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Muscarinic modulation of conductances underlying the afterhyperpolarization in neurons of the rat basolateral amygdala

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The excitability level of pyramidal neurons in the basolateral amygdala (BLA) is greatly increased following muscarinic receptor activation, an effect associated with an increased rate of action potential firing and reduction of the afterhyperpolarization (AHP). We impaled BLA pyramidal neurons in slices of rat ventral forebrain with a single microelectrode to examine the currents underlying the AHP and spike frequency accommodation and determine their sensitivities to muscarinic modulation. In voltage-clamp, depolarizing steps were followed by biphasic outward tail currents, consisting of rapidly decaying (I_{Fast}) and slowly decaying (I_{Slow}) current components. These corresponded temporally with the medium and slow portions of the AHP, respectively. The reversal potential for the I_{Fast} component of the AHP tail current shifted in the depolarizing direction with increases in the extracellular K⁺ concentration. The amplitude of I_{Fast} was reduced during perfusion of 0-Ca²⁺ medium or by superfusion of TEA (1–5 mM) or carbachol (10–40 μM). It is suggested that I_{Fast} was produced by the rapidly decaying Ca²⁺-activated K⁺ current (I_{C}) and the muscarinic-sensitive M-current (I_{M}). The I_{Slow} tail current component reversed at the estimated values for E_{K} in medium containing either normal or elevated K⁺ levels. This component was eliminated by perfusion of 0-Ca²⁺ medium or inclusion of cyclic-AMP in the recording electrode. It was not blocked by TEA (5 mM) or apamin (50–500 nM), but was reduced by carbachol in a dose-dependent manner ($\text{IC}_{50} = 0.5 \mu\text{M}$). Electrical stimulation of cholinergic afferent pathways to the BLA produced inhibition of I_{Slow} , an effect which was enhanced by eserine and prevented by atropine. Loss of the I_{Slow} component was always accompanied by similar reductions in accommodation and the slow AHP. It was concluded that this tail current component resulted from the slowly decaying Ca²⁺-activated K⁺ current, I_{AHP} . Thus, the muscarinic inhibition of I_{AHP} contributes to the enhanced excitability exhibited by BLA pyramidal neurons following cholinergic stimulation.

INTRODUCTION

The basolateral nucleus of the amygdala (BLA) has been described as cortical-like in its cytoarchitecture⁷. In agreement with this, previous work from our laboratory has demonstrated that the major neuronal cell type of the rat BLA is a large, pyramidal projection neuron having many morphological and electrophysiological features similar to those seen in pyramidal neurons of the hippocampus and cerebral cortex²⁹ (see also refs. 20,21). In our experiments, pyramidal neurons of the BLA characteristically had resting potentials near –70 mV and showed pronounced accommodation in the frequency of action potential firing during a prolonged pulse of depolarizing current. Prolonged depolarizations were followed by a prominent, biphasic afterhyperpolarization (AHP) that could be divided

into 2 components, a rapidly decaying medium AHP (mAHP) with a time-course of tens to a few hundred milliseconds, followed by a long-lasting slow AHP (sAHP) that decayed over a period of several seconds²⁹.

The biphasic AHP found in BLA pyramidal neurons closely resembles that exhibited by pyramidal neurons in the mammalian hippocampus. In this latter cell type, it has been determined that the mAHP results from the action of several potassium currents^{27,30}, including a rapidly decaying Ca²⁺-activated K⁺ current (I_{C})^{1,6}, the voltage-activated M-current (I_{M})^{5,13} and a hyperpolarization-activated cation current (I_{H})¹³. In contrast, the sAHP in hippocampal pyramidal neurons is produced by a single current, the slowly decaying Ca²⁺-activated K⁺ conductance, I_{AHP} ¹⁷. The muscarinic inhibition of I_{AHP} in hippocampal neurons results in reductions of both the accommodation response during

a prolonged depolarizing stimulus and the subsequent sAHP^{3,9,10,18,19}.

The BLA receives an extensive cholinergic innervation arising from scattered populations of cholinergic cell bodies located within the nucleus basalis and adjacent regions of the ventral forebrain^{8,14}. Stimulation of these cholinergic amygdalopetal inputs in slices of rat ventral forebrain or exogenous application of muscarinic agonists produces several changes in BLA pyramidal neurons, including a prolonged membrane depolarization associated with a decrease in membrane conductance and reductions of the accommodation response and sAHP²⁹. These changes are associated with a greatly increased level of BLA neuronal excitability. We have previously shown that the muscarinic-induced membrane depolarization resulted from inhibitions of the M-current and a voltage-insensitive K⁺ leak conductance³². In the present study, we used the single-electrode voltage-clamp technique in combination with bath application of carbachol and stimulation of cholinergic afferents to the BLA to identify additional membrane conductances that contribute to production of spike frequency accommodation and the sAHP, and which serve as targets of muscarinic inhibition in BLA pyramidal neurons. Some of these results have appeared in abstract form^{22,31}.

MATERIALS AND METHODS

The experiments were carried out using slices of rat ventral forebrain, prepared as previously described^{29,32}. Briefly, adult male Sprague-Dawley rats (150–200 g; Charles River) were killed by decapitation and the brain rapidly removed to ice-cold artificial cerebrospinal fluid (ACSF), pregassed with 95% O₂/5% CO₂. The ACSF (pH 7.4) consisted of (in mM): NaCl 124; KCl 3.5; CaCl₂ 3.0; MgSO₄ 1.5; NaH₂PO₄ 1.0; NaHCO₃ 26.2; glucose 11.0. Horizontal slices of the ventral forebrain containing the BLA were cut at 500 μm using a Vibraslicer (World Precision Instruments) and transferred to a holding chamber containing room temperature ACSF continuously bubbled with 95% O₂/5% CO₂. Individual slices were transferred to a recording chamber as needed and held submerged with nylon netting under continuously flowing oxygenated ACSF. All experiments were performed at room temperature (24°C). In some experiments, tetrodotoxin (TTX; 1 μM) was added to the perfusion medium prior to voltage-clamping to block action potential generation and eliminate spontaneous synaptic activity. In various experiments, carbachol (carbamylocholine chloride, 0.5–40 μM), atropine sulfate (1 μM), cesium chloride (1 mM), cobalt chloride (2 mM), cadmium chloride (200 μM), tetraethylammonium chloride (TEA, 1–5 mM) or apamin (50–500 nM) were added to the bathing medium. For preparation of calcium-free medium, NaCl was reduced to 118.5 mM and CaCl₂ was replaced with 10 mM MgSO₄. Test substances were dissolved in ACSF to their final concentration and applied to the slice by means of a multi-port valve system. All drugs were obtained from Sigma Chemical Co.

