Tris), pH adjusted to 7.2 with KOH. For voltage-clamp recordings of barium currents through calcium channels, the external solution was as above but with the addition of 1.0 μM tetrodotoxin and equimolar substitution of BaCl2 for CaCl2. The internal solution contained (in mM) 30 tetraethylammonium chloride, 100 CsCH3O3S, 4 NaCl, 1 MgCl2, 0.5 CaCl2, 10 HEPES, 10 EGTA, 3 MgATP, and 0.3 GTP-Tris, pH adjusted to 7.2 with tetraethylammonium hydroxide. In some experiments we substituted 0.1 mM GTP* for GTP in the pipette. Under these recording conditions all inward currents were abolished by the addition of 0.1 mM CdCl2 to the external solution (e.g., see Fig. 2); liquid junction potential was <1 mV.

Stock solutions (100 μM in water) of ω-CgTx (Calbiochem) and ω-AgaIVA (generous gift from Pfizer, Groton, CT) were prepared and added to perfusate containing 0.1% cytochrome C. Final toxin concentrations (1.0 and 0.2 μM for ω-CgTx and ω-AgaIA, respectively) were chosen that completely blocked nonoverlapping components of calcium current (McCleskey et al. 1987; Mintz et al. 1992). Although we found that 100 nM ω-AgaIA was a saturating concentration in preliminary experiments, we chose to use 200 nM because the kinetics of inhibition were faster using the higher concentration. Other reagents were obtained, prepared, and added to the bath as described in the accompanying paper (Bayliss et al. 1997).

**RESULTS**

**General properties of calcium currents in caudal raphe neurons**

The present report is based on recordings from neurons located in ROB and RPa in rat brain stem slices (n = 201). We recorded barium currents through calcium channels under conditions that minimize Na+ and K+ currents. A subset (n = 21) of caudal raphe neurons was processed for immunohistochemical identification of TPH after recording to determine whether the neurons were serotonergic; of those cells, ~86% (n = 18) were found to contain TPH immunoreactivity, suggesting that the majority of recorded neurons was serotonergic. A representative serotonergic caudal raphe neuron and the calcium channel currents obtained from the neuron are shown in Fig. 1. The recorded neuron (arrowhead) contained Lucifer yellow (Fig. 1A) and also showed TPH immunoreactivity (Fig. 1B). Another TPH-immunoreactive cell adjacent to the recorded neuron is also shown (Fig. 1B, →). Depolarizing voltage steps from a holding potential of −70 mV evoked relatively nonactivating inward currents in this cell (Fig. 1C) that became evident at −40 mV and peaked at −10 mV (Fig. 1D). Averaged I-V relationships from eight caudal raphe neurons revealed an activation threshold between −40 and −30 mV and a peak of 952.2 ± 144.2 pA at −10 mV (cf. Fig. 2C, ◊), similar to HVA calcium currents in other cell types. We also tested for the presence of LVA calcium currents in caudal raphe neurons, defining LVA currents as the difference current evoked at −40 mV from holding potentials of −70 and −100 mV; these LVA difference currents were small, averaging...
FIG. 1. Calcium channel currents in a serotonergic caudal raphe neuron. Barium currents through calcium channels were recorded from a caudal raphe neuron. The cell was filled with Lucifer yellow via the recording pipette and subsequently processed for immunohistochemical detection of tryptophan hydroxylase (TPH), the serotonin (5-HT) synthesizing enzyme. A: fluorescence photomicrograph of recorded neuron filled with Lucifer yellow (arrowhead). Scale bar: 25 μm. B: fluorescence photomicrograph of Texas Red-stained TPH-immunoreactive neurons. The recorded neuron (arrowhead) is stained with the TPH antibody, indicating that it is serotonergic. An adjacent TPH-positive raphe neuron is also shown (→). C: calcium channel currents were recorded from the same serotonergic neuron during incrementing (20 mV) voltage steps from a holding potential of −70 mV. D: corresponding current-voltage (I-V) relationship for the calcium channel current in this cell.

22.0 ± 1.9 pA (n = 8), and were not studied further (not shown).

Inhibition of calcium current by 5-HT

We tested the effect of 5-HT on calcium channel currents in caudal raphe neurons, as shown in Fig. 2. Under control conditions, voltage steps to 0 mV from −70 mV evoked a peak inward current of 623 pA; in the presence of 5-HT (1.0 μM), the current (measured isochronally at the control peak current) was reduced to 263 pA, representing a ∼58% reduction (Fig. 2A). The inhibition of current amplitude was invariably associated with a slowing of activation kinetics. The current recovered completely after wash, and a further application of 5-HT caused a second inhibition that was of similar magnitude. The time course of the effect of 5-HT on peak current in this cell is shown in Fig. 2B. Inhibition of calcium channel current was evident in the overwhelming majority of caudal raphe neurons tested with 5-HT (90%; n = 149 of 165).

