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# Metabotropic Glutamate Receptor Agonist ACPD Inhibits Some, but Not All, Muscarinic-Sensitive $K^+$ Conductances in Basolateral Amygdaloid Neurons

MARK D. WOMBLE AND HYLAN C. MOISES
*Department of Physiology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0622*

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*ABSTRACT* Muscarinic agonists produce membrane depolarization and losses of spike frequency accommodation and the slow afterhyperpolarization (AHP) when applied to neurons of the basolateral amygdala (BLA). Underlying these changes are the muscarinic-induced inhibitions of several $K^+$ conductances, including the voltage-activated M-current ($I_M$), a slowly decaying $Ca^{2+}$-activated current ($I_{AHP}$), a voltage-insensitive leak current ($I_{Leak}$), and the hyperpolarization-activated inward rectifier current ($I_{IR}$). Similar depolarizations and losses of the slow AHP have been observed in other neuronal cell types following stimulation of metabotropic glutamate receptors. Therefore, we tested the effects of the metabotropic glutamate receptor agonist, 1-aminocyclopentane-1s,3R-dicarboxylic acid (ACPD), on pyramidal neurons impaled with a single microelectrode for current- and voltage-clamp recordings in a brain slice preparation of the rat BLA. Application of ACPD (20 or 100 μM) to BLA neurons inhibited $I_M$ and $I_{AHP}$, resulting in membrane depolarization and reductions in the amplitude and duration of the slow AHP. However, ACPD did not inhibit the muscarinic-sensitive current $I_{IR}$, nor was $I_{Leak}$ blocked in the majority of neurons examined. These findings suggest the possibility that muscarinic cholinergic and metabotropic glutamatergic receptor agonists may activate separate intracellular transduction pathways which have convergent inhibitory effects onto $I_M$ and $I_{AHP}$ in BLA pyramidal neurons. 

## INTRODUCTION

The basolateral nucleus of the amygdala (BLA) receives a substantial cholinergic innervation from neurons of the basal forebrain region (Carlsen et al., 1985; Hellendall et al., 1986). Stimulation of this pathway or direct application of muscarinic agonists to BLA pyramidal neurons in vitro results in membrane depolarization and losses of spike frequency accommodation and the slowly decaying portion of the afterhyperpolarization (AHP), changes which serve to greatly enhance the level of neuronal excitability (Washburn and Moises, 1992). Similar responses to cholinergic stimulation have also been observed in neurons from several regions of the mammalian brain (Benardo and Prince, 1982; Cole and Nicoll, 1983; Halliwell and Adams, 1982; Madison et al., 1987; McCormick and Prince, 1986; Uchimura and North, 1990). Voltage-clamp analysis of BLA neurons reveals that the muscarinic-induced depolarization results from inhibitions of the voltage-activated M-current ($I_M$) and a voltage-insensitive $K^+$ leak current ($I_{Leak}$) (Womble and Moises, 1992), while the losses of accommodation and the slow AHP are due to the muscarinic inhibition of a slowly decaying, $Ca^{2+}$-activated $K^+$ current ($I_{AHP}$) (Womble and Moises, 1993a). In addition to these currents, BLA neurons also possess another muscarinic-sensitive $K^+$ current, the hyperpolarization-activated inward rectifier current ($I_{IR}$) (Womble and Moises, 1993b). The mechanisms underlying muscarinic inhibition of $K^+$ conductances in BLA pyramidal neurons is unknown. However, in hippocampal pyramidal neurons, muscarinic receptors are positively coupled to the phosphoinositide (PI) transduction pathway (Fisher and Bartus, 1985), activation of which is thought to mediate the muscarinic inhibitions of $I_M$, $I_{Leak}$, and $I_{AHP}$ (Baraban et al., 1985; Dutar and Nicoll, 1988a,b: Malenka et al., 1986).

