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5-Hydroxytryptamine$_4$ Receptors Reduce Afterhyperpolarization in Hippocampus by Inhibiting Calcium-Induced Calcium Release

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SUMMARY

Serotonin acting on 5-hydroxytryptamine$_4$ receptors increases membrane excitability in CA1 hippocampal pyramidal cells by reducing the slow calcium-activated afterhyperpolarization. This effect is mediated through an increase in cAMP and activation of protein kinase A, although subsequent steps have not been elucidated. We now report that a significant portion of the calcium responsible for the generation of the afterhyperpolarization originates from the release of intracellular calcium through a calcium-induced calcium-release mechanism. Thus, the afterhyperpolarization is enhanced by caffeine, whereas it is inhibited by dantrolene and ruthenium red, two blockers of calcium-induced calcium release. The afterhyperpolarization is also inhibited by thapsigargin, which depletes intracellular calcium stores. These observations raised the possibility that serotonin might reduce the afterhyperpolarization by regulating calcium-induced calcium release. Consistent with this possibility, administration of calcium-induced calcium-release blockers, as well as of thapsigargin, occluded the ability of serotonin to inhibit the afterhyperpolarization. Similarly, administration of caffeine, which enhances the contribution of calcium-induced calcium release to the afterhyperpolarization, enhanced the effect of serotonin. These results indicate that serotonin inhibits the afterhyperpolarization in the CA1 region of hippocampus by reducing the ability of extracellular calcium to trigger calcium release from intracellular stores. As such, they identify a physiological role for the calcium-induced calcium release in hippocampus and provide evidence for its regulation by G protein-coupled receptors and, more specifically, 5-hydroxytryptamine$_4$ receptors.

In hippocampal pyramidal cells, action potentials are followed by an AHP (1, 2). This mechanism, which is common to a variety of neuronal cell types, serves to limit firing in response to a sustained excitation in a process known as spike frequency adaptation. Several neurotransmitters, including serotonin (5-HT), reduce this AHP, thus increasing the excitability of the target cells (3). We have shown previously that serotonin acts on serotonin receptors of the 5-HT$_4$ subtype to elicit this effect (4–7) and that the signaling mechanism involves cAMP and PKA (8). The mechanisms by which PKA reduces the AHP, however, have not been elucidated.

In sympathetic neurons and in some central neurons, CICR from intracellular calcium stores can contribute a significant fraction of the intracellular calcium responsible for the generation of the AHP (9, 10). Therefore, agents that increase CICR enhance the AHP, whereas those that inhibit CICR reduce this afterpotential. In the present study, we have used this general approach to examine the role of CICR in the generation of the AHP in the CA1 region of hippocampus and its possible regulation by serotonin.

Materials and Methods

Hippocampal brain slices were prepared using conventional techniques as described previously (8). Recordings were obtained from pyramidal neurons of the CA1 region in a recording chamber in which the slice was held submerged in Ringer solution (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose) bubbled to equilibrium with 95% O_2, 5% CO_2 at 30 ± 1°. Intracellular recordings were obtained generally by using sharp microelectrodes filled with 2M $KMeSO_4$ (80–120 MΩ). Experiments that were intended to test the effect of ruthenium red were conducted using the blind, whole-cell, tight-seal recording technique. In these experiments, the intracellular recording solution was composed of 120 mM $KMeSO_4$, 5 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 μM EGTA, 10 mM HEPES, and the osmolarity

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ABBREVIATIONS: AHP, afterhyperpolarization; 5-HT, 5-hydroxytryptamine; CICR, calcium-induced calcium release; PKA, protein kinase A; ANOVA, analysis of variance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DMPX, 3,7-dimethyl-1-propargylxanthine.

was adjusted to 285 mOsM using $KMeSO_4$. Electrode resistances ranged from 3 to 8 MΩ when filled with this solution.

In most experiments, action potentials were triggered using depolarizing constant current pulses (3 msec, 0.8–1.5 nA) delivered at 50 Hz. The amplitude of the resulting AHP was measured 150–200 msec after the end of the stimulus to minimize contamination by the medium-duration AHP that precedes the slow AHP in these cells (11). In a few experiments, calcium action potentials were used to elicit the AHP. In this case, recordings were obtained in the presence of tetrodotoxin (1 μM) and tetraethylammonium (5 mM) and 70-msec-duration depolarizing constant current pulses were used to trigger an all-or-none calcium spike as described previously (8). All recordings were obtained in the presence of BMY 7378 (3 μM) in the bath to block the 5-HT_{1A} receptors that are also expressed on these cells and that would result in a large hyperpolarization and decrease in input resistance upon administration of serotonin.

In most experiments, a sequence of 1, 2, 3, 5, 10, and 20 action potentials was triggered to determine control AHPs. A drug was then administered and the stimulation was repeated to assess the effect of the drug on the AHP. In this experimental design, each cell served as its own control and comparisons between groups were accomplished using a factorial ANOVA as implemented in GB-STAT (version 5.0 for Windows; Dynamic Microsystems, Silver Spring, MD). This procedure takes into consideration the experimental design, in which repeated measures were obtained along both factors. In a few experiments, such as those focused on examining the effects of ruthenium red and thapsigargin, it was necessary to compare groups of cells recorded in the presence or absence of these drugs. Statistical analysis of these experiments was accomplished using a repeated measures factorial ANOVA using the number of spikes in the burst as the repeated measure. To compare the effectiveness of a treatment (e.g., caffeine) under two conditions (e.g., control and dantrolene), the factorial ANOVA was conducted on the difference between control and treatment.

