The Journal of Neuroscience, July 1993, 13(7): 3041–3050

Modulation of Calcium Currents by a Metabotropic Glutamate Receptor Involves Fast and Slow Kinetic Components in Cultured Hippocampal Neurons

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The modulation of high-threshold Ca^{2+} currents by the selective metabotropic glutamate receptor (mGluR) agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), was investigated in cultured hippocampal neurons using whole-cell voltage-clamp recording. ACPD reduced high-threshold Ca^{2+} currents carried by Ba^{2+} with an EC_{50} of 15.5 μM. The inhibition was reversible, voltage dependent, and blocked by L-2-amino-3-phosphonopropionic acid (1 mM) or by pretreatment with pertussis toxin. Inhibition by ACPD was greatly enhanced, and became irreversible, when the non-hydrolyzable GTP analog GTPγS was included in the whole-cell pipette. In some neurons, the Ba^{2+} current was inhibited by L(+)-2-amino-4-phosphonobutanoic acid (L-AP4) as well as ACPD while most cells were insensitive to L-AP4, suggesting that these agonists activate distinct receptors. The inhibition of Ca^{2+} currents was reduced but not eliminated in the presence of either ω-conotoxin GVIA or nifedipine, suggesting that both N- and L-type Ca^{2+} currents were affected. The degree and kinetics of inhibition were dependent on intracellular calcium. With $[Ca]_i < 1$ nM, inhibition had a fast onset ($t \approx 1$–2 sec) and a rapid recovery, consistent with a membrane-delimited pathway. However, a slow component of inhibition appeared when the steady state $[Ca]_i$ was increased to 100 nM (t onset ≈ 3 min). The slow component did not require transient Ca^{2+} influx or release of intracellular Ca^{2+}. We suggest that Ca^{2+} channel modulation by ACPD involves either two mGluR subtypes with separate coupling mechanisms or a single mGluR that couples to both mechanisms.

[Key words: metabotropic glutamate receptors, calcium channels, G-proteins, L-2-amino-4-phosphonobutanoic acid, hippocampus, trans-ACPD]

Glutamate activates two types of receptors, ligand-gated ion channels and G-protein–coupled (metabotropic) receptors (mGluRs). The ligand-gated ion channels have well established roles in excitatory synaptic transmission (for review, see Mayer and Westbrook, 1987; Collingridge and Lester, 1989), but the physiological functions of mGluRs are much less defined (Schoepp et al., 1990a). mGluRs were first identified in mRNA-injected *Xenopus* oocytes as coupled to inositol phospholipid metabolism (Sugiyama et al., 1987). Recently, five mGluRs have been cloned that have several intracellular transduction mechanisms and distinctive expression patterns (Houamed et al., 1991; Masu et al., 1991; Abe et al., 1992; Tanabe et al., 1992).

Pharmacological and physiological studies have just begun to characterize mGluRs, although this has been hampered by a lack of high-affinity ligands or effective antagonists. One major action of mGluRs in hippocampal neurons appears to be modulation of potassium and calcium channels (Charpak et al., 1990; Lester and Jahr, 1990; Desai and Conn, 1991). In addition, L-2-amino-4-phosphonobutanoic acid (L-AP4), a glutamate analog that does not activate mGluR1 (Houamed et al., 1991), inhibits synaptic transmission and voltage-sensitive Ca^{2+} currents in cultured olfactory bulb neurons via a G-protein–coupled receptor (Trombley and Westbrook, 1992). Thus, more than one G-protein–coupled glutamate receptor may be involved in modulation of Ca^{2+} channels.

It has been proposed that modulation of Ca^{2+} channels by glutamate or L-AP4 is membrane delimited (Lester and Jahr, 1990; Trombley and Westbrook, 1992). However, the existence of multiple mGluR subtypes and the activation of three diffusible second messengers even by a single mGluR receptor (Aramori and Nakanishi, 1992) provide multiple possible coupling mechanisms. The best-studied biochemical action of mGluR activation in hippocampal neurons is inositol trisphosphate (IP_3) production and mobilization of Ca^{2+} from intracellular stores (Murphy and Miller, 1988; Furuya et al., 1989; Schoepp et al., 1990a).