The effect of 5-HT on calcium channel current was evaluated at different membrane potentials in eight neurons. As shown in the I-V plots of Fig. 2C, 5-HT caused an inhibition at all potentials at which the current was activated. In 5-HT, a slight shift in the I-V relationship was consistently observed, with the peak occurring at 0 mV rather than at −10 mV. Inhibition of calcium channel current was dependent on the concentration of 5-HT (Fig. 2D). Concentration-response data were fitted with a logistic equation that predicted an EC50 of 0.1 μM and a maximal inhibition of ∼49%. In a population of 101 neurons tested with a saturating concentration of 5-HT (1.0 μM) the current inhibition averaged 48.8 ± 1.2%. Fourteen neurons tested with 5-HT were recovered after histological processing for TPH immunoreactivity; 12 of those 14 neurons were identified as serotonergic (86%), and the averaged current inhibition in those identified serotonergic neurons was 49.1 ± 3.9%. Thus 5-HT inhibits nearly half of the calcium channel current in the majority of serotonergic caudal raphe neurons.

Pharmacology of the 5-HT receptor mediating calcium current inhibition

Pharmacological experiments were performed to determine the 5-HT receptor subtype that mediates calcium current inhibition in caudal raphe neurons. The results of these experiments, illustrated in Fig. 3, indicate that a 5-HT1A receptor is involved. As shown in Fig. 3A, the 5-HT1A agonist R(+)-8-hydroxydipropylaminotetralin hydrobromide (8-OH-DPAT) (0.1 μM) mimicked the current inhibition by 5-HT, causing
FIG. 2. 5-HT inhibits calcium current in caudal raphe neurons. A: voltage steps to 0 mV from a holding potential of −70 mV (top trace) were used to elicit calcium channel currents (bottom traces) in control and during 2 applications of 1.0 μM 5-HT. Peak current was reversibly inhibited by 5-HT and the current inhibition was associated with a slowing of activation kinetics. B: peak currents (from A) are plotted as a function of time. The effect of 5-HT was reversible and could be repeated. C: averaged I-V relationships (means ± SE) obtained under control conditions and in the presence of 1.0 μM 5-HT (n = 8) show that peak inward current was inhibited by 5-HT at all potentials and 5-HT caused a slight shift in the I-V relationship. D: effect of different concentrations of 5-HT on calcium channel current inhibition. The data were fitted with a logistic equation (——) that predicted an EC₅₀ of 0.1 μM and a peak inhibition of 49.2%. Each point represents data from 3–8 cells.

Calcium channel subtypes in caudal raphe neurons and their inhibition by 5-HT

Multiple components of HVA calcium current have been identified in central neurons (Dunlap et al. 1995; Randall and Tsien 1995). We performed pharmacological experiments using drugs and toxins that discriminate among the current components to 1) characterize the complement of currents in caudal raphe neurons and 2) determine the current components sensitive to 5-HT.

The effects of various calcium channel antagonists on the I-V relationship in a representative caudal raphe neuron are shown in Fig. 4A. Control currents were recorded before application of the antagonists (●). Application of ω-AgaIVA (200 nM), an inhibitor of P/Q-type calcium channels, resulted in a 37% decrease in current (▲); subsequent application of ω-CgTx (2 μM), an inhibitor of N-type channels, resulted in a further 31% decrease in current (□). The current inhibition induced by these toxins was not associated with any apparent shift in the I-V relationship or any appreciable change in current kinetics (Fig. 4A, inset). We next...
FIG. 3. Inhibition of calcium current is mediated by a 5-HT\textsubscript{1A} receptor. Currents were evoked during voltage steps from −70 to 0 mV. A: the 5-HT\textsubscript{1A} agonist 8-OH-DPAT decreased current amplitude and activation kinetics. B: peak currents evoked during voltage steps are plotted as a function of time. 5-HT and NAN-190, a 5-HT\textsubscript{1A} antagonist (both at 1.0 \textmu M), were applied as indicated (horizontal bars). NAN-190 diminished the effect of 5-HT on calcium channel current. C: 5-HT and ketanserin (both at 1.0 \textmu M) were applied as indicated (horizontal bars). The inhibition of current by 5-HT was unaffected by bath application of the 5-HT\textsubscript{2A/C} antagonist ketanserin. D: averaged (mean ± SE) inhibition of current (\% of control) induced by 5-HT (\(n = 101\)) and 8-OH-DPAT (black bars; \(n = 6\)) and by 5-HT in the presence of 5-HT receptor antagonists NAN-190 (\(n = 8\)), (+) WAY 100135 (\(n = 8\)), and ketanserin (\(n = 3\)). The 5-HT\textsubscript{1A} agonist 8-OH-DPAT mimicked and the 5-HT\textsubscript{1A} antagonists NAN-190 and (+) WAY 100135 attenuated the effects of 5-HT. The inhibition of current by 5-HT was not different in the presence of the 5-HT\textsubscript{2A/C} antagonist ketanserin. Differences among groups were highly significant \(F(4,117) = 44.2; P < 0.0001\). Asterisks: significantly different from 5-HT (\(P < 0.01\)).

tested the effect of nimodipine (10 \textmu M), a dihydropyridine antagonist of L-type calcium channels (\(\triangle\)), in this cell. Nimodipine had very little effect on the current, reducing it by only 1.4%. The current resistant to all three calcium channel blockers (29%) was completely suppressed by 0.1 mM CdCl\textsubscript{2} and displayed voltage-dependent and kinetic properties similar to those of the total current.