The BLA also receives a substantial excitatory glutamatergic innervation (Rainnie et al., 1991). Recently, a new subtype of glutamate receptor, the metabotropic receptor, has been identified (Sugiyama et al., 1987). Activation of metabotropic glutamate receptors in-

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creases PI hydrolysis in several cell types, leading to production of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate ($IP_3$) and mobilization of intracellular $Ca^{2+}$ stores (Desai and Conn, 1990; Furuya et al., 1989; Sladeczek et al., 1985; Sugiyama et al., 1987). Stimulation of these receptors on hippocampal pyramidal neurons mimics several of the actions produced by muscarinic agonists, including membrane depolarization and reductions in accommodations and the slow AHP (Desai and Conn, 1991; Stratton et al., 1989). Several studies have shown that these changes result, at least in part, from the metabotropic glutamatergic-induced inhibitions of $I_M$ and $I_{AHP}$ (Baskys, 1992; Charpak et al., 1990; Ito et al., 1992). The effect of metabotropic receptor activation on $I_{Leak}$ has not been examined. Although the intracellular steps linking stimulation of PI hydrolysis to the inhibition of $I_M$ or $I_{AHP}$ remain unclear, these findings suggest the possibility that a common intracellular transduction pathway may be activated by muscarinic and metabotropic glutamatergic agonists.

The presence of metabotropic glutamate receptors which are functionally coupled to increased PI hydrolysis have been demonstrated in the rat amygdala (Akiyama et al., 1992; Martin et al., 1992). Therefore, we have voltage-clamped BLA pyramidal neurons in a slice preparation of the basal forebrain to examine the possibility that application of the metabotropic receptor agonist, 1-aminocyclopentane-1s,3R-dicarboxylic acid (ACPD) (Desai and Conn, 1990; Palmer et al., 1989), might produce inhibition of the muscarinic-sensitive currents $I_M$, $I_{Leak}$, $I_{AHP}$, or $I_{IR}$. Our findings demonstrate that ACPD inhibited $I_M$ and $I_{AHP}$, resulting in membrane depolarization accompanied by reductions of spike frequency accommodation and the slow AHP. However, $I_{Leak}$ and $I_{IR}$ were unaffected by ACPD treatment. Some of these results have previously been reported in abstract form (Womble et al., 1992).

## MATERIALS AND METHODS

Horizontal slices of the ventral forebrain (500 μm) were prepared from adult male rats and maintained in a recording chamber perfused with physiological saline saturated with 95% $O_2$-5% $CO_2$ at room temperature. The saline consisted of (in mM) NaCl, 124; KCl, 3.5; $CaCl_2$, 3.0; $MgSO_4$, 1.5; $NaH_2PO_4$, 1.0; $NaHCO_3$, 26.2; glucose, 11.0, pH 7.3. In various experiments, 1s,3R-ACPD (20 or 100 μM), 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX, 20 μM), 2-amino-5-phosphonovaleric acid (APV, 50 μM), atropine (2 μM), or tetrodotoxin (TTX, 1 μM) were dissolved in saline to their final concentration and applied to the slice via a multiport perfusion system. ACPD was acquired from Tocris Neuramin, CNQX was from Research Biochemicals Inc. and all other drugs were from Sigma Chemical Co. (St. Louis, MO).

Pyramidal neurons in the BLA were impaled with a single microelectrode filled with 2.7 M KCl/0.4 M potassium acetate (30–100 MΩ) for current- and voltage-clamp recording using an Axoclamp 2A sample and hold amplifier, as previously described (Womble and Moises, 1992, 1993a,b). The data were digitized using pClamp software (Axon Instruments) and analyzed off-line with DAOS software (Laboratory Software Associates, Victoria, Australia), equipped with a cursor-based, least-squares exponential curve fitting routine. Fitted curves were extrapolated back to the preceding voltage change and used for estimation of the instantaneous and steady-state current values. Statistical differences were determined using the paired t-test.