The Ca^{2+} channel subtypes that are modulated by mGluRs are also unclear. Although N-type channels, the predominant Ca^{2+} channel subtype in neurons of the PNS, are a major target of neurotransmitter modulation (for review, see Tsien et al., 1988; Hess, 1990), in hippocampal neurons, N-, L-, and P-type Ca^{2+} channels contribute approximately equally to the whole-cell current (Regan et al., 1991; Mintz et al., 1992). In acutely dissociated cortical neurons, L-type channels were inhibited by an mGluR (Sayer et al., 1992) whereas N-type channels were inhibited in acutely dissociated CA3 neurons (Swartz and Bean, 1992a).

In order to elucidate the mechanisms of the Ca^{2+} channel modulation by mGluRs, we focused on three issues: (1) whether (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and L-AP4 mediated inhibition of Ca^{2+} channels involves distinct receptors, (2) whether Ca^{2+} channel modulation by ACPD is

Received Oct. 15, 1992; revised Jan. 21, 1993; accepted Feb. 25, 1993.

We thank J. Volk for preparation of cell cultures and Dr. Bruce Bean for a preprint of a manuscript in press. This work was supported by grants from the National Institutes of Health (NS26494) and the Klingenstein Fund for the Neurosciences.

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dependent on $[Ca^{2+}]_i$, and (3) which Ca^{2+} channel subtypes are affected. Our results suggest that ACPD and L-AP4 act via separate receptors to reduce voltage-dependent Ca^{2+} currents in cultured hippocampal neurons. The action of ACPD involves fast and slow kinetic components resulting in a decrease in both N- and L-type Ca^{2+} channel activity.

A preliminary report has appeared in abstract form (Sahara and Westbrook, 1991).

Materials and Methods

Cell culture. Primary cultures of rat hippocampal neurons were prepared from neonatal rat pups (Sprague–Dawley). Pups were anesthetized with halothane and decapitated, and the hippocampi removed and cut into 1 mm slices. The slices were incubated in activated papain (20 U/ml, 45–60 min), dissociated by gentle trituration, and plated (10,000 cells/cm^2) on a confluent layer of hippocampal astrocytes as previously described (Legendre and Westbrook, 1990). The culture medium contained Minimum Essential Medium (GIBCO), 0.6% glucose, 5% heat-inactivated horse serum (Hyclone), and a supplement including 200 μg/ml transferrin, 200 μM putrescine, 60 nM sodium selenite, 40 nM progesterone, 40 ng/ml corticosterone, 20 ng/ml triiodothyronine, and 10 μg/ml insulin. Half of the medium was changed twice weekly.

Electrophysiology. Currents were recorded in the whole-cell patch-clamp mode using a patch-clamp amplifier (PC-501, Warner Instrument Co., Hamden, CT). Recordings were made from well-isolated pyramidal-shaped neurons after 5–7 d in culture. All experiments were done at room temperature. The extracellular recording solution contained (in mM) 165 NaCl, 2.5 KCl, 2.5 $BaCl_2$, 1 $MgCl_2$, 10 *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), and 10 glucose; pH was adjusted to 7.3 with NaOH and osmolarity to 325 mOsm. Tetrodotoxin (TTX; 1 μM) and tetraethylammonium chloride (5 mM) were added to block voltage-dependent sodium channels and potassium channels; strychnine (2 μM), picrotoxin (50 μM), and 7-chlorokynurenic acid (500 nM) were added to block glycine, γ-aminobutyric acid, and *N*-methyl-*D*-aspartate (NMDA) channel activity. The chamber was continuously perfused at 1 ml/min with extracellular solutions in which Ca^{2+} was substituted for Ba^{2+}. This prevented Ba^{2+}-induced depolarization of other neurons in the culture with subsequent increases in synaptic activity.