Pyramidal neurons within the BLA were impaled with a single microelectrode pulled from 1.2 mm o.d. capillary tubing (WP Instruments) and filled with a solution containing 2.7 M potassium chloride and 0.4 M potassium acetate, yielding electrode resistances of 30–100 MΩ. In some experiments, 100 mM cyclic-AMP was also included in the electrode filling solution. Only cells with resting potentials more

negative than –55 mV and overshooting action potentials greater than 70 mV in height were included in this study. Intracellular discontinuous current-clamp and single-electrode voltage-clamp recordings were obtained using an Axoclamp 2A amplifier with a 30% duty cycle, as described in Womble and Moises³². The sampling frequency in both modes was approximately 3.0 kHz. Amplified current and voltage signals were displayed on a storage oscilloscope, while a separate oscilloscope was used to continuously monitor the headstage output to verify that the voltage drop across the recording electrode had dissipated completely during the interval between current injection and voltage sampling. In voltage-clamp mode, gain settings of 3–8 nA/mV produced clamp settling times of ≤ 3 ms. Voltage clamp records were discarded if the voltage response during the command pulse showed a sag of more than 1–2 mV in the recorded membrane potential, indicating loss of voltage control. This problem was only encountered in some cells during the peak outward current elicited by a depolarizing voltage-step. The membrane potential actually recorded during the voltage step, rather than the designated command potential, is illustrated in all figures and was used for the construction of current–voltage relationships.

For all current-clamp experiments, the neuron was held at a constant membrane potential of –60 mV by application of steady DC current prior to action potential production. Similarly, –60 mV was used as the standard holding potential level in all voltage-clamp experiments, unless otherwise noted. The use of a standard membrane potential allowed for direct comparison of the AHP between cells and controlled for the voltage-dependent conductance changes which are normally associated with the depolarizing action of carbachol^{29,32}. The level of –60 mV was chosen as a constant reference potential in order to enhance the AHP amplitude and to avoid activation of the H-current^{13,26}, an inward-going current which activates in BLA neurons with hyperpolarization below –60 mV²². In some experiments, the hybrid-clamp technique was utilized to evoke an AHP tail current^{17,19,24}. In this technique, the cell was held at –60 mV in current-clamp mode prior to injection of a depolarizing current pulse to evoke a burst of action potentials. Upon termination of the current pulse, the amplifier was rapidly switched into voltage-clamp mode at a holding potential of –60 mV to record the currents underlying the AHP. Activation of cholinergic afferents in the slice was accomplished by brief tetanic stimulation of the substantia innominata or the external capsule, delivered via a bipolar stimulating electrode placed on the surface of the slice.

Current and voltage records were filtered at 300 Hz and collected for storage by microcomputer using pClamp software (Axon Instruments). The data records were analyzed off-line with DAOS software (Laboratory Software Associates, Vic., Australia) using a cursor controlled least-squares procedure for exponential curve fitting. The complex AHP tail current was separated into individual components using a curve ‘peeling’ technique. In this analysis, a single exponential curve was first fitted to the late, slowly decaying portion of the tail current, beginning approximately 300 ms after termination of the depolarizing voltage step, to obtain an estimate of the *I*_{Slow} component. This curve was extrapolated back to the end of the preceding depolarizing step and then subtracted from the total tail current. A second exponential curve was fitted to the residual tail current to yield an estimate of the *I*_{Fast} component. Peak amplitudes for each of these tail current components were obtained from the point at which the extrapolated exponential curves intersected the preceding depolarizing voltage step (time 0).

Mean values are given in the text together with the S.E.M. The S.E.M. was also included as error bars with graph points that refer to the means of 3 or more measurements.

RESULTS

The data presented here were obtained from stable, long-term recordings in 97 BLA pyramidal neurons. These cells had a mean resting potential of -68.6 ± 0.5 mV ($n = 97$) and exhibited pronounced accommoda-

tion in the rate of spike discharge during passage of a prolonged (500 ms) depolarizing current pulse through the recording electrode. In the vast majority of cases (92%), termination of a current-evoked burst of action potentials was followed by a long-lasting, biphasic AHP which we have found to be characteristic of BLA pyramidal neurons²⁹. In the example shown in Fig. 1A, a pyramidal neuron was held in current-clamp mode at a membrane potential of -60 mV by the application of steady DC current. Passage of a 500 ms depolarizing current pulse (0.5 nA) through the recording electrode caused the cell to fire an initial burst of action potentials at high frequency followed by a gradual decrease in the rate of spike firing. Termination of the current pulse revealed a prolonged AHP that decayed over a period of several seconds. It was possible by visual inspection to resolve the AHP into 2 distinct components, a medium-duration mAHP, lasting for approximately 200 ms, followed by a slowly decaying sAHP that persisted for over 3 seconds.