Most drugs were administered in the bath, dissolved at known concentrations, as indicated in the text. Ruthenium red was injected passively into the cell by dissolving it at a concentration of 30 μM in the intracellular recording solution. All tests were conducted at least 10 min after obtaining the whole-cell configuration to allow for diffusion of the ruthenium red to the cell interior. Control whole-cell

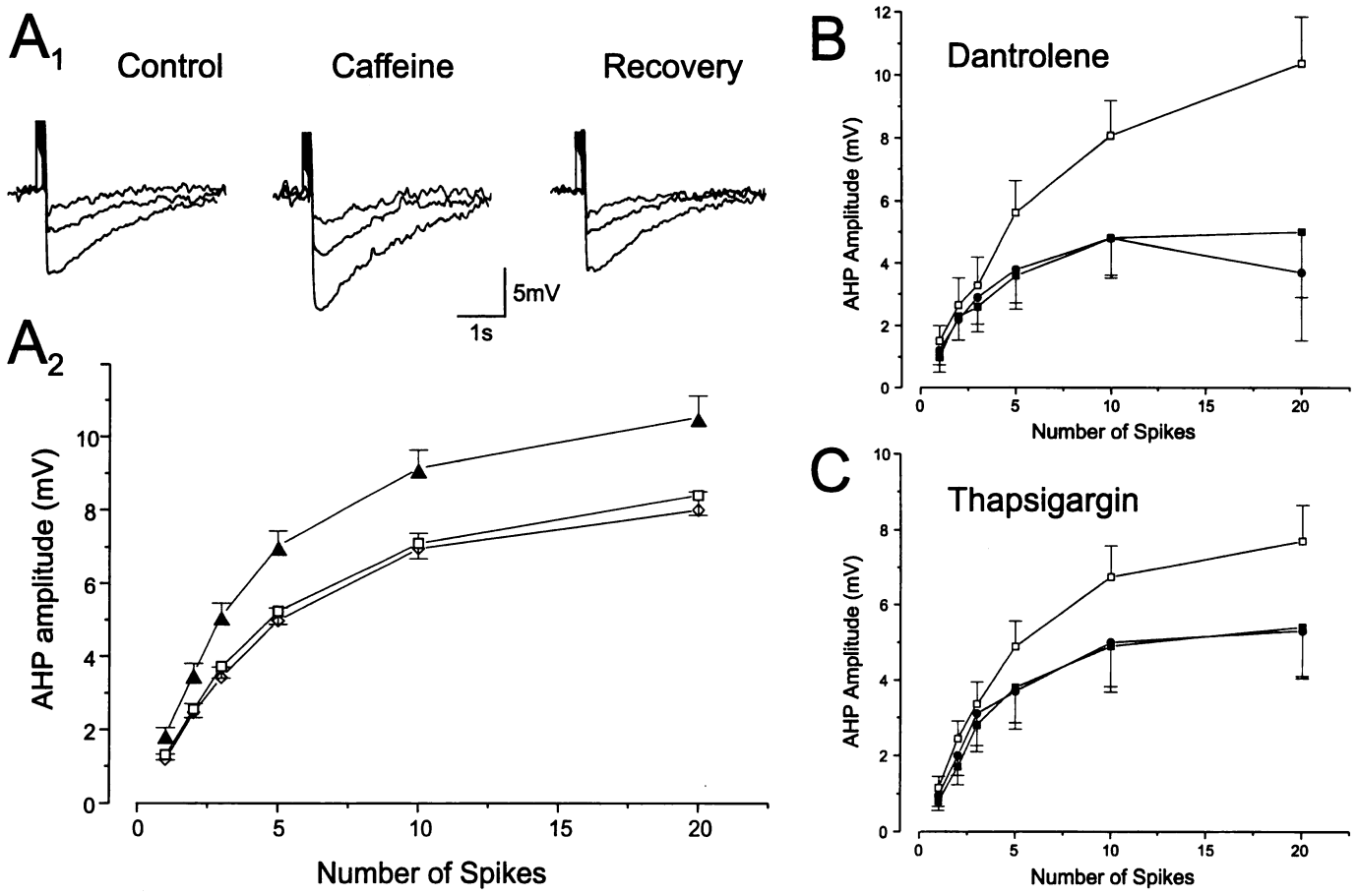

Fig. 1. Effect of caffeine and blockers of CICR on the AHP in CA1 pyramidal neurons. A, Constant current pulses were used to trigger 1–20 spikes, which were followed by an AHP with an amplitude that depended on the number of spikes in the burst ($F = 231$, $p < 0.001$). Previous studies have shown that this AHP is mediated by calcium-activated potassium currents (18). Administration of caffeine in the bath (1 mM) elicited an increase in the amplitude of the AHP. This effect recovered upon removal of the caffeine from the bath. A_1, Representative traces corresponding to AHPs after 1, 3, and 10 spikes under control conditions (*left*), 1–2 min after administration of 1 mM caffeine (*center*), and 10 min after removal of caffeine from the bath (*right*). A_2, Summary graph of the effects of caffeine on the amplitude of the slow AHP. □, Control; ▲, 1 mM caffeine; ◇, recovery. *Error bars*, mean ± standard error. B, Bath application of the calcium-release channel blocker dantrolene (30 μM, 60 min) elicited a significant reduction of the amplitude of the AHP ($n = 10$ cells tested) and blocked the effect of caffeine ($n = 6$ cells tested). □, Control; ■, dantrolene 30 μM; ●, caffeine in the presence of dantrolene. *Error bars*, mean ± standard error. Note that in the group of cells tested with caffeine, this compound was tested before administration of dantrolene and was found to produce significant enhancements of the AHP ($F = 65$, $p < 0.001$). For the sake of clarity, these data are not plotted in this graph. C, Pretreatment with the intracellular-type calcium pump inhibitor thapsigargin also results in a reduction in the amplitude of the AHP and a blockade of the action of caffeine. □, Control; ■, thapsigargin 1–10 μM; ●, caffeine in the presence of thapsigargin. *Error bars*, mean ± standard error. Note that caffeine was also tested in the control group and found to produce a significant increase in the AHP ($F = 197$, $p < 0.001$). These data are not illustrated in this figure, again for the sake of clarity.