Patch pipettes were fabricated from thin-wall borosilicate glass (1.5 mm o.d.; WPI Inc., Sarasota, FL), coated with Sylgard (Dow-Corning Corp., MI), and fire polished, and had DC resistances of 2–5 MΩ. Recordings pipettes contained 160 mM cesium methanesulfonic acid (Aldrich Chemical Co. Inc., WI), 10 mM HEPES, 11 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetra-acetic acid (EGTA), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM adenosine 5′-triphosphate (ATP; disodium salt), and 0.5–1 mM guanosine 5′-triphosphate (GTP; sodium salt); pH was adjusted to 7.3 with CsOH and osmolarity to 315 mOsm. ATP and GTP were diluted into the intracellular solutions for each experiment and stored on ice until needed. In some experiments, the pipette Ca^{2+} concentration was adjusted by substituting 11 mM bis-(*o*-amino-phenoxy-ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA) for EGTA; Ca^{2+} was added to give the desired buffered Ca^{2+} concentration based on the BAPTA dissociation constant reported by Tsien (1980). These were 11 mM BAPTA + 0 mM Ca^{2+} ($[Ca^{2+}] < 10^{-9}$ M), 11 BAPTA + 2.5 Ca^{2+} ($[Ca^{2+}] = 3 \times 10^{-8}$ M), and 11 BAPTA + 5 mM Ca^{2+} ($[Ca^{2+}] = 10^{-7}$ M).

Drug applications. Drugs were diluted in the Ba^{2+} control solution and delivered via a linear array of six glass capillary tubes (400 μm diameter) mounted on a micromanipulator, and positioned within ≈200 μm of the cell. Solution flow was controlled with three-way latching solenoid valves. To assess the kinetics of (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) modulation, solution exchange was performed by simultaneously opening one valve and closing the adjacent valve. This allowed solution exchange within 100 msec (see, e.g., Lester et al., 1990). ACPD and L-2-amino-3-phosphonopropionic acid (L-AP3) were purchased from Tocris Neuramin (Bristol, U.K.); L(+)-2-amino-4-phosphonobutanoic acid (L-AP4) and 7-chlorokynurenic acid were from Cambridge Research Biochemical Inc. (Wilmington, DE); guanosine 5′-*o*-(3-thiotriphosphate) (GTPγS; tetralithium salt), picrotoxin, TTX, DL-2-amino-5-phosphonovaleric acid (DL-AP5), ω-conotoxin (ω-CgTx), and nifedipine from Sigma (St. Louis, MO); and BAPTA from Molecular Probes Inc. (Eugene, OR). Nifedipine and 7-chlorokynurenic acid were dissolved in ethanol and then diluted in water and stored at −20°C.; the highest final concentrations of ethanol were 0.01% and 0.002%, respectively. Other drug were dissolved in water. In some experiments, neuronal cultures were incubated at 37°C with pertussis toxin (PTX; Sigma, 0.5 μg/ml) for 24–30 hr.

Data acquisition and analysis. Following establishment of whole-cell recording, 5 min was allowed for equilibration of patch pipette contents with the cytoplasm before the beginning of data collection. During this time the neuron was perfused with the extracellular Ca^{2+} solution. However, following the initiation of data collection, the neuron was continuously perfused with Ba^{2+} solution from one of the flowpipes. Depolarizing voltage jumps (30 msec) were delivered at regular intervals (3/min) under computer control to elicit Ba^{2+} currents. Current records were corrected for linear leakage and capacitance using a *P*/4 procedure. For kinetic experiments, 10 msec voltage steps from −60 mV to 0 mV at 1 sec intervals were used, the current remaining in Cd^{2+} (100 μM) was used for leak subtraction. Currents records were low-pass filtered with an 8-pole Bessel filter at 2 kHz, digitized at 5 kHz, and stored on an IBM-AT using pCLAMP (version 5.5). Data were analyzed on a Macintosh using AXOGRAPH software (Axon Instruments). For display, the initial 500–1000 μsec following a voltage step were blanked to eliminate uncompensated capacitance transients. There were small differences between the time to peak for different neurons; thus, the amplitude of Ba^{2+} currents was measured by averaging a short data segment 15–20 msec following onset of the voltage jump; comparison with measurement made at peak current revealed no systematic differences. Currents that showed obvious changes in the slope of activation, suggesting poor space clamp, were discarded. Although ATP was included in the whole-cell pipette, most cells still showed significant current rundown within 15–30 mins; thus, to measure current inhibition, the current in the presence of drug was compared to the average of the current amplitude immediately before drug application and following washout. This procedure underestimated the initial inhibition by approximately 10% in most cells. Data are expressed as mean ± SD. The significance of the results was determined using Student's *t* test or, where applicable, paired *t* test.