After a series of current-evoked afterhyperpolarizations had been recorded in normal medium, the cell was voltage-clamped at a holding potential of -60 mV (Fig. 1B). Stepping the neuron to -30 mV for 900 ms resulted in a brief inward current in the absence of

TTX, followed by activation of an increasingly large outward current which corresponded temporally with the development of the accommodation response seen in the unclamped neuron. Termination of the depolarizing voltage step revealed a long-lasting, outward tail current, with a rapidly decaying portion that corresponded in duration to the mAHP and a slowly decaying segment that showed a close temporal correspondence with the occurrence of the sAHP. Plotting tail current amplitude as a function of time on a semi-logarithmic scale (Fig. 1C, circles) revealed that the tail current could be separated into 2 current components, each of which decayed along a single exponential time-course. We have designated the initial, rapidly decaying tail current component as I_{Fast} , and the later, slowly decaying component as I_{Slow} . In the neuron shown in Fig. 1, I_{Fast} had a time 0 peak amplitude of 224 pA and decayed with a tau of 151 ms, while I_{Slow} had a time 0 peak amplitude of 72 pA and a decay tau of 1,638 ms. Overall, tail currents recorded upon returning to a holding potential of -60 mV following a 900 ms depolarizing voltage step showed an I_{Fast} component with an average peak amplitude of 165 ± 11 pA and a decay tau of 138 ± 9 ms ($n = 77$). The I_{Slow} tail current component of BLA pyramidal neurons had a

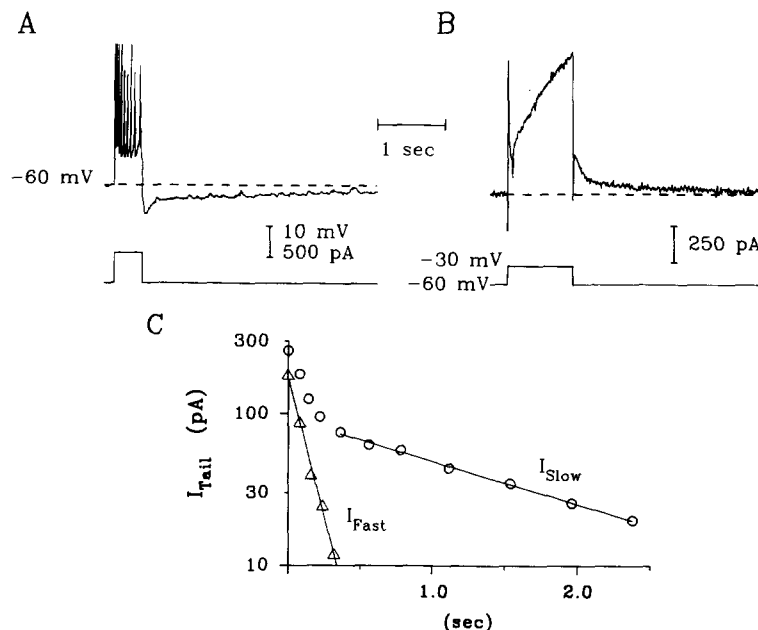


Fig. 1. The AHP and its underlying currents. A: current-clamp record obtained in normal medium from a pyramidal neuron held at -60 mV by the application of steady DC current. The resting potential prior to application of DC current was -68 mV. Passage of a 500 ms depolarizing current pulse (0.5 nA) evoked a series of action potentials the rate of firing of which accommodated with time. Termination of the current pulse revealed the presence of a prolonged biphasic AHP, consisting of medium and slow components. Action potentials were truncated by the digitization process. B: voltage-clamp record obtained from the same neuron in the absence of TTX. Application of a 900 ms voltage step to -30 mV from a holding potential of -60 mV activated a brief inward current and then an increasingly large outward current. This was followed upon termination of the step by a prolonged, biphasic tail current. The durations of the rapidly and slowly decaying components of the tail current corresponded with the durations of the mAHP and sAHP, respectively. C: semi-logarithmic plot of the total tail current (○) recorded in B as a function of time. The late portion of the tail current showed a slow rate of decay that followed a single exponential time-course (I_{Slow}). Extrapolation and subtraction of this curve from the total current allowed for the isolation of a rapidly decaying current component (I_{Fast}), which also followed a single exponential time-course (△).

peak amplitude of 106 ± 12 pA and displayed a much slower mean rate of decay ($2,030 \pm 197$ ms, $n = 71$).

Reversal potentials of I_{Fast} and I_{Slow}

The reversal potentials (E_{Rev}) for the rapidly and slowly decaying components of the tail current were determined to characterize the ionic basis for I_{Fast} and I_{Slow} . During these measurements, Cs^+ (1 mM) was included in the bathing saline to prevent contamination of the tail current by the H-current, a voltage-activated inward current that develops during hyperpolarizations beyond -60 mV^{13,22}. The protocol used for determination of E_{Rev} is illustrated in Fig. 2A. In this experiment, a pyramidal neuron in normal ACSF containing 3.5 mM extracellular K^+ was voltage-clamped to a holding potential of -60 mV and stepped to -35 mV for 900 ms. The decay of the tail current that followed this step depolarization was then examined upon returning to a series of holding potentials between -59 and -101 mV. In Fig. 2C, we have plotted the peak amplitudes of the I_{Fast} and I_{Slow} components of the tail

current as a function of the membrane potential to which the cell was clamped upon termination of the depolarizing step. As this figure shows, the 2 components of the tail current reversed at different membrane potentials, -63 mV for the I_{Fast} component (filled circles) and -88 mV for the I_{Slow} component (open circles). Overall, the mean reversal potentials for I_{Fast} and I_{Slow} were -74.5 ± 2.4 mV ($n = 8$) and -95.5 ± 2.6 mV ($n = 4$), respectively, when determined in normal ACSF containing 3.5 mM extracellular K^+ . The reversal potential determined for I_{Slow} agreed well with a theoretical calculation of -97 mV for the potassium equilibrium potential (E_K), assuming an internal K^+ concentration in BLA neurons of 165 mM, a value derived from measurements of E_K in hippocampal pyramidal neurons².