## RESULTS

Stable intracellular recordings were obtained from 16 BLA pyramidal neurons, with an average resting membrane potential of $-66.1 \pm 1.3$ mV (±S.E.M.), similar to previous descriptions of these cells (Washburn and Moises, 1992; Womble and Moises, 1992, 1993a,b). Injection of a prolonged (500 ms) depolarizing current pulse through the recording electrode caused the cell to fire a burst of action potentials whose rate of firing accommodated with time (Fig. 1A). Termination of the current pulse was followed by a long-lasting, biphasic AHP, consisting of an initial, rapidly decaying component (medium AHP) and a prolonged, slowly decaying component (slow AHP) (Fig. 1B), as previously described (Washburn and Moises, 1992; Womble and Moises, 1993a). Rapid switching of the amplifier to voltage-clamp mode upon termination of the depolarizing current pulse (hybrid-clamp) revealed the presence of a biphasic outward tail current that corresponded in time with the AHP (Fig. 1C). Similar biphasic tail currents were also observed in voltage-clamped BLA neurons following a 500 ms depolarizing voltage step to −30 mV from a −60 mV holding potential (not shown). We have previously shown that the later, slowly decaying component of the AHP tail current consists of a slowly decaying $Ca^{2+}$-activated $K^+$ current ($I_{AHP}$). (Lancaster and Adams, 1986; Madison et al., 1987), which is responsible for spike frequency accommodation and production of the slow AHP in BLA neurons (Womble and Moises, 1993a). In the present study, $I_{AHP}$ was measured by extrapolating a single exponential curve fitted to the slowly decaying portion of the AHP tail current back to the end of the previous depolarizing step. This analysis yielded for $I_{AHP}$ an average peak amplitude of $54 \pm 9$ pA and decay tau of $2{,}139 \pm 407$ ms ($n = 15$), values which are similar to those previously reported for BLA neurons (Womble and Moises, 1993a).

Within minutes of changing the bath perfusate to medium containing ACPD (20 or 100 μM), BLA pyramidal neurons depolarized by an average of $3.0 \pm 0.9$ mV ($P < 0.01$; $n = 16$), from their normal resting level of −66.1 mV to an average of $-63.1 \pm 1.7$ mV. The depo-