Results

Whole-cell voltage-clamp recordings were made from hippocampal neurons after 5–7 d culture. High-threshold Ca^{2+} currents were evoked by depolarizing voltage steps from −60 to 0 mV using Ba^{2+} (2.5 mM) as the charge carrier. The inward currents (I_{Ba}) had negligible inactivation during the 30 msec pulse and were completely blocked by Cd^{2+} (100 μM). In some neurons, especially those older than 7 d in culture, the activation of I_{Ba} showed evidence of poor space clamp; these neurons were not further analyzed.

ACPD reversibly inhibits high-threshold Ca^{2+} currents

To examine mGluR modulation of I_{Ba}, (1*S*,3*R*)-ACPD, the active stereoisomer of *trans*-ACPD, was applied by rapid perfusion. ACPD inhibited I_{Ba} in the majority of neurons tested. Figure 1*A* shows current traces from immediately before and after application of 200 μM ACPD. For neurons dialyzed with 11 mM EGTA (pCa = 8) and GTP, the reduction of I_{Ba} was 21.2 ± 8.5% (n = 27). As is apparent in Figure 1*A*, the inhibition was gradually relieved during the depolarizing voltage step. The inhibition could not be attributed to NMDA receptor activation (see Chernevskaya et al., 1991), as NMDA receptors were blocked by adding Mg^{2+} (1 mM) and 7-chlorokynurenic acid (500 nM), although on some neurons ACPD produced small inward holding currents (5–10 pA). These were not investigated further. ACPD reduced I_{Ba} rapidly and was completely reversible following return to the control solution (Fig. 1*B*). Although I_{Ba} ran down despite inclusion of ATP in the pipette, repeated applications of ACPD were still effective. However in most cells the percentage inhibition was largest following the first application and gradually decreased on subsequent exposures to ACPD. For example, the inhibition decreased from (20.5 ± 6.1% to 15.9