Averaged data illustrating the effects of these antagonists on calcium channel currents are provided in Fig. 4B. The percentage of current sensitive to each toxin was independent of the order of presentation of the toxins (not shown), indicating that the toxins each blocked distinct current components at the concentrations used. We found that most of the current (>70%) in caudal raphe neurons was contributed by the combination of \(\omega\)-CgTx-sensitive N-type and \(\omega\)-AgAlIVA-sensitive P/Q-type channels, approximately equally distributed between the two types (35.9 ± 2.1% and 36.8 ± 2.3%, respectively). There was essentially no dihydropyridine-sensitive, L-type current in caudal raphe neurons, because nimodipine inhibited only 1.8 ± 1.6% of the total calcium channel current. Finally, a substantial component of calcium current was resistant to the three antagonists, representing 27.0 ± 3.3% of the total current.

To determine which component(s) of calcium current were sensitive to 5-HT, we tested effects of 5-HT under control conditions and in the presence of the calcium channel antagonists, as shown in Fig. 4C. Application of 5-HT to this cell caused a marked inhibition of current (63%). After the P/Q-type current was blocked with 200 nM \(\omega\)-AgAlIVA, 5-HT inhibited a smaller percentage of current (37%), and after N-type current was blocked with 1 \mu M \(\omega\)-CgTx, only 7% of current was sensitive to 5-HT. Thus, of the 5-HT-sensitive current in this cell, 41% was P/Q type [i.e., (63 − 37)/63%], 48% was N type [i.e., (37 − 7)/63%], and 11% was resistant to the toxins (i.e., 7/63%). The residual current was completely blocked by 0.1 mM Cd\textsuperscript{2+}.

Averaged data revealed that of the ~49% of total current sensitive to 5-HT, 26.7 ± 2.6% was blocked by \(\omega\)-CgTx, 17.6 ± 2.2% was blocked by \(\omega\)-AgAlIVA, and only 3.7 ± 1.3% remained after application of both toxins. Of the 5-HT-sensitive current, 53.7 ± 3.7% was \(\omega\)-CgTx sensitive, N type, 43.3 ± 3.4% was \(\omega\)-AgAlIVA sensitive, P/Q type, and 6.3 ± 3.3% was resistant to the two blockers (Fig. 4D, top). Also, as expected from the lack of effect of nimodipine on total calcium channel current, the current inhibited by 5-HT was the same in the presence and absence of nimodipine (\(n = 3\); not shown). N-type current was somewhat more sensitive to 5-HT than was P/Q-type
5-HT inhibits predominantly N- and P/Q-type calcium currents. A: calcium channel currents were evoked at a number of membrane potentials under control conditions (•) and in the presence of the P/Q- and N-type calcium channel toxins ω-agatoxin IVA (ω-AgaIVA) (0.2 μM; ▲) and ω-conotoxin GVIA (ω-CgTx) (2 μM; □). Both toxins reduced the calcium current. The L-type channel antagonist nimodipine had no effect on current (●); the residual current remaining after application of these calcium channel antagonists was blocked by CdCl₂ (0.1 mM; ○). Inset: calcium channel currents evoked at 0 mV under the indicated conditions. B: averaged percentage of total calcium current that is N type, P/Q type, L type, and residual is plotted. C: peak currents were measured during voltage steps from −70 to 0 mV and plotted as a function of time. 5-HT (1.0 μM), ω-AgaIVA (0.2 μM), ω-CgTx (2.0 μM), and Cd²⁺ (0.1 mM) were applied at the times indicated (horizontal bars). After application of ω-AgaIVA, 5-HT inhibited a smaller fraction of current than in control, and after ω-CgTx, 5-HT had only a small effect on the residual current. D, top: percentage of 5-HT-sensitive calcium current that was N type, P/Q type, and residual. D, bottom: percentage of N-type, P/Q-type, and residual current inhibited by 5-HT. Numbers in parentheses: number of cells.