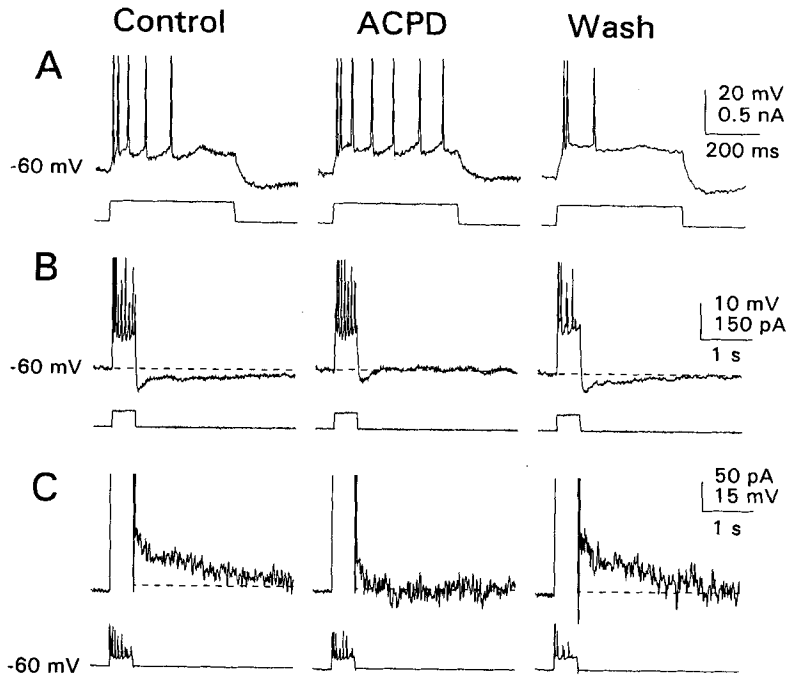


Fig. 1. Effects of ACPD on accommodation and the slow AHP. A: Injection of a 500 ms depolarizing current pulse (+0.2 nA) elicited a series of action potentials in control medium whose rate of firing declined with time before firing ceased. This accommodation response was decreased during perfusion of 20 μM ACPD, an effect that was reversed after washing for 30 min with control saline. B: Termination of a 500 ms depolarizing current pulse was followed in control medium by a long-lasting, biphasic AHP. Application of 20 μM ACPD inhibited the slowly decaying portion of the AHP, an effect reversed by washing with control saline for 30 min. C: Hybrid-clamp records obtained from the same neuron as B. The cell was depolarized in current-clamp mode from a resting level of −60 mV to elicit a burst of action potentials (lower records). Upon termination of the depolarization, the amplifier was rapidly switched into voltage-clamp mode at a holding potential of −60 mV and the current underlying production of the AHP recorded (upper records). Application of 20 μM ACPD inhibited the slowly decaying portion of the outward tail current ($I_{AHP}$), which was restored upon washing with control saline. (Control $I_{AHP}$: decay tau = 2,700 ms, peak amplitude = 58 pA.) In A–C, cells were maintained in current-clamp mode at a membrane potential of −60 mV by injection of steady DC current. All bathing media contained CNQX (20 μM) and APV (50 μM) to block ionotropic glutamate receptors.

larization produced by 20 μM or 100 μM ACPD was similar in magnitude, suggesting that the lower concentration produced a maximal effect. However, the 3.0 mV resting membrane depolarization produced by ACPD was less than half of the depolarization produced in BLA neurons by the cholinergic agonist carbachol (6.5 ± 1.0 mV, n = 13, $P < 0.05$; data from Womble and Moises, 1992). This provided us with the first indication that ACPD does not fully replicate the effects of muscarinic receptor stimulation in BLA neurons.

In three neurons examined in the absence of TTX, application of ACPD decreased the accommodation response normally observed during a 500 ms depolarizing current pulse (Fig. 1A) and reduced the amplitude and duration of the subsequent slow AHP (Fig. 1B). Recordings obtained from these cells using the hybrid-clamp protocol revealed a concomitant inhibition of the underlying $I_{AHP}$ (Fig. 1C). The inhibitory effects of ACPD on accommodation, the slow AHP and $I_{AHP}$ were reversed within 30 min of switching to normal ACSF bathing solution (Fig. 1). Similar reductions in $I_{AHP}$ amplitude were observed in 12 other neurons in the presence of TTX in which $I_{AHP}$ was activated in voltage-clamp mode by a depolarizing voltage step to −30 mV from a holding potential of −60 mV (not shown). Overall, ACPD (20 or 100 μM) reduced $I_{AHP}$ amplitude by 63%, from 54 ± 9 pA to 20 ± 6 pA (n = 15; $P < 0.005$). The inhibitory action of ACPD on $I_{AHP}$ was not prevented in the presence of the muscarinic receptor antagonist atropine (2 μM; n = 4) or a combination of the ionotropic glutamate receptor antagonists CNQX (20 μM) and APV (50 μM) (n = 4) (Fig. 1).

The voltage- and muscarinic-sensitive M-current (Brown and Adams, 1980; Halliwell and Adams, 1982; Womble and Moises, 1992) was identified in voltage-clamped BLA neurons as a slow inward current relaxation elicited during a 1 s hyperpolarizing voltage step to −55 mV from a holding potential of −40 mV (Fig. 2). The current relaxation followed a single exponential time course, with an average decay tau of 220 ± 39 ms and peak amplitude of 82 ± 11 pA (n = 12), in agreement with our previous measurements of $I_M$ in BLA neurons (Womble and Moises, 1992). The inward current relaxation observed during the voltage step to −55 mV was not due to decay of $I_{AHP}$, since it was unchanged after blockade of $I_{AHP}$ by bath application of