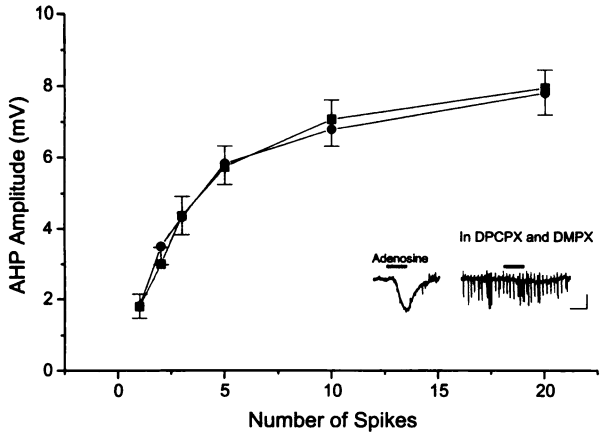

Fig. 2. Effect of adenosine receptor antagonists on the AHP. Bath administration of the adenosine antagonists DPCPX (1 μM) and DMPX (10 μM) had no effect on the amplitude of the AHP in CA1 pyramidal neurons (n = 7 cells tested). However, they blocked the hyperpolarization induced by bath administration of adenosine (50 μM, *inset*). *Error bars*, mean ± standard error. Administration of these antagonists resulted in an increase in membrane noise. A similar increase in membrane noise was observed after caffeine (see Fig. 1A$_1$), which suggests that it was the result of adenosine receptor blockade. Because this noise was inhibited by administration of tetrodotoxin, it presumably reflected increased synaptic activity.

recordings were obtained over a comparable time period. Slices were incubated in 1 μM thapsigargin in an oxygenated static bath for 2–4 hr to inhibit intracellular calcium pumps. Once in the recording chamber, slices were perfused with 10 μM thapsigargin for 40–60 min.

Results

As illustrated in Fig. 1A$_1$, when a pyramidal cell of the CA1 region is stimulated to trigger one or more action potentials, the resulting calcium influx elicits a slow AHP, the amplitude of which is dependent on the number of action potentials in the burst (Fig. 1A$_2$). We first tested whether CICR is present in these neurons and contributes to the AHP by using caffeine, which at low millimolar concentrations enhances the ability of calcium to open the calcium-release channels (12). As illustrated in Fig. 1A, bath administration of caffeine (1 mM) enhanced the AHP elicited by 1–20 action potentials (n = 34 cells, F = 136.1, $p < 0.001$) and this effect recovered upon removal of caffeine from the bath. A second administration of caffeine resulted in a comparable enhancement of the AHP, which indicated that there was no desensitization to this effect of caffeine (n = 2 cells tested; data not shown).