In the experiment shown in Fig. 2, perfusion of the slice with medium containing high external K^+ (15 mM) shifted the reversal potentials for both I_{Fast} and I_{Slow} in the depolarizing direction. Under these conditions, measurements of tail current decay made upon

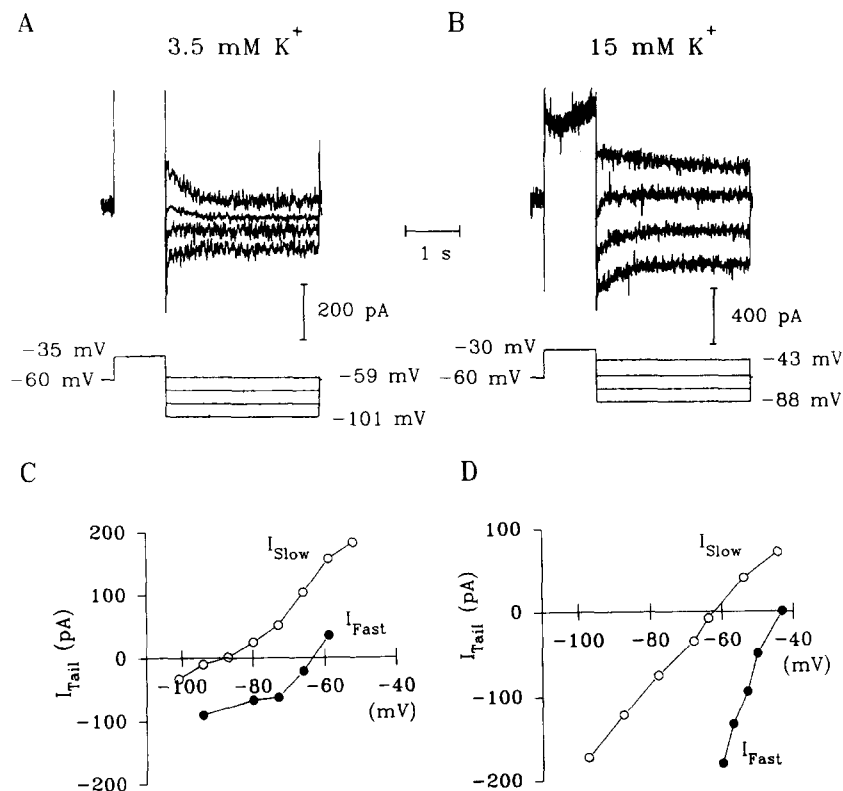


Fig. 2. Determination of the reversal potentials for I_{Fast} and I_{Slow} during perfusion of ACSF containing normal and elevated levels of potassium. For these experiments, $1 \mu M$ TTX was included in all bathing media, along with 1 mM Cs^+ to block the hyperpolarization-activated H-current. A: voltage-clamp records from a pyramidal neuron in normal (3.5 mM K^+) ACSF. Tail currents were evoked by a 900 ms depolarizing voltage step to -35 mV from a holding potential of -60 mV and examined upon returning to different holding potentials between -59 and -101 mV. Resting potential of the cell was -65 mV. B: tail currents recorded at holding potentials between -43 and -88 mV from the same cell after switching the bathing medium to ACSF containing 15 mM K^+ . The resting potential was -48 mV. C, D: plots of peak amplitudes of the isolated fast and slow tail current components (I_{Tail}), determined from individual single exponential curve fits, as a function of holding potential after the depolarizing step. The reversal potential for each component was estimated from the zero current level. The reversal potential of I_{Fast} (\bullet) was shifted from -63 mV in normal ACSF to -43 mV in ACSF containing elevated K^+ . The reversal potential for I_{Slow} (\circ) was shifted from -88 mV to -58 mV by the same 4.3-fold increase in extracellular K^+ concentration.

returning to holding potentials between -88 and -43 mV (Fig. 2B) yielded reversal potentials of -43 for I_{Fast} and -58 mV for the I_{Slow} component (Fig. 2D). Measurements obtained from 5 pyramidal neurons exposed to 15 mM K^+ ACSF yielded a mean reversal potential for I_{Fast} of -55.6 ± 4.0 mV, while that for I_{Slow} was -59.0 ± 2.6 mV. The 37 mV positive shift in the reversal potential of I_{Slow} , from -96 to -59 mV, produced by an increase in extracellular K^+ concentration from 3.5 to 15 mM K^+ is exactly that predicted by the Nernst equation, and indicates that the I_{Slow} component of the tail current was a pure K^+ conductance. On the other hand, the finding that the same 4.3-fold increase in external K^+ concentration yielded only a 19 mV positive shift in the reversal potential of I_{Fast} indicates that this portion of the tail current was substantially, but not exclusively, a K^+ conductance.

During this series of experiments, we also examined the effect that changes in the holding potential had on the decay rates of the fast and slow components of the AHP tail current. These experiments were carried out in normal ACSF containing 3.5 mM K^+ . In 5 pyramidal neurons, the rate of decay for I_{Slow} was unaffected by changes in holding potential over the range of -50 to -70 mV. The effect of varying membrane potential on the I_{Fast} rate of decay was less clear. In 3 neurons, the rate appeared to accelerate with hyperpolarization, but this was not observed in the other 2 neurons.

Ca^{2+} -dependence of I_{Fast} and I_{Slow}

Results from an earlier current-clamp study of BLA pyramidal neurons suggested that Ca^{2+} -activated K^+ currents may be involved in the accommodation response and production of the medium and slow AHP in these cells. Thus, addition of Cd^{2+} to the bathing medium or inclusion of EGTA in the recording electrode reduced the amplitude of the current-evoked mAHP and eliminated both accommodation and the sAHP²⁹. The involvement of Ca^{2+} -sensitive outward currents in the production of the AHP was confirmed in voltage-clamped BLA neurons. Fig. 3 shows the results of an experiment in which we examined the sensitivity of AHP tail currents elicited by depolarizing voltage steps to changes in external Ca^{2+} concentration. Switching the bathing solution from normal ACSF containing 3 mM Ca^{2+} to ACSF in which Ca^{2+} was replaced with 10 mM Mg^{2+} virtually eliminated the I_{Slow} portion of the tail current, reducing it from 441 to 28 pA. At the same time, the peak amplitude of the I_{Fast} component was also reduced, from 417 to 189 pA. In addition, perfusion of 0- Ca^{2+} ACSF substantially reduced the amplitude of the peak outward current recorded during the depolarizing voltage step. Current