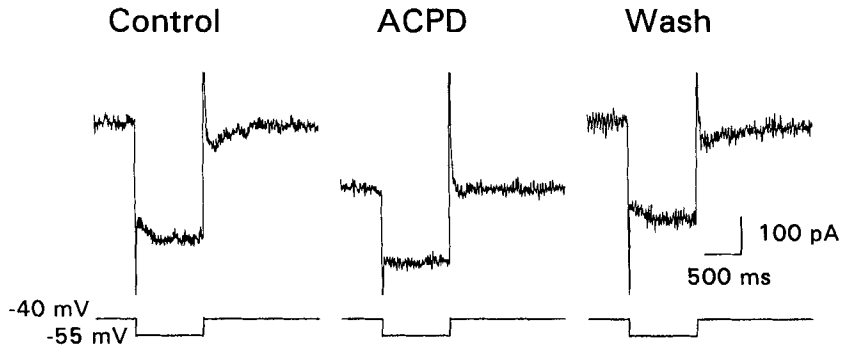


Fig. 2. Blockade of the M-current by ACPD. In control medium, a 1 s hyperpolarizing voltage step to −55 mV largely deactivated the sustained $I_M$ present at the −40 mV holding potential, producing a slow inward-going current relaxation. Perfusion of ACPD (20 μM) blocked $I_M$, eliminating the slow current relaxation and producing an inward shift in holding current level. These effects were reversed upon washing with control medium for 30 min. All bathing media contained 1 μM TTX. (Control $I_M$: decay tau = 151 ms, amplitude = 70 pA.)

200 μM $Cd^{2+}$ (n = 2) or 2 mM $Co^{2+}$ (n = 1), or when 100 μM cyclic-AMP was included in the recording electrode (n = 3) (Womble and Moises, 1993a). In these experiments, following blockade of the slow AHP and $I_{AHP}$ by the calcium channel blockers or intracellular perfusion of cyclic AMP, hyperpolarizing voltage steps to −55 mV from a holding potential of −40 mV revealed M-currents with an average decay tau of 191 ± 22 ms and amplitude of 107 ± 22 pA (n = 6) (data not shown).

Application of ACPD (20 μM or 100 μM) blocked the M-current, as evidenced by the loss of the inward current relaxation during the hyperpolarizing voltage step (Fig. 2), an effect that was reversed by washing for 30 min with normal bathing medium. Overall, ACPD reduced the amplitude of the M-current relaxation by 61%, from 82 ± 11 pA to 32 ± 9 pA (n = 12; $P < 0.005$). The inhibitory action of ACPD on $I_M$ was not prevented in the presence of atropine (2 μM; n = 4) or a combination of CNQX (20 μM) and APV (50 μM) (n = 2) (data not shown).

*Muscarinic agonists* produce an inward shift in the level of steady-state current recorded at a holding potential of −40 mV in hippocampal and BLA pyramidal neurons, due to the inhibition of $I_M$ and a voltage-insensitive $K^+$ leak current ($I_{Leak}$) (Benson et al., 1988; Madison et al., 1987; Womble and Moises, 1992). Since perfusion of ACPD blocked $I_M$ and produced a similar inward shift in holding current (Fig. 2), we examined the possibility that ACPD might also block $I_{Leak}$ in BLA neurons. To test this, cells were voltage-clamped at a holding potential of −70 mV, where $I_M$ is largely inactive (Womble and Moises, 1992), and a series of hyperpolarizing voltage steps was applied to command potentials of −75 to −140 mV (Fig. 3A). Current-voltage (I-V) relationships were constructed by plotting the instantaneous current response derived from exponential curves fitted to the current elicited during a hyperpolarizing voltage step as a function of the membrane potential obtained during the step (Fig. 3B). These plots were linear over the tested range of voltages, reflecting the presence of the voltage-insensitive leak conductance. In the majority of cells tested (64%; 9 of 14), application of 20 or 100 μM ACPD had no effect on this I-V relationship, indicating that $I_{Leak}$ was unaffected by the drug in these neurons. In the remaining five neurons, ACPD appeared to produce a small decrease in conductance, suggesting a possible reduction in $I_{Leak}$.

However, several other lines of evidence support the conclusion that $I_{Leak}$ was not blocked by ACPD. The level of steady-state current measured at a holding potential of −70 mV was not significantly altered by ACPD (control level of 80 ± 20 pA vs. 57 ± 30 pA in the presence of ACPD; n = 14; $P > 0.5$). Similarly, membrane conductance measurements obtained from the instantaneous current response recorded at the onset of a voltage step from −70 mV to −77 mV were unchanged by ACPD (9.2 ± 0.8 nS in controls vs. 8.7 ± 0.6 nS with ACPD; n = 14). These findings stand in contrast to the muscarinic inhibition of $I_{Leak}$ in BLA neurons, where carbachol (40 μM) produced an average inward current shift of 53 ± 9 pA (n = 15) in steady-state current level recorded at a holding potential of −70 mV (Womble and Moises, 1992) and an average decrease in instantaneous membrane conductance measured during a voltage step from −70 mV to −77 mV of 2.4 ± 0.9 nS (n = 8; data not shown).