PTX-sensitive G proteins mediate calcium channel inhibition by 5-HT

In many neuronal cells, inhibition of calcium current is mediated by GTP-binding proteins that are sensitive to PTX (Anwyl 1991; Hille 1994). Two types of experiments were performed to test whether this was the case in caudal raphe neurons. In the first, cells were recorded with the use of pipette solutions in which GTP was replaced with the nonhydrolyzable analogue GTPγS. Under these recording conditions, as shown in Fig. 5A, 5-HT caused inhibition of current associated with kinetic slowing of the remaining current. However, in contrast to its effects with GTP in the pipette, the effect of 5-HT did not reverse on washing (cf. Fig. 2, A and B). In addition, a second application of 5-HT to this cell had little further effect on calcium channel current. In fact, when recorded with GTPγS in the pipette, the second response to 5-HT was reduced to 4.2% of the first response in the same cells (59.4 ± 1.9% vs. 2.5 ± 0.9%, n = 5; P < 0.0001). To test whether the G protein mediating effects of 5-HT in caudal raphe neurons was sensitive to PTX, interleaved recordings were made from alternate slices incubated in the presence or absence of PTX (1–2 μg/ml) at 37°C for ≥2
h before recording. As shown in Fig. 5B, the effect of 1.0 μM 5-HT on calcium channel current was completely blocked by pretreatment with PTX; 5-HT inhibited only 1.3 ± 1.1% of current in cells from slices incubated with PTX (n = 10), whereas it inhibited 46.2 ± 4.3% of current in cells from adjacent slices not treated with PTX (n = 11; P < 0.0001). The current inhibition in cells from those untreated adjacent slices was not different from that measured in the population of caudal raphe neurons (48.8 ± 12.2%, n = 101; see also Fig. 3D).

Voltage dependence of calcium current inhibition by 5-HT

Some forms of G protein-mediated inhibition of calcium currents by transmitters are voltage dependent, being relieved by strong depolarizations (Hille 1994; Penington and Kelly 1990; Penington et al. 1991). To determine whether this was the case in caudal raphe neurons, we tested the effects of 5-HT on calcium channel current with the use of the protocol shown in Fig. 6A (Elmslie et al. 1990). From a holding potential of −70 mV, currents were evoked during test pulses to 0 mV, before and after a depolarizing pulse to +70 mV. The time course for the effect of 5-HT is shown in Fig. 6B, which plots the current amplitude of both the first test pulse (●) and the second test pulse (■) in the top plot and the ratio of the two in the bottom plot (●). As seen in the sample traces of Fig. 6A, the currents induced by the first and second test pulse were similar in amplitude under control conditions, i.e., the ratio of current amplitudes during the test pulse before and after the depolarizing step (post/pre ratio) was close to 1.0. By contrast, in the presence of 1.0 μM 5-HT, the current during the first test pulse was diminished to a greater extent than during the test pulse following the depolarizing step, and the post/pre ratio increased to 1.61. On average, 1.0 μM 5-HT increased the post/pre ratio from 1.05 ± 0.02 to 1.52 ± 0.07. Thus the depolarizing step partially relieved the current inhibition by 5-HT. The effects of different concentrations of 5-HT on the post/pre ratio were well fitted with a logistic equation that predicted an EC50 of 0.1 μM, similar to that for current inhibition (cf. Fig. 2D), and a maximal post/pre ratio of 1.56 (Fig. 6C).

Effect of calcium current inhibition on repetitive firing behavior

We performed whole cell current-clamp experiments to investigate the role of N- and P/Q-type calcium channels, and their modulation by 5-HT, on the firing behavior of caudal raphe neurons. After application of a combination of ω-CgTx and ω-AgaIVA (2.0 and 0.2 μM, respectively), action potentials were smaller in amplitude and increased in duration (not shown); in addition, as shown in Fig. 7A, the AHP was reduced in amplitude. Averaged data revealed that the toxins diminished the AHP by ~25% (from −30.8 ± 0.2 mV to −23.1 ± 1.4 mV; P < 0.005, n = 5). Action potentials were also measured in the presence of 5-HT, after compensation with DC injection for the 5-HT-induced hyperpolarization (~15.2 ± 3.0 mV, n = 10) (see Bayliss et al. 1997). As shown in Fig. 7B, the AHP was diminished in 5-HT; averaged data showed that 5-HT inhibited the AHP by ~24% (from −37.2 ± 2.1 mV to −28.3 ± 1.7 mV; P < 0.0001). Effects of 5-HT on other characteristics of the spike (i.e., amplitude, duration, etc.) were variable. Thus the effect common to both calcium channel toxins and 5-HT was a decrease in the AHP amplitude.