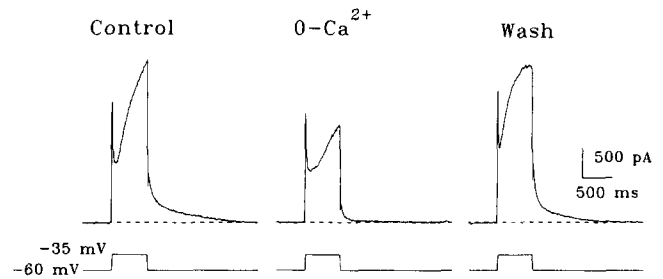


Fig. 3. Dependence of depolarization-evoked outward currents on the presence of extracellular calcium. In this pyramidal neuron, a 500 ms depolarizing step to -35 mV from a holding potential of -60 mV elicited a large outward current during the step, followed by a biphasic tail current. Repetition of the voltage step protocol 15 min after changing from normal bathing medium to ACSF containing 0- Ca^{2+} revealed large reductions in the I_{Fast} and I_{Slow} components of the tail current, as well as in the amplitude of the outward current generated during the voltage step. These changes were reversed after washing with normal ACSF for 15 min. TTX ($1 \mu M$) was included in all bathing media. Each trace is an average of 3 successive sweeps.

amplitudes returned to control levels upon washing with normal ACSF. Loss of I_{Slow} and reductions in I_{Fast} amplitude were seen in all experiments with BLA neurons exposed to 0- Ca^{2+} ACSF ($n = 2$), or to normal ACSF containing the Ca^{2+} channel blockers Cd^{2+} ($200 \mu M$, $n = 2$) or Co^{2+} (2 mM, $n = 2$). Overall, treatments designed to reduce or block Ca^{2+} influx produced a $84.8 \pm 6.1\%$ ($n = 6$) reduction in the I_{Slow} component of the AHP tail current and a $63.5 \pm 5.7\%$ ($n = 6$) reduction in the I_{Fast} component. These findings suggest that I_{Slow} resulted from the action of a slowly decaying Ca^{2+} -dependent conductance, while a more rapidly decaying Ca^{2+} -sensitive outward conductance contributed in part to production of I_{Fast} .

Characterization of I_{Slow}

The data presented above indicate that the I_{Slow} portion of the AHP tail current was produced by a slowly decaying, Ca^{2+} -activated K^+ current. A current with these characteristics, termed I_{AHP} , has been shown to be responsible for production of the sAHP in several neuronal cell types, including bullfrog sympathetic neurons^{24,28}, hippocampal pyramidal neurons^{17,19}, sensorimotor cortical neurons²⁵ and olfactory cortical neurons¹¹. Intracellular injection of cyclic-AMP or bath application of its membrane permeate analogues has been shown to block I_{AHP} and the sAHP in hippocampal neurons^{18,19}. These treatments also block accommodation and the sAHP in BLA pyramidal neurons²⁹, suggesting the presence of a current similar to I_{AHP} in BLA neurons. To test the ability of intracellular administration of cyclic-AMP to block the I_{Slow} component of AHP tail currents recorded in BLA pyramidal neurons, we impaled neurons with recording electrodes containing 100 mM cyclic-AMP. Within a few minutes after

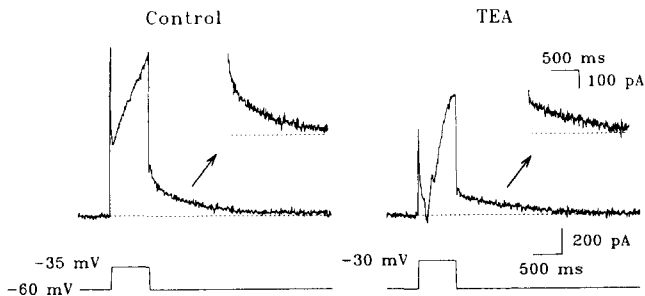


Fig. 4. Inhibition of the I_{Fast} portion of the tail current by TEA. Current responses were evoked by 900 ms depolarizing steps from a holding potential of -60 mV to the indicated command potentials. Superfusion of TEA (5 mM) selectively inhibited the I_{Fast} component of the tail current, reducing its peak amplitude from 196 to 40 pA. The I_{Slow} tail current component was unaffected. The initial portions of the tail currents are shown at a higher gain in the inserts. Records are averages of 3 sweeps each.

the initial impalement, the accommodation response, the sAHP and I_{Slow} were all largely eliminated ($n = 4$). In comparison, the mAHP and I_{Fast} were unaffected by intracellular iontophoresis of the nucleotide. These data support the hypothesis that the slowly decaying portion of AHP tail currents recorded in BLA pyramidal neurons was produced by I_{AHP} , and suggests that this current contributes to production of the accommodation response and the sAHP. Interestingly, the bee-venom toxin, apamin (50–500 nM), a selective blocker of I_{AHP} in bullfrog ganglion cells²⁴ and rat supraoptic neurons⁴, did not effect either the fast or slow portions of AHP tail currents recorded in BLA pyramidal neurons ($n = 4$). It should be noted, however, that apamin does not block I_{AHP} in hippocampal pyramidal neurons²⁷ or olfactory cortical neurons¹¹ and thus is not a definitive test for this current.

Characterization of the conductances underlying I_{Fast}

Our findings indicate that a substantial portion of the I_{Fast} component of the AHP tail current was Ca^{2+} sensitive, suggesting the involvement of a rapidly decaying Ca^{2+} -activated K^+ current. A current of this type, termed I_C , has been shown to be involved in the production of the mAHP in bullfrog sympathetic neurons²⁴, hippocampal pyramidal neurons^{6,27,30} and cortical pyramidal neurons²⁵. In these cell types, I_C is inhibited by TEA^{1,6,27}. We have previously shown that TEA (0.2–1 mM) selectively reduces the current-evoked mAHP while sparing the sAHP in BLA pyramidal neurons²⁹, suggesting a possible involvement of I_C in the production of the mAHP. Thus, we examined the TEA sensitivity of the I_{Fast} portion of AHP tail currents recorded in BLA pyramidal neurons. As illustrated in Fig. 4, TEA (5 mM) selectively blocked the I_{Fast} portion of the tail current, reducing its peak amplitude in this case from 196 to 40 pA, with little or no effect on the I_{Slow} component. Overall, lower concentrations of TEA (1–2 mM) reduced I_{Fast} by $55 \pm 5\%$ ($n = 3$), while 5 mM TEA largely eliminated this portion of the tail current, reducing it by an average of $93 \pm 6\%$ ($n = 3$). These findings are consistent with the possible contribution of I_C to the rapidly decaying portion of the AHP tail current.