In addition to acting on CICR, caffeine also antagonizes

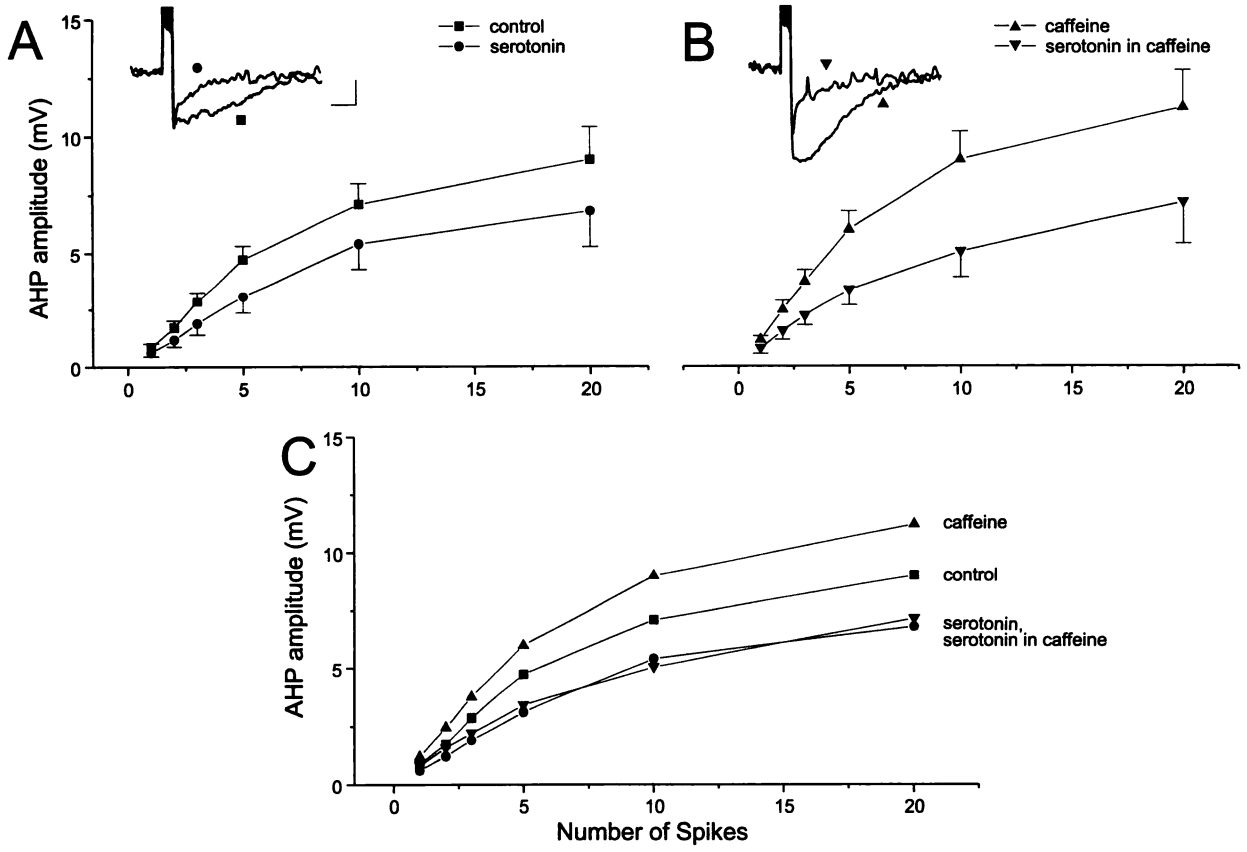

Fig. 3. Effect of serotonin on the AHP elicited in the presence of caffeine. A, Bath administration of serotonin (10 μM) to a group of five cells elicits a significant reduction in the amplitude of the AHP (F = 9.4, $p < 0.05$). B, As illustrated previously, administration of caffeine (1 mM) to the same group of cells results in an enhancement of the AHP. In the presence of caffeine, the efficacy of serotonin in reducing the AHP is significantly increased (F = 48.3 $p < 0.05$). C, This increase in the effect of serotonin can be seen most clearly when the *curves* (minus the *error bars*) obtained for this group of cells under the different conditions are superimposed. In the presence of caffeine (1 mM), the effect of serotonin was essentially doubled. Notice that serotonin reduced the AHPs obtained in the absence and presence of caffeine to the same absolute level. ■, Control; ●, serotonin; ▲, caffeine; ▼, serotonin in the presence of caffeine. *Error bars*, mean ± standard error. *Insets*, AHP recordings obtained from a single cell after a burst of 10 action potentials under control conditions and after serotonin (A) and in caffeine and after serotonin in caffeine (B). *Calibration bar*, 500 msec, 2.5 mV.

adenosine receptors. To control for this effect, we tested whether adenosine antagonists could also enhance the AHP. Concurrent bath administration of the A_1 receptor antagonist DPCPX (1 μM) and the A_2 antagonist DMPX (10 μM) greatly reduced or blocked the membrane hyperpolarization elicited by bath-administered adenosine (50 μM; Fig. 2, *inset*) but had no detectable effect on the AHP (n = 7 cells, F = 0.016, p = 0.89; Fig. 2). Caffeine is also an effective phosphodiesterase inhibitor. However, an effect at the level of the phosphodiesterase is also unlikely because phosphodiesterase inhibitors reduce rather than enhance the AHP (4, 13).

To examine further the possible contribution of CICR to the AHP, we determined the effects of the calcium-release channel blockers dantrolene and ruthenium red (14). As illustrated in Fig. 1B, bath administration of dantrolene (30 μM) markedly reduced the amplitude of the AHP (n = 10 cells, F = 11.6, $p < 0.01$) and blocked its potentiation by caffeine (n = 6 cells tested). Similarly, intracellular injection of ruthenium red, a second blocker chemically unrelated to dantrolene, also elicited a significant reduction in the amplitude of the AHP (F = 262, $p < 0.001$, n = 6 cells) and again blocked the effect of caffeine (n = 4 cells tested; data not shown). Ryanodine, a third inhibitor of CICR, was also tested in these experiments. No consistent reduction of the AHP or the effect of caffeine was observed in the majority of cells tested (n = 9 cells). We have no explanation for the failure of ryanodine to consistently inhibit the AHP.