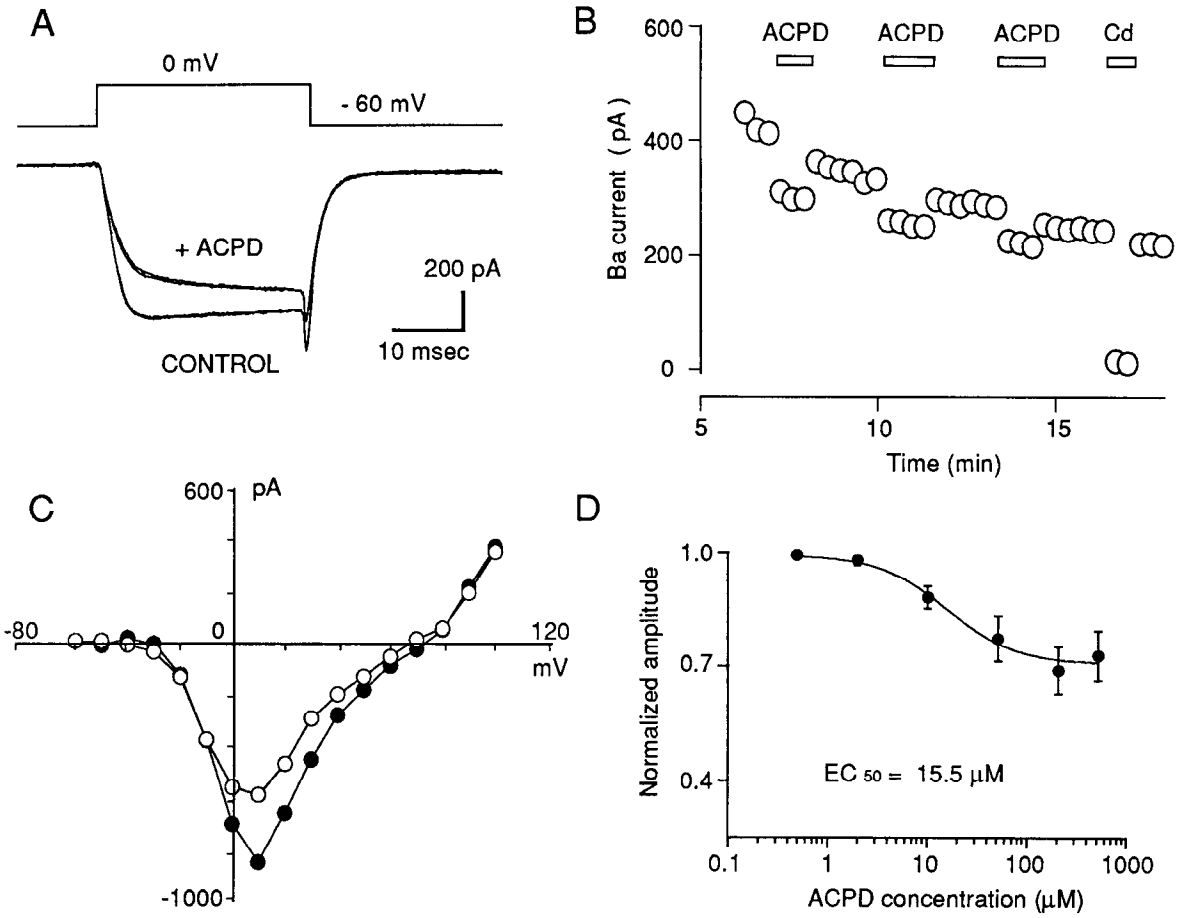

Figure 1. ACPD inhibits high-threshold Ca^{2+} currents. *A*, Rapid perfusion of ACPD reversibly inhibited high-threshold Ca^{2+} currents, evoked by 30 msec depolarizing pulses from −60 to 0 mV. Ba^{2+} (2.5 mM) was used as the charge carrier. Patch pipettes were buffered to pCa = 8 with 11 mM EGTA and 1 mM Ca^{2+}. Leak-subtracted currents immediately before and after ACPD application (200 μM) are superimposed. In the presence of ACPD, a slow relief of inhibition was observed during the voltage step. *B*, Inhibition produced by repeated applications of ACPD gradually declined during 20 min of whole-cell recording. I_{Ba} was evoked at 20 sec intervals. *C*, The *I–V* relationship in Ba^{2+} solution in the absence (*solid circles*) and presence (*open circles*) of 200 μM ACPD. I_{Ba} was unaffected at positive holding potentials. *D*, Concentration–response curve for ACPD inhibition of I_{Ba}. The data set includes only neurons tested with at least three agonist concentrations. Data was fitted to the logistic equation $I = 1 - I_{max}/(1 + (EC_{50}/[ACPD])^n)$, where I is the normalized current amplitude, I_{max} is the response at saturating [ACPD], EC_{50} is the [ACPD] that produces 50% of the maximum response, and n is the slope factor. I_{max} was 0.38.

± 4.3% during three sequential applications of ACPD (n = 7). As not all neurons were ACPD sensitive, subsequent analysis is based on neurons (n = 138) that demonstrated >10% inhibition. We also occasionally observed small potentiation of I_{Ba} by ACPD in otherwise typical neurons, but this did not occur with sufficient frequency to allow further analysis.

Figure 1*C* shows the current–voltage relationship measured in control solution and in the presence of 200 μM ACPD. Relief of I_{Ba} inhibition was observed at positive holding potentials as has been observed for several neurotransmitter receptors (e.g., Bean, 1989; Elmslie et al., 1990). The concentration–response curve for ACPD modulation of I_{Ba} (n = 6) is shown in Figure 1*D*. ACPD had little effect at 2 μM and was maximally effective at 200 μM. The data were fitted to the logistic equation with an EC_{50} = 15.5 μM and a slope factor (n) = 1.25. This closely parallels the EC_{50} reported for activation of mGluRs by *trans*-ACPD in other preparations (Schoepp et al., 1990a).

A PTX-sensitive G-protein mediates the action of ACPD

PTX has been reported to block L-AP4 modulation of Ca^{2+} currents (Trombley and Westbrook, 1992), but PTX was ineffective in blocking mGluR modulation of Ca^{2+} channels (e.g., Lester and Jahr, 1990; Swartz and Bean, 1992a). We first confirmed that Ca^{2+} channel modulation by ACPD required a G-protein by including the nonhydrolyzable GTP analog GTPγS (100–200 μM) in the recording pipette. GTPγS markedly increased the inhibition of I_{Ba} by ACPD compared to GTP, and as expected the inhibition was irreversible (Fig. 2*A*). For GTPγS the inhibition of I_{Ba} was 44.8 ± 12.5% (n = 5) compared to 23.4 ± 8.7%; n = 9; $P < 0.01$) for GTP (Fig. 2*B*). Subsequent applications of ACPD produced little or no additional inhibition.