Two hyperpolarization-activated currents, the H-current ($I_H$) (Halliwell and Adams, 1982; Mayer and Westbrook, 1983) and the inward rectifier current ($I_{IR}$) (Constanti and Galvan, 1983; Uchimura and North, 1990), have also been observed in BLA pyramidal neurons (Womble and Moises, 1993b). These currents differ in their rates and voltage ranges of activation, as illustrated in Figure 3. Stepping a BLA neuron from a holding potential of −70 mV to −99 mV for 1 s activated only $I_H$, producing a slowly developing inward current

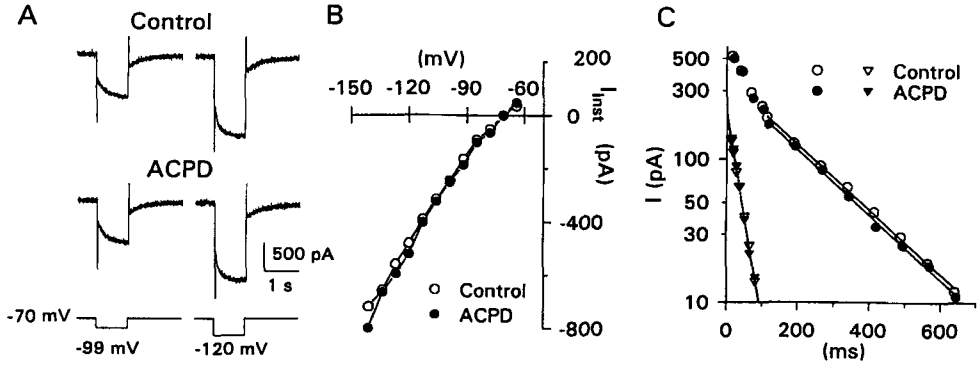


Fig. 3. Effects of ACPD on the voltage-insensitive $K^+$ leak current and the hyperpolarization-activated currents, $I_H$ and $I_{IR}$. A: From a holding potential of −70 mV, a 1 s hyperpolarizing voltage step to −99 mV elicited the H-current, while a step to −120 mV evoked both the rapidly activating $I_{IR}$ and the more slowly developing $I_H$. Addition of 20 μM ACPD to the bathing medium did not reduce the instantaneous current response or alter the hyperpolarization-evoked currents. B: The instantaneous current responses ($I_{Inst}$) recorded from this cell during a series of hyperpolarizing voltage steps from the holding potential of −70 mV plotted as a function of the membrane potential obtained during the step. The linear nature of the plots indicated the presence of a voltage-insensitive leak conductance which was unaffected by ACPD. C: Semi-logarithmic plots of the hyperpolarization-activated currents elicited in A during the voltage steps to −120 mV as a function of time. The steady-state current level obtained at the end of the voltage step was adjusted to zero to show current amplitudes. A single exponential was fitted to the late, slowly activating portion of the current record to provide an estimate for the $I_H$ component (circles). Extrapolation of this curve and subtraction from the total current record revealed that the rapidly activating $I_{IR}$ component also followed a single exponential time course (triangles). Neither the $I_H$ or $I_{IR}$ components were affected by ACPD treatment. ($I_H$: activation tau = 170 ms, amplitude = 380 pA; $I_{IR}$: activation tau = 35 ms, amplitude = 192 pA.)

that followed a single exponential time course (Fig. 3A). With a larger voltage step to a command potential of −120 mV, the H-current component was preceded by activation of the rapidly developing $I_{IR}$. Plotting the total current evoked at −120 mV on a semi-logarithmic scale (open symbols, Fig. 3C), enabled us to separate the $I_{IR}$ and $I_H$ current components by exponential curve peeling. During a step from −70 mV to −120 mV, the $I_H$ component had an average amplitude of 199 ± 30 pA and activation tau of 188 ± 47 ms (n = 9), while the amplitude of the $I_{IR}$ component was 125 ± 16 pA, with an activation tau of 49 ± 9 ms (n = 8), in agreement with our previous measurements of these current components in BLA neurons (Womble and Moises, 1993b).