Inhibition of I_{Fast} by TEA is not a definitive test for I_C , however. The mAHP in hippocampal and olfactory cortical neurons is also produced in part by the M-current (I_M)^{11,27,30}, a voltage- and muscarinic-sensitive current^{5,13} that is inhibited by similar concentrations of TEA^{11,27}. This current is also present in BLA pyramidal neurons³². We have found that the amplitude of I_M in BLA neurons, measured during a 1 s hyperpolariz-

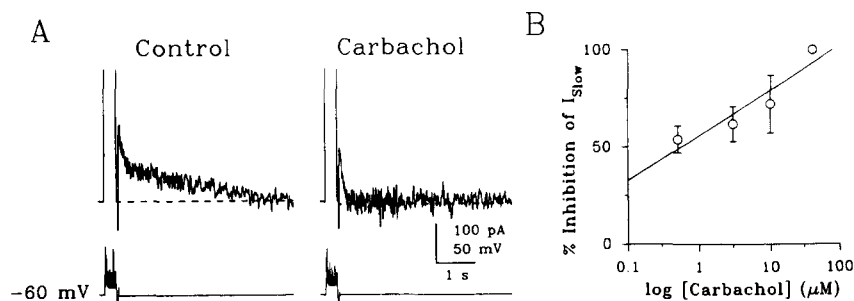


Fig. 5. Inhibition of the I_{Slow} component of the AHP tail current by carbachol. A: an unclamped BLA neuron was maintained at a resting potential level of -60 mV with steady DC current injection. In the absence of TTX, a 500 ms depolarizing current pulse elicited a burst of action potentials (lower records). Switching to voltage-clamp mode at a holding potential of -60 mV upon termination of the current pulse (hybrid-clamp) revealed a biphasic tail current underlying the AHP (control, upper record). Addition of carbachol (10 μ M) to the perfusate eliminated the I_{Slow} portion of the tail current and produced a small reduction in the peak amplitude of the I_{Fast} component, from 133 to 112 pA. Dashed lines indicate baseline current levels at the -60 mV holding potential. Each trace represents the average of 4 sweeps. B: concentration–response relationship for the inhibition of I_{Slow} by carbachol, determined from a total of 21 neurons. The percent reduction in I_{Slow} peak amplitude, measured at a holding potential of -60 mV, is plotted against the log concentration of carbachol. The fitted line yielded an approximate IC_{50} of 0.5 μ M. Each point represents the mean of measurements from 4–7 pyramidal neurons. At the highest concentration of agonist tested (40 μ M), I_{Slow} was completely inhibited in all cases and thus the size of the point obscures the error bar.

ing voltage step to -55 mV from a holding potential of -40 mV, was reduced by an average of 59% ($n = 2$) during perfusion of 1 mM TEA and 86% ($n = 2$) by 5 mM TEA (Womble and Moises, unpublished observations), indicating that this current could also contribute to the TEA-sensitivity of I_{Fast} . Therefore, to test for the possible involvement of I_M in the production of I_{Fast} , we examined this portion of the AHP tail current during perfusion of carbachol, a cholinergic agonist that is a potent inhibitor of $I_M^{13,19,32}$ but not of $I_C^{6,18}$. As illustrated by the hybrid-clamp experiment in Fig. 5A, perfusion of 10 μ M carbachol resulted in a small decrease in I_{Fast} amplitude, in this case reducing it from 133 to 112 pA. Overall, administration of 10–40 μ M carbachol, concentrations which have previously been found to largely eliminate I_M in BLA neurons³², reduced the I_{Fast} portion of the AHP tail current by $29 \pm 9\%$ ($n = 7$). In two other neurons impaled with cyclic-AMP-containing electrodes to block the I_{Slow} portion of the tail current, application of carbachol reduced I_{Fast} by an average of 44%, indicating that the observed reduction in I_{Fast} was not due to inhibition of I_{Slow} . These findings suggest that I_M contributes a small but significant portion to the I_{Fast} component of the AHP tail current.

Muscarinic modulation of I_{Slow}

Muscarinic receptor activation results in inhibition of the sAHP and its underlying I_{AHP} in several neuronal cell types, including frog sympathetic neurons²⁴, olfactory cortical neurons¹¹, sensorimotor cortical neurons²⁵ and hippocampal pyramidal neurons^{3,9,10,18,19}. Similarly, we have previously reported that application of muscarinic agonists reduces the accommodation response and inhibits the sAHP in BLA pyramidal neurons²⁹. Therefore we sought to determine the muscarinic sensitivity of the I_{Slow} portion of AHP tail currents recorded in BLA neurons. As illustrated in Fig. 5A, application of carbachol (10 μ M) selectively inhibited the I_{Slow} portion of the AHP tail current. In other experiments, when I_{Slow} was not completely blocked, it could be seen that the rate of decay for the residual I_{Slow} component was unchanged. The inhibitory effect of carbachol on I_{Slow} could be reversed by washing with ACSF containing 1 μ M atropine (not shown), suggesting that this effect was mediated by muscarinic receptor activation. The concentration–response relationship for the inhibition of I_{Slow} by carbachol was determined in 21 BLA pyramidal neurons using agonist concentrations of 0.5–40 μ M (Fig. 5B). The inhibitory effect of carbachol was dose-dependent, with an IC_{50} of approximately 0.5 μ M, similar to that obtained for inhibition of the sAHP in BLA pyramidal

neurons²⁹ and I_{AHP} in hippocampal pyramidal neurons¹⁹.