To test the PTX sensitivity of ACPD responses, hippocampal neurons were incubated for 24–30 hr at 37°C with PTX (0.5 μg/ml). Following PTX treatment, ACPD (with GTP in the pipette) produced only a 4.1 ± 2.0% (n = 5) inhibition of I_{Ba}. This was significantly less that inhibition in untreated sister cultures (21.3 ± 2.6%; n = 5; $P < 0.01$) or in sister cultures treated with heat-inactivated PTX (17.8 ± 6.3%; n = 4).

L-AP3 antagonizes ACPD

L-AP3 has been reported to be a noncompetitive antagonist of mGluRs (Schoepp et al., 1990b), but L-AP3 was ineffective in antagonizing mGluR1 and mGluR5 in CHO cells (Abe et al.,

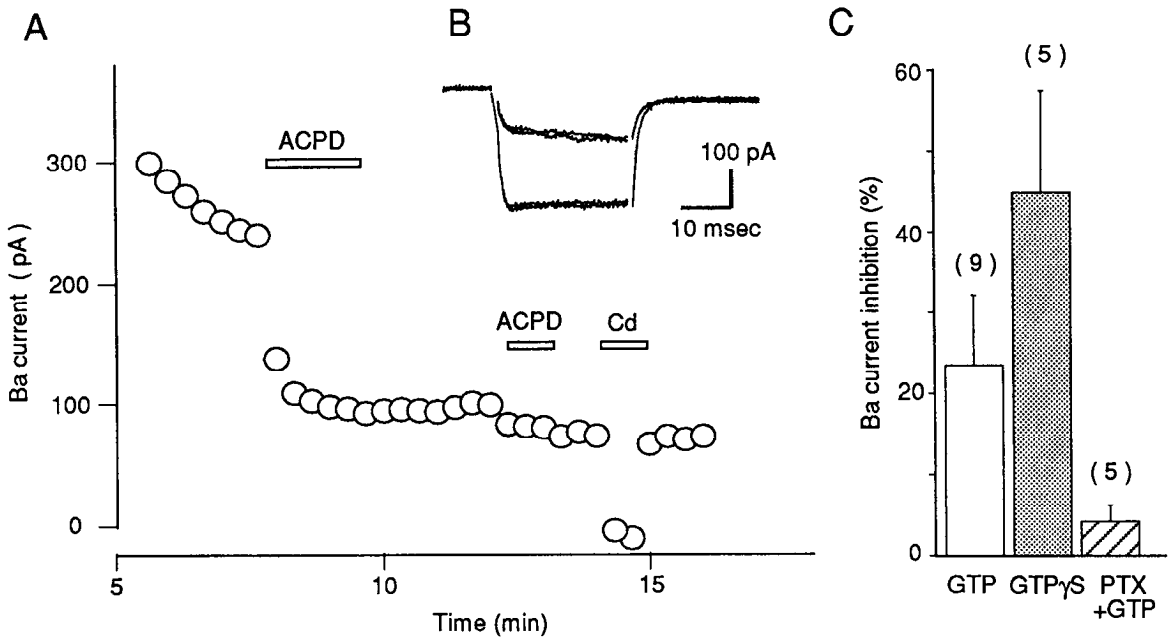

Figure 2. A PTX-sensitive G-protein mediates the fast inhibition of I_{Ba}. *A*, In neurons dialyzed with GTPγS (100–200 μM), ACPD (200 μM) produced an irreversible inhibition of I_{Ba}. *B*, Current was evoked by a 30 msec voltage step from −60 mV to 0 mV in the presence and absence of ACPD. Initial 1 msec following the voltage step was blanked. *C*, The reduction in I_{Ba} was significantly larger in cells dialyzed with GTPγS compared to GTP ($P < 0.01$). However, following preincubation with PTX (0.5 μg/ml, 24–30 hr at 37°C), the modulation of I_{Ba} by ACPD was blocked.