The consequence on repetitive firing behavior of blocking N- and P/Q-type channels is shown in Fig. 8, A and B.
Long depolarizing rectangular current pulses of increasing amplitude were delivered via the recording electrode during control conditions and after application of ω-CgTx and ω-AgaIVA and the effects on instantaneous firing frequency during the first (▲, ●) and last interspike intervals (∆, ○) were determined. As described in the accompanying paper (Bayliss et al. 1997) and as evident in the sample traces (Fig. 8A), both initial and steady-state firing frequency increased with increased current injection. A decrementing pattern of firing was consistently observed during the current pulse, particularly with the larger current pulses, such that the initial firing frequency increased with current proportionally more than the steady firing rate, which tended to saturate. This characteristic decrementing firing pattern was apparent under both control conditions (Fig. 8A, left) and in the presence of the calcium channel toxins (Fig. 8B, right). The major effect of the toxins on firing behavior was to increase the slope of the f-I curve (Fig. 8B; cf. ▲ and ●); the steady-state firing frequency was not substantially increased by the toxins (Fig. 8B; cf. △ and ○). Thus, after N- and P/Q-type calcium channels were blocked with the calcium channel toxins, depolarizing current pulses caused increased firing in caudal raphe neurons especially early in the pulse. We tested the effects of 5-HT on repetitive firing behavior with the use of similar current pulses, but with DC injected to compensate for the 5-HT-induced hyperpolarization (Fig. 8, C and D). The effects of 5-HT were strikingly similar to those of the toxins: 5-HT increased the slope of the f-I curve (Fig. 8D; ■ and ○) without much effect on the steady-state f-I curve (Fig. 8D; □ and ○). So, inhibition of N- and P/Q-type calcium current in caudal raphe neurons, whether by application of calcium channel toxins or 5-HT, caused a decrease in the spike AHP and increased the early spike frequency response to current injection.

**DISCUSSION**

We characterized the calcium channel currents in serotonergic caudal raphe neurons of the neonatal rat and tested the effect of 5-HT on calcium currents and firing behavior in those cells. We found that caudal raphe neurons have a prominent HVA and a small LVA calcium current. The HVA current comprised ω-CgTx-sensitive N-type and ω-AgaIVA-sensitive P/Q-type currents in roughly equal proportions together with a smaller, toxin-insensitive residual component; caudal raphe neurons did not express an appreciable dihydropyridine-sensitive L-type current. We found that 5-HT acted via the 5-HT1A receptor through a PTX-sensitive G protein to inhibit N- and P/Q-type calcium channel current, with N-type channels being slightly more sensitive than P/Q-type channels; only a small fraction of calcium current remained sensitive to 5-HT after application of ω-CgTx and ω-AgaIVA. Current inhibition elicited by 5-HT was associated

**FIG. 6.** Current inhibition was partially relieved by strong depolarizations. A: voltage pulse protocol (top trace) that included a test pulse to 0 mV before and after a large depolarizing step to +70 mV was applied to test whether current inhibition by 5-HT was alleviated by strong depolarizations. Bottom traces: currents evoked under control conditions and during application of 5-HT. B: peak currents were measured during the test pulses before (pre; ◆) and after (post; ■) the depolarizing step and plotted as a function of time (top plot). Bottom plot: ratio of current amplitudes during the test pulse before and after the depolarizing step (post/pre ratio), plotted as a function of time. The ratio increased from near 1.0 in control to ~1.6 in the presence of 5-HT, before recovering again after wash. C: averaged (mean ± SE) post/pre ratio measured with the use of this protocol with different concentrations of 5-HT. The data were fitted with a logistic equation that predicted an EC50 of 0.1 μM and a maximal ratio of 1.56. Each point represents data from ≥3 cells.
with kinetic slowing and was relieved by strong depolarizing pulses, indicating that it was voltage dependent. Inhibition of N- and P/Q-type calcium channels, whether induced by calcium channel toxins or 5-HT, substantially decreased the AHP amplitude. These changes in the AHP were associated with an increase in the spike firing response to injected depolarizing current that was especially prominent early in the current pulse. Together these data suggest that 5-HT$_{1A}$ receptor-mediated inhibition of calcium current may act to increase the firing response to suprathreshold current inputs. Combined with the 5-HT-induced hyperpolarization described in the accompanying paper (Bayliss et al. 1997), the effect of 5-HT would be to increase the firing response to strong, suprathreshold inputs while inhibiting weaker inputs.