Effects of synaptic stimulation

The BLA receives an extensive cholinergic innervation from the nucleus basalis and adjacent regions of the ventral forebrain^{8,14}. Cholinergic afferents arise in the substantia innominata (SI) portion of this region and course through the external capsule (EC) on their way to innervate the BLA^{15,23}. We have previously shown that activation of these afferents by direct electrical stimulation of the SI or EC in slices of the ventral forebrain produces inhibitions of both accommodation and the sAHP, effects which were prevented in the presence of atropine, indicating mediation by muscarinic receptors²⁹. Therefore, we sought to determine whether stimulation of this afferent pathway would mimic the inhibitory effects of carbachol on the slow component of the AHP tail current recorded in BLA neurons. For these experiments, the tail current was examined before and several seconds after tetanic activation of amygdalopetal afferents (30 Hz repetitive stimulation for 500 ms with 0.2 ms stimuli). We found that afferent pathway stimulation produced an average reduction in I_{Slow} amplitude of $28 \pm 8\%$ ($n = 4$). Stimulation applied in the presence of the anticholinesterase agent eserine (5 μ M) increased the magnitude of I_{Slow} inhibition to $51 \pm 16\%$ ($n = 5$). Fig. 6 illustrates the largest change in I_{Slow} amplitude that we observed following afferent stimulation. The perfusing medium for this experiment contained 5 μ M eserine, and 20 μ M picrotoxin to block the fast inhibitory

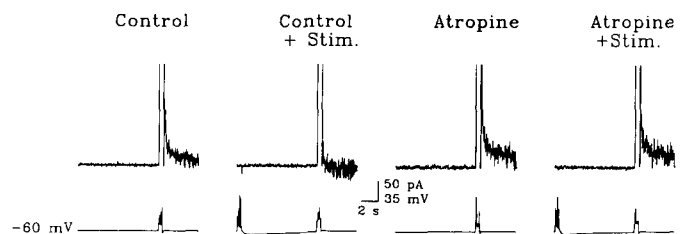


Fig. 6. Hybrid-clamp records obtained in the absence of TTX, showing inhibition of the AHP tail current following stimulation of the cholinergic afferent pathway to the BLA. The unclamped neuron was held at a membrane potential of -60 mV with steady DC current prior to application of a 500 ms depolarizing current pulse to elicit a burst of action potentials (lower traces). Upon termination of the current pulse, a rapid switch to voltage-clamp mode at a holding potential of -60 mV revealed the biphasic AHP tail current (Control, upper record). Tail current amplitude was greatly reduced following tetanic stimulation (30 Hz for 500 ms with 0.2 ms stimuli) of the external capsule pathway to the BLA (Control + Stimulation). After recovery of the tail current and addition of atropine (1 μ M) to the perfusing medium, a tail current was again elicited (Atropine). Afferent stimulation in the presence of atropine failed to reduce tail current amplitude (Atropine + Stimulation). Eserine (5 μ M) and picrotoxin (20 μ M) were included in all bathing media.

post-synaptic potential normally evoked by synaptic stimulation²⁹. A biphasic AHP tail current was evoked at a holding potential of -60 mV using the hybrid-clamp technique. Afferent activation reduced the peak amplitude of the I_{Slow} component in this cell from 42 pA to 2 pA. At the same time, the I_{Fast} portion of the tail current was also reduced, from 80 to 25 pA. Similar stimulations applied to the unclamped neuron were accompanied by reductions in spike frequency accommodation and the sAHP (not shown). The perfusing saline was then switched to ACSF containing $1 \mu\text{M}$ atropine, in addition to eserine and picrotoxin. Afferent stimulation under these conditions failed to produce a reduction in tail current amplitude, an action of atropine that was also observed in 2 other neurons. These findings confirm that activation of the cholinergic afferent pathway running from the ventral fore-brain region to the BLA inhibits I_{Slow} and results in reductions of both spike frequency accommodation and the sAHP, suggesting that activation of this pathway *in vivo* may have a significant impact on the functioning of BLA pyramidal neurons.

DISCUSSION

Pyramidal neurons of the BLA exhibit a characteristic biphasic AHP following a current-evoked burst of action potentials. The AHP can be divided into an initial short-lived medium AHP, that may last up to 100–200 ms, followed by a long-lasting slow AHP that decays over a period of several seconds. Voltage-clamp analysis showed that depolarization of BLA neurons was followed by a prolonged outward tail current. This tail current was responsible for generation of the AHP and could be divided into 2 current components on the basis of their different rates of decay. The initial portion of the AHP tail current, termed I_{Fast} , decayed rapidly and corresponded in duration to the mAHP, while the I_{Slow} component of the tail current decayed much more slowly and was responsible for production of the sAHP.

The I_{Slow} portion of the AHP tail current appeared to reflect the exponential decay of a single, slowly decaying Ca^{2+} -activated K^+ current. Thus, I_{Slow} was highly sensitive to treatments which caused reductions in voltage-gated Ca^{2+} influx and was a pure K^+ conductance, as evidenced by having a reversal potential close to E_{K} . It was not blocked by apamin or low concentrations of TEA and showed no voltage sensitivity in its rate of decay. However, I_{Slow} was markedly reduced during intracellular loading of the cell with cyclic-AMP and was inhibited by carbachol in a dose-dependent and atropine-sensitive manner. Therefore

we conclude that the I_{Slow} component of the AHP tail current recorded in BLA pyramidal neurons is similar to the slowly decaying Ca^{2+} -activated K^+ conductance, termed I_{AHP} , previously described in other neuronal cell types, including bullfrog sympathetic neurons^{24,28}, hippocampal pyramidal neurons^{17,19}, sensorimotor cortical neurons²⁵ and olfactory cortex neurons¹¹.

Since we found that reductions in Ca^{2+} influx during the depolarizing voltage step greatly decreased the amplitude of the ensuing slow portion of the AHP tail current, this raised the possibility that the carbachol-induced inhibition of I_{Slow} was secondary to inhibition of the voltage-dependent Ca^{2+} current. Indeed, muscarinic receptor activation has been shown to block the Ca^{2+} current of pyramidal neurons in cultured hippocampal slices¹². The finding that the carbachol-induced reductions in the amplitude of I_{Slow} were not associated with changes in the decay rate of this current component, however, suggested that decreases in the underlying I_{AHP} conductance did not result from reductions in Ca^{2+} influx. This conclusion is supported by our previous current-clamp study which demonstrated that the amplitude and duration of Ca^{2+} spikes elicited by BLA neuron depolarization in the presence of TTX and TEA were unaffected by carbachol²⁹. A similar conclusion was also reached by Knopfel et al.¹⁶ who used intracellular recording techniques combined with microfluorometric measurements of intracellular Ca^{2+} levels in hippocampal pyramidal neurons to show that muscarinic inhibition of I_{AHP} occurs without a corresponding reduction in internal Ca^{2+} levels.