1992; Aramori and Nakanishi, 1992). However, L-AP3 did antagonize ACPD-induced mGluR1 responses in *Xenopus* oocytes (Houamed et al., 1991) and quisqualate-induced IP_3 synthesis and Ca^{2+} mobilization in neurons (Irving et al., 1990; Schoepp et al., 1990b). The ability of L-AP3 to block ACPD modulation of I_{Ba} was examined as shown in Figure 3. At submaximal concentrations of ACPD (50–100 μM), L-AP3 (1 mM) blocked I_{Ba} inhibition (Fig. 3*A,B*). For five neurons, inhibition by ACPD was 18.0 ± 8.8%, and 6.2 ± 1.8% in the absence and presence of L-AP3 ($P < 0.05$; Fig. 3*C*). However, at high concentrations of ACPD (200 μM), I_{Ba} modulation was completely unaffected by L-AP3 ($n = 8$). This suggests that L-AP3 is a low-affinity competitive mGluR antagonist. In addition, higher concentrations of L-AP3 (1 mM) directly reduced the amplitude of the Ca^{2+} current. This was abolished by DL-AP5 (100 μM) in combination with Mg^{2+} (1 mM) and 7-chlorokynurenic acid (500 nM), consistent with an agonist action of L-AP3 on NMDA receptors.

L-AP4 and ACPD modulate Ca^{2+} currents via different receptors

L-AP4 has been reported to activate a G-protein–coupled glutamate receptor, and to inhibit Ca^{2+} influx and synaptic transmission in olfactory bulb neurons (Trombley and Westbrook, 1992). No antagonists are known to block the action of L-AP4, but L-AP4 does not activate mGluR1 (Houamed et al., 1991). Thus, to determine whether ACPD and L-AP4 activate the same receptor, ACPD (200 μM) and L-AP4 (50 μM) were sequentially applied to 66 hippocampal neurons. In the majority of the neurons (58 of 66), I_{Ba} was inhibited only by ACPD, as shown for one neuron in Figure 4*A*. However, in eight neurons, both agonists were equally effective in reducing I_{Ba}. Inhibition was 23.0 ± 10.2% for ACPD and 26.4 ± 8.1% ($n = 8$) for L-AP4 (Fig. 4*B*). L-AP4 also reduced I_{Ba} even after several applications of ACPD had resulted in a progressively smaller ACPD response. These data suggest that L-AP4 and ACPD activate distinct G-protein–coupled receptors, but that only a minority of cultured hippocampal neurons express AP4 receptors that are functionally coupled to calcium channels.

I_{Ba} modulation by ACPD is dependent on $[Ca^{2+}]_i$

The best-characterized action of mGluR activation is IP_3-mediated release of intracellular Ca^{2+} (Sugiyama, 1987; Furuya et al., 1989), and $[Ca^{2+}]_i$ may also affect modulation of K^+ and Ca^{2+} currents by muscarinic receptors (Marrion et al., 1991; Beech et al., 1992). The dependence of ACPD modulation on intracellular calcium was tested by buffering the patch pipette to <1 nM, 30 nM, or 100 nM $[Ca^{2+}]_i$. We waited 5 min after the establishment of whole-cell recording before application of ACPD to allow equilibration of the patch pipette with the cytoplasm. ACPD became more effective as Ca^{2+} was increased in the patch pipette. The I_{Ba} inhibition was 15.1 ± 5.3% ($n = 5$) at <1 nM, 25.4 ± 4.7% ($n = 6$) at 30 nM, and 29.6 ± 10.2% ($n = 5$) at 100 nM $[Ca^{2+}]i$. The inhibition at 100 nM $[Ca^{2+}]_i$ was significantly greater than at <1 nM $[Ca^{2+}]_i$ ($P < 0.05$). There was a larger variance in inhibition at higher $[Ca^{2+}]_i$, perhaps due to variations in cell dialysis that depend on cell size and access resistance (see Pusch and Neher, 1988; Mathias et al., 1990). We attempted to determine whether release of Ca^{2+} from intracellular stores contributed to the modulation using thapsigargin (1 μM; $n = 6$), a selective inhibitor of the microsomal Ca^{2+}-ATPase (Thastrup et al., 1990). However, thapsigargin caused an irreversible inhibition of I_{Ba} even in the absence of ACPD (not shown). Release of intracellular Ca^{2+} from caffeine-sensitive store can also decrease Ca^{2+} currents (Thayer et al., 1988; Kramer et al., 1991), and the effects of caffeine (10 mM; $n = 5$) were additive to those of ACPD (not shown). These results suggest that I_{Ba} modulation by ACPD is dependent on