We have previously shown that $I_{IR}$, but not $I_H$, is inhibited following muscarinic receptor activation (Womble and Moises, 1993b). However, as shown in Figure 3, ACPD (20 μM) did not alter the time courses of current activation or the amplitudes of either $I_H$ or $I_{IR}$. Overall, ACPD (20 or 100 μM) did not reduce the amplitudes of either $I_H$ (199 ± 30 pA versus 177 ± 32 pA with ACPD; n = 9) or $I_{IR}$ (125 ± 16 pA vs. 125 ± 12 pA with ACPD; n = 8) when measured during a step from −70 mV to −120 mV.

## DISCUSSION

Muscarinic receptor activation produces several changes in BLA pyramidal neurons, including prolonged membrane depolarization and losses of spike frequency accommodation and the slow AHP (Washburn and Moises, 1992). These effects have been shown to result from the muscarinic inhibition of several $K^+$ conductances, including $I_M$, $I_{Leak}$, $I_{AHP}$, and $I_{IR}$ (Womble and Moises, 1992, 1993a,b). The present data demonstrate that the metabotropic glutamate receptor agonist, 1s,3R-ACPD, mimicked several of these muscarinic effects. We found that ACPD inhibited $I_{AHP}$ in BLA pyramidal neurons and thus reduced the spike frequency accommodation response normally observed during a current-induced burst of action potentials, as well as the slowly decaying portion of the AHP which followed. This agonist also blocked the M-current, producing a sustained depolarization of the cell. However, unlike the actions of muscarinic agonists, ACPD did not appear to inhibit $I_{Leak}$ or $I_{IR}$. The lack of effect on $I_{Leak}$ may account for the finding that the depolarization produced in BLA neurons by a saturating concentration of ACPD was significantly less than that produced by carbachol (Womble and Moises, 1992).

Cloning and expression studies have identified a family of metabotropic receptor subtypes (Aramori and Nakanishi, 1992; Houamed et al., 1991; Masu et al., 1991; Tanabe et al., 1992). These receptors have been shown to be linked to the stimulation of PI hydrolysis and generation of diacylglycerol and $IP_3$ (Desai and Conn, 1990; Furuya et al., 1989; Sladeczek et al., 1985; Sugiyama et al., 1987), although effects on cyclic-AMP, arachidonic acid and phospholipase D metabolism have also been observed (Boss and Conn, 1992; Dumuis et al., 1990; Schoepp et al., 1992; Winder and Conn, 1992). In hippocampal pyramidal neurons, activation of PI turnover has been implicated in the inhibitions of $I_{AHP}$ and $I_M$ induced by application of either muscarinic receptor agonists (Baraban et al., 1985; Dutar and

Nicoll, 1988a,b; Malenka et al., 1986) or metabotropic glutamate receptor agonists (Baskys, 1992; Charpak et al., 1990). However, more recent evidence suggests that the direct excitatory effects of ACPD on hippocampal pyramidal neurons may be mediated by a separate class of metabotropic receptors which are positively linked to phospholipase D activation (Boss and Conn, 1992; Desai et al., 1992). Our results demonstrate that, like muscarinic cholinergic receptors, the metabotropic glutamate receptors of BLA pyramidal neurons which are activated by ACPD are functionally coupled to the inhibition of $I_{AHP}$ and $I_M$. However, metabotropic receptor activation did not result in blockade of the muscarinic-sensitive current $I_{IR}$, nor was $I_{Leak}$ inhibited in the majority of BLA neurons examined. One possible interpretation of these results is that activation of the PI transduction system following muscarinic receptor activation results in the blockade of all four muscarinic-sensitive $K^+$ currents, while activation of the phospholipase D system by metabotropic glutamatergic agonists produces inhibitions of only $I_M$ and $I_{AHP}$. Thus, there may be a partial convergence of action between two intracellular transduction systems in pyramidal neurons of the basolateral amygdala.

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