Previous work with BLA pyramidal neurons has shown that accommodation in the frequency of action potential firing during a prolonged depolarizing current pulse was reduced by treatments which also produced a concurrent reduction in the sAHP, including superfusion of medium containing Cd^{2+} or carbachol, elevated intracellular cyclic-AMP concentrations, or activation of cholinergic afferent fibers, leading to the suggestion that accommodation and the sAHP were produced by the action of the same current²⁹. In the present study, voltage-clamp recordings demonstrated that these types of treatments all specifically inhibited the I_{Slow} portion of the AHP tail current. These findings indicate that I_{AHP} is largely responsible for production of both the accommodation response and the sAHP in BLA pyramidal neurons, in agreement with similar findings from frog sympathetic neurons²⁴, hippocampal pyramidal neurons^{17,18} and olfactory cortical neurons¹¹.

In contrast to the slowly decaying portion of the AHP tail current, the I_{Fast} component appeared to consist of more than a single current. Part of I_{Fast} was

due to the action of a rapidly decaying Ca^{2+} -dependent outward conductance, since the peak amplitude of this component was consistently diminished by treatments which reduced or abolished voltage-dependent Ca^{2+} influx during the preceding depolarization. The I_{Fast} component was predominantly a K^+ conductance, as evidenced by its reversal potential of -75 mV when measured from an initial holding potential of -60 mV, a value that agrees with the reversal potential obtained for the mAHP in hippocampal pyramidal neurons³⁰. A similar reversal potential has been obtained in hippocampal neurons for the rapidly decaying, Ca^{2+} -activated K^+ current, I_{C} , which partially underlies production of the mAHP in these cells^{6,30}. These findings suggest that a portion of the I_{Fast} component of the AHP tail current recorded in BLA neurons might be attributable to a rapidly decaying Ca^{2+} -activated K^+ current similar to the I_{C} of bullfrog sympathetic neurons^{1,24} and hippocampal pyramidal neurons^{6,17,18}.

A second, carbachol-sensitive current appeared to also contribute to the I_{Fast} portion of the AHP tail current. Since I_{C} is not inhibited by carbachol^{6,18}, the portion of I_{Fast} which was blocked by this agonist may be due to inhibition of the muscarinic-sensitive M-current^{5,13,19}. Studies have demonstrated that I_{M} contributes to production of the mAHP in hippocampal pyramidal neurons^{27,30} and olfactory cortical neurons¹¹. We have previously shown that this current is also present in BLA pyramidal neurons³², where it has many characteristics that make it a likely contributor to the I_{Fast} component of the AHP tail current. Thus, I_{M} is a K^+ conductance activated by membrane depolarization, with a decay tau of approximately 150 ms at a holding potential of -60 mV³², characteristics that were also associated with the I_{Fast} portion of the AHP tail current. The involvement of both I_{M} and I_{C} in the production of I_{Fast} is consistent with the finding that this portion of the AHP tail current was largely eliminated by 5 mM TEA, a drug concentration that inhibits both I_{M} ^{11,27} and I_{C} ^{1,6,27}. This conclusion is also supported by the finding that cholinergic afferent pathway stimulation in the presence of eserine resulted in a reduction of the I_{Fast} tail current component, an action that was prevented by atropine.

The present experiments were performed at a holding potential of -60 mV to prevent contamination of the AHP tail current by the H-current (I_{H}), a mixed cationic inward current activated by hyperpolarization^{13,22,26}. In separate experiments, we have found that 2 mM extracellular Cs^+ inhibited I_{H} in BLA neurons and reduced the amplitude of the mAHP when the preceding burst of action potentials was evoked from the normal resting potential level of -70 mV (Womble

and Moises, unpublished observations). This finding suggests that I_{H} also contributes to production of the mAHP normally observed in unclamped BLA neurons. Thus, the data we have obtained suggests that several currents contribute to the mAHP in BLA pyramidal neurons, indicating that these cells are similar to hippocampal pyramidal neurons, in which a mixture I_{C} , I_{M} and I_{H} all contribute to generation of the mAHP^{27,30}.

We have previously shown that stimulation of afferent pathways from the ventral forebrain region to the BLA reduces both accommodation and the slow AHP²⁹. These actions were prevented in the presence of TTX or atropine, suggesting they were mediated by synaptic release of acetylcholine acting on muscarinic receptors²⁹. In the present study, similar stimulations inhibited the I_{Slow} portion of the AHP tail current, an effect that was enhanced by eserine and prevented by atropine. These findings confirm the cholinergic nature of this afferent pathway. Our results suggest that the loss of I_{Slow} produced following the activation of cholinergic fibers *in vivo* may have a significant impact on the size and duration of the AHP and on the rate of action potential firing in BLA pyramidal neurons.

Stimulation of forebrain cholinergic inputs to the BLA or application of muscarinic agonists to neurons in the *in vitro* slice preparation induces several changes in unclamped BLA pyramidal cells, including a slow membrane depolarization associated with an increase in input resistance and blockade of the slow AHP and accommodation response²⁹. The net effect of these cholinergic actions is to greatly increase the responsiveness of BLA neurons to subsequent excitatory stimuli. The increased input resistance and membrane depolarization have previously been shown to be produced by the muscarinic inhibitions of the M-current and a voltage-insensitive K^+ leak conductance (I_{Leak})³². In the present study, we have demonstrated that the losses of accommodation and the sAHP are due to the muscarinic inhibition of the I_{Slow} component of the AHP tail current. This current component is more sensitive to the inhibitory actions of carbachol ($\text{IC}_{50} = 0.5 \mu\text{M}$) than are either I_{M} or I_{Leak} (each with $\text{IC}_{50} = 2 \mu\text{M}$)³², suggesting that inhibition of I_{Slow} and the accompanying reductions in accommodation and the slow AHP may represent the major actions of synaptically released acetylcholine. Thus, inhibition of I_{AHP} may be the most important factor in the cholinergic regulation of BLA neuronal excitability.

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