**Calcium currents in caudal raphe neurons**

The profile of calcium current recorded in caudal raphe neurons was similar to that of dorsal raphe neurons inasmuch as RDo cells also have very little LVA and L-type current and a prominent N-type component (Penington and Fox 1995; Penington et al. 1991). However, unlike the caudal raphe neurons reported here, in which ~37% of whole cell calcium current was $\omega$-AgAIVA sensitive, dorsal raphe neurons apparently have no $\omega$-AgAIVA-sensitive, P/Q-type current and instead express a relatively large residual calcium current component (Penington and Fox 1995). The reason for such disparate findings in these different raphe cell groups is unclear. Quantitative data regarding P/Q-type currents reported here were based on experiments in which 200 nM $\omega$-AgAIVA was used, whereas in the study of dorsal raphe neurons a concentration of 100 nM was employed; however, both concentrations should be expected to block completely the P/Q-type current component, and therefore it is unlikely that this accounts for the difference. Two other methodological differences between the studies are noteworthy in this context. Dorsal raphe neurons were isolated enzymatically from young adult rats, whereas caudal raphe neurons were studied in situ in a thin slice preparation from neonatal rats. Although it is possible that protease treatment affected the toxin sensitivity of the dorsal raphe neuronal calcium current, a number of investigators have recorded $\omega$-AgAIVA-sensitive, P/Q-type channels from protease-treated, acutely isolated neurons (Foehring 1996; Ishibashi and Akaike 1995a,b; Randall and Tsien 1995; Rusin and Moises 1995; Surmeier et al. 1995; Yan and Surmeier 1996). Alternatively, perhaps the differences reflect the postnatal age of the animals studied. This would imply the interesting possibility that expression of P/Q-type channels, presumably $\alpha_{1A}$ calcium channel subunits (Stea et al. 1994), is developmentally regulated in raphe neurons, being present in neonates and absent in older animals. To our knowledge, there is no information at present regarding the developmental pattern of expression of $\alpha_{1A}$ calcium channel subunits in brain stem raphe neurons. Finally, it is also possible that the complement of channels expressed in the different raphe cell groups is truly distinct, irrespective of postnatal age. Certainly the phenotypes of caudal and dorsal raphe neurons are different in many other respects, e.g., peptide expression, projection pattern, etc. (Jacobs and Azmitia 1992; Skagerberg and Bjorklund 1985).

**Calcium current inhibition by 5-HT**

We found that 5-HT inhibited approximately half of the calcium channel current in ~90% of caudal raphe neurons tested through a 5-HT$_{1A}$ receptor-mediated mechanism. In this respect the current inhibition by 5-HT in ROb and RPa was similar to that reported in RDo, in which 5-HT$_{1A}$ receptor activation caused ~49% inhibition in 83% of the cells (Penington et al. 1991). It is noteworthy that the degree of current inhibition by 5-HT when rectangular voltage steps are used may actually underestimate the magnitude of 5-HT-induced calcium current inhibition that occurs during a more physiologically relevant voltage waveform, such as an action potential (Penington et al. 1992).

The major difference in the calcium current inhibition by 5-HT in caudal and dorsal raphe neurons was the contribution of P/Q-type current to the 5-HT-sensitive current, a difference that reflects the relative absence of any P/Q-type current in RDo neurons (Penington and Fox 1995). Thus ~37% of total calcium current and ~43% of the 5-HT-sensitive current was sensitive to $\omega$-AgAIVA in ROb and RPa neurons, whereas none of the calcium current (5-HT-sensitive or otherwise) was blocked by $\omega$-AgAIVA in RDo neurons (Penington and Fox 1995).

The receptor and calcium channel pharmacology of the inhibitory effect of 5-HT on calcium current that we report here (i.e., 5-HT$_{1A}$ receptor inhibition of N- and P/Q-type channels) is similar in many respects to that reported for 5-HT in other central neurons studied to date (Bayliss et al. 1995; Foehring 1996; Koike et al. 1994). For example, 5-HT$_{1A}$ receptors mediate calcium current inhibition in hypoglossal motoneurons (Bayliss et al. 1995), cortical neurons (Foehring 1996), and ventromedial hypothalamic neurons (Koike et al. 1994). In addition, 5-HT targeted both N- and P/Q-type channels in hypoglossal motoneurons and in cortical pyramidal neurons (Bayliss et al. 1995; Foehring 1996). Similar to caudal raphe neurons reported here, N-type channels in hypoglossal motoneurons were more potently inhibited by 5-HT than were P/Q-type channels (Bayliss et al. 1995). In ventromedial hypothalamic neurons, N-type channels were shown to be inhibited by 5-HT; involvement of P/Q-type channels was not tested in those cells (Koike et al. 1994).

Inhibition of both N- and P/Q-type channels by a number of neurotransmitter receptors e.g., $\gamma$-aminobutyric acid-B (GABA$_B$), muscarinic (m$_2$/m$_4$), D$_1$ dopaminergic, and $\alpha_2$-adrenoceptors has also been reported in other cell systems,
Inhibition of N- and P/Q-type calcium channels enhances the initial firing response to current injection. The effect of 2-s rectangular current pulses of increasing amplitude on firing behavior of caudal raphe neurons was determined. A: representative records of firing responses to 3 current pulses (20, 40, and 60 pA) during control and after application of a cocktail of channel toxins (ω-CgTx and ω-AgaIVA, 2.0 and 0.2 μM, respectively). The toxins increased the current-induced firing frequency response, especially early in the pulse. Dotted line: maximum level of the AHP under control conditions, showing the reduction in AHP amplitude induced by the toxins.

B: averaged (mean ± SE; n = 7) firing frequency response to current injection for the initial (▲, ●) and the final interspike intervals (●, ○) was determined in control (●, ○) and in the presence of the calcium channel toxins (▲, △). Membrane potential before the current pulses averaged ~54 ± 2.7 mV and ~53 ± 2.8 mV in control and toxins, respectively. 