If some of the calcium contributing to the calcium rise that was responsible for the generation of the AHP originated from intracellular calcium release, depleting intracellular calcium stores should also reduce the AHP. This was tested using thapsigargin, a selective inhibitor of the endoplasmic

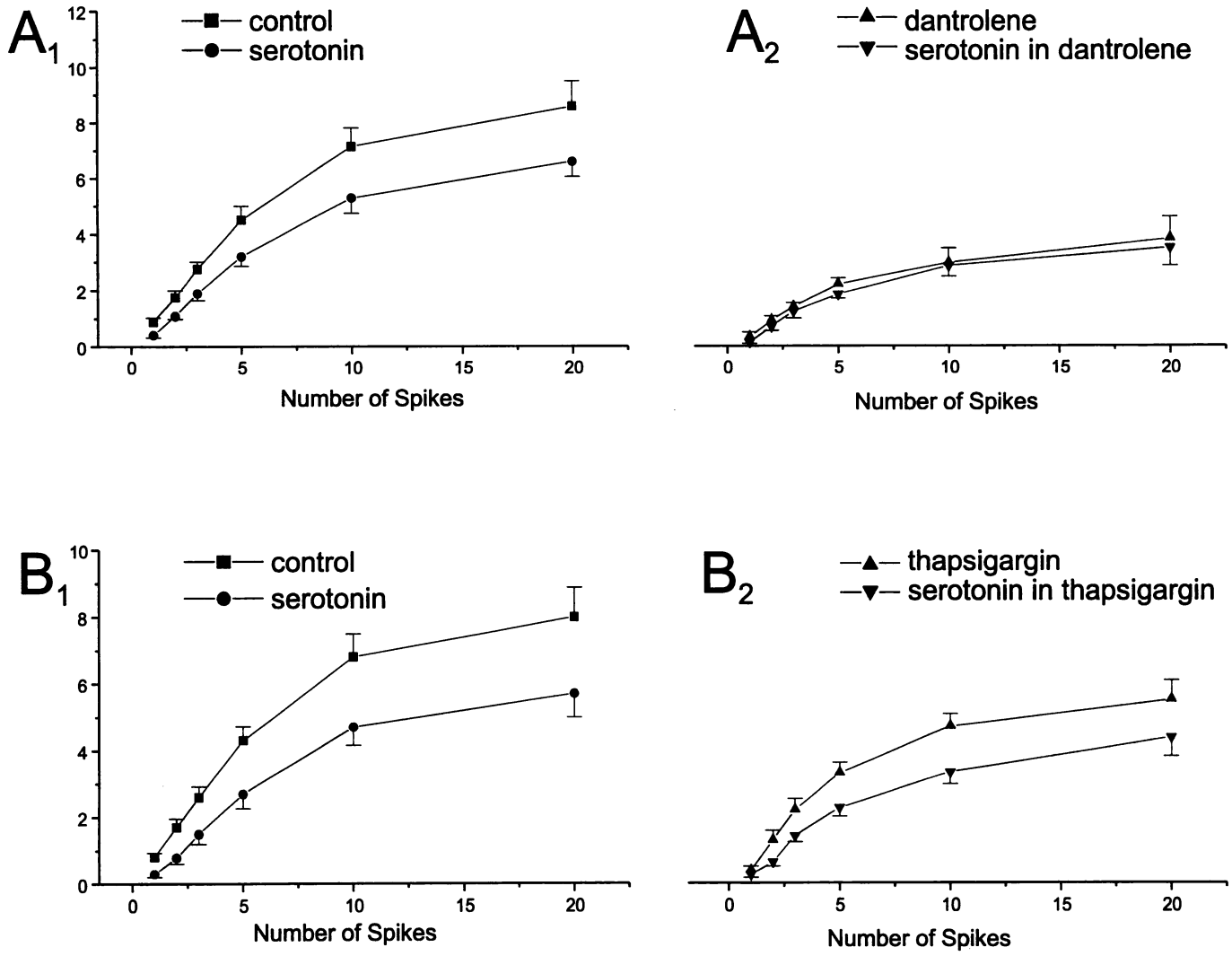

Fig. 4. Effect of inhibitors of intracellular calcium release on the ability of serotonin to reduce the AHP. A, Effect of dantrolene on the serotonin response. A_1, Bath administration of serotonin (10 μM) reduces the amplitude of the AHP in a group of six cells tested. A_2, Administration of dantrolene (30 μM) to these cells results in a reduction in the amplitude of the AHP. A second administration of serotonin in the presence of dantrolene has little effect on the amplitude of the AHP. This failure to reduce the AHP does not reflect desensitization because, as illustrated in Fig. 2, serotonin can be applied repeatedly without loss of the response. Statistical comparison of the effects of serotonin under control conditions and in the presence of dantrolene indicated that dantrolene significantly reduced the ability of serotonin to reduce the AHP (F = 22.5, $p < 0.01$). ■, Control; ●, serotonin; ▲, dantrolene; ▼, serotonin in dantrolene. B, Effect of thapsigargin on the serotonin response. B_1, Bath administration of serotonin (10 μM) reduces the amplitude of the AHP in a group of 12 cells. B_2, In a parallel group of seven cells pretreated with thapsigargin, serotonin was found to be significantly less effective in reducing the AHP (F = 5.1, $p < 0.05$). ■, Control; ●, serotonin; ▲, thapsigargin; ▼, serotonin in thapsigargin.

reticulum calcium pump that can deplete intracellular calcium stores (15). As illustrated in Fig. 1D, thapsigargin pretreatment elicited a significant reduction in the amplitude of the AHP ($F = 22$, $p < 0.001$, $n = 11$ cells). As with the calcium-release channel blockers, thapsigargin pretreatment also blocked the enhancement of the AHP elicited by caffeine ($n = 11$ cells tested). These results indicated that, in addition to extracellular calcium influx, CICR contributes a significant proportion of the calcium responsible for the intracellular calcium rise that triggers the AHP.

Serotonin acting through receptors of the 5-HT_4 subtype increases membrane excitability in the CA1 region by reducing the amplitude of the AHP (4, 6). Although cAMP and PKA are necessary mediators for this effect (8), the precise mechanism underlying this response is not known. The observation that a component of the AHP is mediated through CICR and that the intracellular calcium-release channels are targets for PKA phosphorylation (16) raised the possibility that serotonin might reduce the AHP by acting on CICR. If this were the case, serotonin should be more effective when tested in the presence of caffeine, which increases the relative contribution of CICR to the AHP. As illustrated in Fig. 3, the reduction of the AHP elicited by serotonin was almost doubled in the presence of caffeine ($n = 5$ cells, $F = 48.3$, $p < 0.05$). Moreover, as illustrated in Fig. 3C, the AHPs observed in the presence of serotonin under control conditions or after caffeine were superimposable. This suggested that serotonin selectively inhibited only a portion of the AHP, that which is enhanced by caffeine and hence involves CICR.

If serotonin reduced the AHP by inhibiting CICR, then after inactivation of this mechanism, serotonin should be much less effective in reducing the remaining AHP, which would then presumably result predominantly or solely from extracellular calcium influx. In agreement with this prediction, serotonin was significantly less effective in reducing the AHP in the presence of dantrolene than under control conditions (Fig. 4A; $n = 6$ cells tested, $F = 22.5$, $p < 0.01$). A similar reduction in the effectiveness of serotonin was seen after intracellular injection of ruthenium red ($F = 57$, $p < 0.001$, $n = 5$ cells; data not shown). Finally, as illustrated in Fig. 4B, the ability of serotonin to reduce the AHP was also significantly reduced after depletion of intracellular calcium stores with thapsigargin ($F = 5.1$, $p < 0.05$, $n = 7$ cells tested).

Dantrolene, ruthenium red, and thapsigargin could block the effects of serotonin simply by inhibiting the function of the 5-HT_4 receptor. We have shown previously that 5-HT_4 receptors, in addition to reducing the AHP, also elicit a slow membrane depolarization (5–7). This serotonin-induced depolarization could still be observed in the presence of dantrolene, ruthenium red, or thapsigargin. This is illustrated for dantrolene in Fig. 5 in a cell that had a particularly robust and stable depolarizing response to serotonin. Unfortunately, over all, the serotonin depolarization is variable from cell to cell and difficult to generate repeatedly on a given neuron. This made it impractical to attempt a quantitative analysis of the effects of the calcium-release channel blockers and thapsigargin on the depolarization. Nevertheless, qualitatively, more than half of the cells (four of six) used for the dantrolene experiment illustrated in Fig. 4A responded initially to serotonin with a clear membrane depolarization. Of those four cells, three were still depolarized by serotonin after dantrolene administration. A similar proportion of the cells treated with thapsigargin responded to serotonin with a detectable depolarization (four of seven cells). Finally, three of the six cells injected with ruthenium red responded to serotonin with a depolarization, whereas three of the five cells tested in the parallel whole-cell controls were similarly depolarized. These results suggested that the calcium-release channel blockers and the thapsigargin did not inhibit the 5-HT_4-induced depolarization and, hence, did not reduce the ability of serotonin to inhibit the AHP simply by impairing 5-HT_4 receptor functioning.

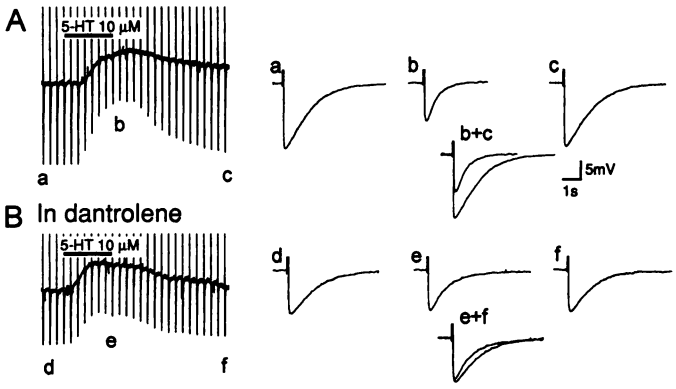

Fig. 5. Effect of dantrolene on the 5-HT_4 induced depolarization. A, Administration of serotonin under control conditions elicits a membrane depolarization and a reduction of the AHP after a calcium spike (*upward deflections*). *Traces a, b,* and *c*, AHP before, during, and after serotonin using an expanded time scale. B, Administration of dantrolene (30 μM) results in a reduction in the amplitude of the AHP. In the presence of dantrolene, serotonin administration still elicits a membrane depolarization, although it fails to reduce the AHP.