C: firing responses to the same 3 current pulses before and during application of 5-HT (1.0 μM). In these experiments, depolarizing DC was injected to compensate for the 5-HT-induced hyperpolarization and return the membrane potential to control levels (~52 ± 2.5 mV and ~50 ± 2.5 mV in control and 5-HT, respectively). Similar to the effects of the toxins, 5-HT caused an increase in the current-induced firing frequency response that was associated with a decrease in the AHP (dotted line). D: averaged (mean ± SE; n = 10) firing frequency response to current injection for the initial (●, ●) and the final interspike intervals (●, ○) was determined in control (●, ○) and in the presence of 5-HT (●, ○). The effects of toxins and 5-HT were similar; both increased the initial firing frequency response with little effect on steady-state firing frequency at the end of the pulse. All sample traces are from the same serotonergic raphe neuron; 5-HT was applied before toxins in this cell.

and in general a greater fraction of N- than P/Q-type current was targeted for inhibition by the neurotransmitters (Amico et al. 1995; Ishibashi and Akaiki 1995a; Mintz and Bean 1993; Rusin and Moises 1995; Surmeier et al. 1995; Yan and Surmeier 1996). By contrast, in rat hippocampal neurons, which express both N- and P/Q-type channels, only the N type was modulated by somatostatin (Ishibashi and Akaiki 1995b) and in CA3 neurons adenosine A1 receptors inhibited N-type current, whereas A2 receptors enhanced P/Q-type current (Mogul et al. 1993). Thus joint inhibition of N- and P/Q-type channels by neurotransmitters, as we have reported here, is not an uncommon phenomenon although differential effects on N- and P/Q-type channels have also been observed.

Involvement of a PTX-sensitive G protein in calcium current inhibition by 5-HT

We found that the inhibition of calcium current by 5-HT was mediated by a PTX-sensitive G protein, indicating that the heterotrimeric G protein coupling 5-HT1A receptors to calcium channels includes a Go-protein of the Gi/Go class. Previously, the inhibition of calcium current by 5-HT1A receptors was found to involve G proteins, but the PTX sensi-
tivity of the G protein was not directly demonstrated (Foehring 1996; Penington et al. 1991). In cortical neurons, the inhibition of calcium current by 5-HT$\textsubscript{1A}$ receptors was blocked by n-ethylmaleimide (Foehring 1996), a sulphhydryl alkylating agent that preferentially interferes with effects involving $G_i/G_o$ (Shapiro et al. 1994). Although this result suggested involvement of $G_i/G_o$ proteins, intracellular application of the a protomer of PTX in that same study did not attenuate 5-HT-induced inhibition of calcium current (Foehring 1996). Our work demonstrates clearly that a PTX-sensitive G protein is involved in mediating calcium current inhibition by 5-HT in caudal raphe neurons. These results are not surprising; calcium current inhibition that involves PTX-insensitive G proteins has occasionally been reported, but far less commonly than current inhibition mediated by PTX-sensitive G proteins (Anwyl 1991; Hille 1994).

Voltage dependence of calcium current inhibition by 5-HT

Inhibition of calcium current by 5-HT was associated with a slowing of activation kinetics, partially relieved after large depolarizing pre pulses and associated with a slight depolarizing shift in the peak of the $I$-$V$ curve. These characteristics of current inhibition have been attributed to a transmitter-induced shift in the voltage dependence of activation of calcium channels from less (willing) to more (reluctant) depolarized potentials (Bean 1989; Hille 1994). Slowing of calcium current activation kinetics was evident at all effective concentrations of 5-HT and the $E_{\text{Ca}}$ for voltage-dependent relief of current inhibition was identical to that for current inhibition (cf. Figs. 2 and 6). Thus 5-HT-induced kinetic slowing and relief of current inhibition by depolarizing pre pulses developed with a concentration dependence similar to that of current inhibition. In some cell types, such voltage-dependent phenomena were not apparent at low agonist concentrations with modest levels of current inhibition, but required higher agonist concentrations (Diversé-Pelfrissi et al. 1995; Foehring 1996; but see Kasai 1992). The voltage dependence of calcium current inhibition is similar to that induced by 5-HT in dorsal raphe neurons and cortical neurons (Foehring 1996; Penington and Kelly 1990; Penington et al. 1991) and is often, but not exclusively, associated with calcium current inhibition mediated by PTX-sensitive G proteins (Hille 1994). It has been suggested that the voltage dependence of inhibition may reflect direct binding of a G protein probably the $G_{\beta\gamma}$-heterodimer (Herlitze et al. 1996; Ikeda 1996) to calcium channels through a membrane-delimited mechanism (Bean 1989; Boland and Bean 1993; Hille 1994). In this respect, a membrane-delimited transduction pathway has been proposed to mediate current inhibition by 5-HT in dorsal raphe neurons and in cortical neurons (Foehring 1996; Penington et al. 1991). Although it was not directly tested in the present experiments, it seems likely that a similar membrane-delimited mechanism may account for inhibition of calcium current by 5-HT in caudal raphe neurons.

Effect of 5-HT and calcium current inhibition on firing behavior

We showed that inhibition of N- and P/Q-type calcium currents was associated with changes in AHP amplitude and with changes in the relationship between current input and firing frequency output. This was true regardless of whether the calcium current inhibition was obtained by application of channel toxins or 5-HT, suggesting that effects of 5-HT on the AHP were mediated secondary to calcium current inhibition. Thus we propose that 5-HT-induced inhibition of the AHP is due to inhibition of calcium current and a resultant decrease in the calcium entry required for activation of the AHP conductance. The decrease in AHP amplitude, which is a primary determinant of repetitive firing behavior (Baldissera and Gustafsson 1974), likely accounts for the 5-HT-induced increase in firing frequency response to current injection that we observed. These data are similar to those reported by Bayliss et al. (1995), who also showed that 5-HT$\textsubscript{1A}$ receptor activation inhibits calcium current and the AHP in hypoglossal motoneurons. Furthermore, our data provide experimental support for the hypothesis of Penington and Kelly (1990), who suggested that inhibition of calcium current could decrease the AHP amplitude and thereby modify the firing behavior of 5-HT-sensitive raphe neurons.

It is important to point out that we have not ruled out a direct effect of 5-HT on the calcium-activated $K^+$ conductance that underlies the AHP. In this respect, a direct inhibition of the AHP conductance by 5-HT has been reported in rat hippocampal neurons and in lamprey spinal neurons (Andrade and Chaput 1991; Andrade and Nicoll 1987; Pedarzani and Storm 1993; Wallén et al. 1989; Wikström et al. 1995). In the hippocampus, a distinctly different mechanism involving 5-HT$\textsubscript{1A}$ receptors and activation of PKA was shown to mediate inhibition of the AHP (Andrade and Chaput 1991; Pedarzani and Storm 1993). In lamprey spinal neurons, 5-HT$\textsubscript{1A}$ receptor activation caused inhibition of the AHP (Wikström et al. 1995). In these other neurons, a direct effect on the calcium-activated $K^+$ conductance was proposed because the AHP was inhibited by 5-HT in the absence of any measurable change in calcium current (inferred from the calcium action potential waveform) (Andrade and Nicoll 1987; Wallén et al. 1989). However, our demonstration that the effects of 5-HT on the AHP and on repetitive firing behavior were indistinguishable from those of the calcium channel toxins (which have no direct effect on the AHP conductance) suggests that an additional direct effect on the calcium-activated $K^+$ conductance need not be invoked to explain effects of 5-HT on the AHP and firing behavior in caudal raphe neurons.

Functional implications of 5-HT effects on caudal raphe neurons

The question arises as to the potential functional consequences of the effects of 5-HT on serotonergic caudal raphe neurons described in this and the accompanying paper (Bayliss et al. 1997). Together these two mechanisms may provide a feedback inhibition of caudal raphe neuronal activity, and thus limit the serotonergic tone imposed on neurons in target areas (e.g., ventral and intermediolateral horns of the spinal cord). According to this scheme 1) activation of inwardly rectifying $K^+$ channels by 5-HT would inhibit the firing of caudal raphe neurons, thereby decreasing activity-dependent 5-HT release, and 2) inhibition of calcium current by 5-HT, if present at serotonergic terminals, would directly decrease calcium-dependent transmitter release (i.e., presyn-
aptic inhibition). It would appear that decreased spontaneous activity of caudal raphe neurons via 5-HT$_{1A}$ receptor-mediated hyperpolarization is probable, by analogy with the well-documented tonic autoinhibition of neuronal activity by 5-HT$_{1A}$ receptors in RDo (Fornal et al. 1996; reviewed in Jacobs and Azmitia 1992). However, the role of 5-HT$_{1A}$ receptors in mediating presynaptic inhibition by 5-HT is less clear. In fact, the predominant homonymous presynaptic receptor on serotonergic nerve terminals in the rat may be the 5-HT$_{1B}$ subtype (Hoye et al. 1994). If the 5-HT$_{1A}$ receptor does not contribute to presynaptic inhibition at serotonergic terminals, our results suggest another possible function of calcium current inhibition by 5-HT. The decrease in the AHP and increased firing response to current inputs evoked by 5-HT would enhance effects of brief suprathreshold synaptic inputs. Coupled with the 5-HT$_{1A}$-induced hyperpolarization, which would gate out smaller synaptic inputs, the cumulative effect would be to enhance strong inputs and inhibit weak inputs onto caudal raphe neurons (i.e., to increase the signal-to-noise ratio of the synaptic input–firing frequency transfer function).

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