The above experiments indicated that the calcium for the AHP has two sources, extracellular calcium influx and intracellular calcium release, and that serotonin reduces the AHP by preferentially inhibiting the ability of calcium influx to trigger intracellular calcium release. This interpretation, however, requires that the AHP that remains after inactivation of intracellular calcium release be dependent on calcium influx. Therefore, we tested the calcium dependence of the AHP that remains after inactivation of intracellular calcium release. In four cells tested, dantrolene reduced the AHP and blocked the serotonin-induced reduction of the AHP as indicated above (Fig. 6). Subsequent administration of the calcium channel blocker cadmium (100 μM) abolished the remaining hyperpolarization. This observation is consistent with the known sensitivity of the AHP to calcium channel blockers (1, 17, 18). These results indicated that serotonin selectively inhibited a component of the calcium-activated AHP that was dependent on intracellular calcium release.

Discussion

Previous studies have shown that the AHP plays an important role in determining the firing properties of hippocampal pyramidal cells (19). One of the fundamental actions of serotonin in hippocampus is to reduce the AHP, thus modifying the encoding properties of these cells (4–6). We have shown previously by exclusion of known receptors (4–6), as well as through the use of selective ligands (6, 7), that the receptor mediating this response belongs to the 5-HT_4 subtype. Like 5-HT_4 receptors elsewhere, these receptors signal

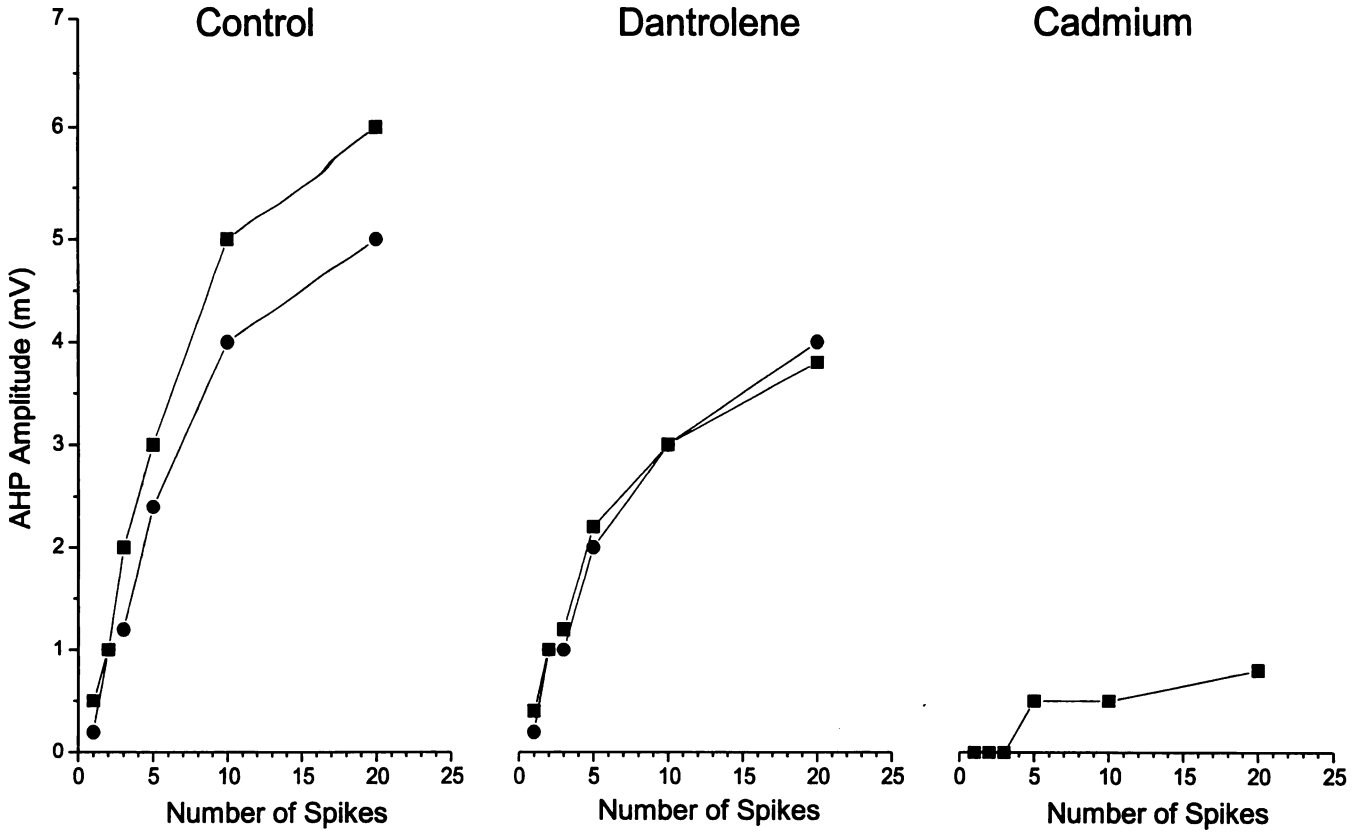

Fig. 6. Effect of dantrolene and cadmium on the amplitude of the AHP. Bath administration of serotonin (10 μM) elicits a reduction in the amplitude of the AHP (*left*). Administration of 30 μM dantrolene to this cell results in a marked reduction in the amplitude of the AHP and a loss of the effect of serotonin (*center*). Administration of the calcium channel blocker cadmium (100 μM) inhibits the AHP that remains after dantrolene (*right*). This indicates that although the entire slow AHP is calcium dependent, only a fraction of it is sensitive to dantrolene; this fraction is regulated by serotonin. Results similar to those illustrated in this figure were obtained in three additional cells. ■, Control; ●, 10 μM serotonin.

the reduction in the AHP through cAMP and PKA (4, 8), although their specific molecular target downstream from the kinase has not been elucidated. The results of the present study indicate that calcium-induced release from intracellular calcium stores plays an important role in the generation of the AHP and that this step is specifically targeted by serotonin acting through receptors of the 5-HT_4 subtype.

The evidence for an involvement of CICR in the generation of the AHP is severalfold. First, the AHP is enhanced by caffeine, a drug that increases the sensitivity of the intracellular calcium-release channel to calcium (12) and therefore should increase CICR. Second, the AHP is inhibited by dantrolene and ruthenium red, two blockers of the calcium-activated intracellular calcium-release channel. Finally, the AHP is also inhibited by thapsigargin, a blocker of the endoplasmic reticulum calcium pump that leads to the depletion of intracellular calcium stores. These results indicate that CICR contributes to the rise in intracellular calcium responsible for the generation of the AHP in the CA1 region of hippocampus. Similar observations have been made in other central (10) and peripheral neurons (9), which suggests that this might be a widespread role for CICR in neurons.

Administration of serotonin reduces the AHP through a mechanism that involves cAMP and the activation of PKA. Because all of the isoforms of the intracellular CICR channel exhibit PKA consensus phosphorylation sites, we tested the possibility that serotonin might regulate the AHP by modulating intracellular calcium release. Consistent with this possibility, the effect of serotonin was found to be enhanced when the CICR contribution to the AHP was maximized in the presence of caffeine. Similarly, dantrolene and ruthenium red reduced the AHP and occluded the effect of serotonin. Finally, depletion of intracellular calcium stores with thapsigargin also partly occluded the ability of serotonin to reduce the AHP. Thapsigargin, however, failed to completely occlude the effect of serotonin. This might reflect insufficient blockade of the calcium pump under the current experimental conditions. Our test protocol, by necessity, promotes loading of intracellular calcium stores, and any residual pump activity might have been able to partly refill these stores. However, we cannot rule out the alternative possibility that serotonin could also act at other sites to reduce the AHP.

Several neurotransmitters, including norepinephrine and histamine, also reduce the AHP by activating PKA (20–22). This suggests that CICR could be a common target for regulation by a variety of neurotransmitter receptors that couple to G_s. However, it seems likely, given the present study and previous studies (8), that serotonin is unique in selectively targeting intracellular calcium release. Other neurotransmitters [norepinephrine, for example (8)], can completely inhibit the AHP and thus must also act on additional targets beyond CICR to reduce the AHP. 5-HT_4 receptors seem to be weakly coupled to adenylate cyclase in rat hippocampus (23, 24) in comparison with β-adrenergic receptors. These observations suggest that the selective effect of 5-HT might be explained by the simple mechanism of low cAMP concentra-

tions selectively targeting CICR. However, other mechanisms are also possible, and we are currently addressing this question. Interestingly, it has been reported recently that norepinephrine, hence PKA, reduces the AHP in these cells by lowering the open probability of the AHP channel (17). Such an effect is consistent with a reduction in the availability of calcium in the immediate vicinity of the AHP channel or a direct effect on the channel itself.

Three distinct forms of the CICR channel have been identified (16). Although the molecular identity of the calcium-release channel involved in the generation of the AHP is presently unknown, all three subtypes identified so far are present in the CA1 region (25) and contain consensus phosphorylation sites for PKA (16). Because 5-HT_4 receptors reduce the AHP by stimulating PKA (8), it is tempting to speculate that the direct phosphorylation of the calcium-release channel by this enzyme could underlie the ability of serotonin to reduce the AHP.

It has been proposed that CICR functions as a mechanism to amplify calcium transients in neurons. As such, it has been shown to participate not only in the control of membrane excitability (9, 10, 26, 27) but also neuronal differentiation (28), glutamate neurotoxicity (29), and possibly synaptic plasticity (30). The ability of 5-HT_4 receptors to regulate CICR therefore suggests a wider role for these receptors, beyond the control of excitability and extending into neuronal development and plasticity.

Acknowledgments

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