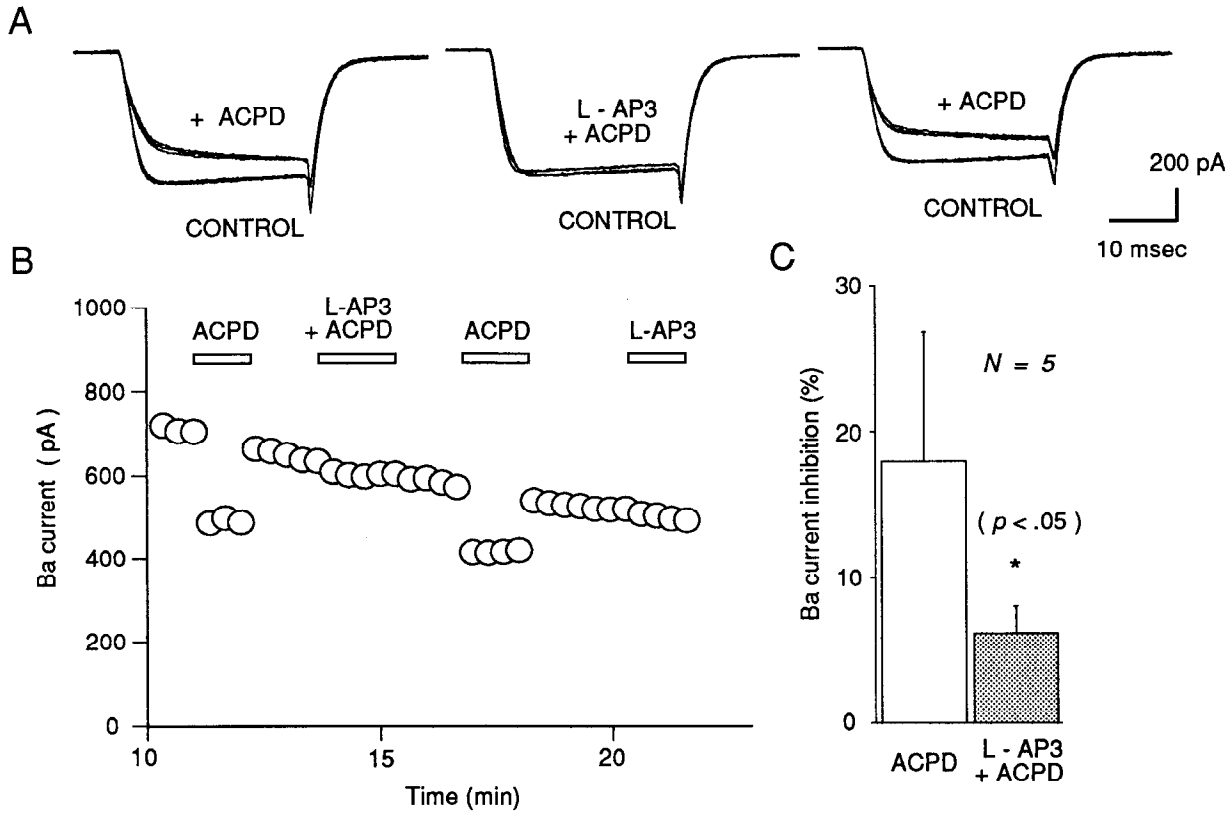

Figure 3. L-AP3 antagonizes ACPD inhibition of I_{Ba}. *A*, I_{Ba} before, during, and after washout of 50 μM ACPD are superimposed. The addition of 1 mM L-AP3 completely blocked the reduction in I_{Ba} by ACPD (*middle*), and was rapidly reversible (*right*). Holding potential was −60 mV. *B*, Plot of I_{Ba} for the neuron shown in *A*. Applications of 50 μM ACPD and 1 mM L-AP3 are indicated by *bars*. L-AP3 had no direct effect on I_{Ba} at 1 mM in the presence of DL-AP5 (100 μM) and 7-chlorokynurenic acid (500 nM) to block NMDA receptors. *C*, Percentage inhibition of I_{Ba} by ACPD (50 μM) before and after L-AP3 (1 mM) application.

the steady state $[Ca^{2+}]_i$, although our data are not sufficient to exclude an additional contribution from intracellular Ca^{2+} stores.

The kinetics of I_{Ba} modulation were also dramatically affected by the Ca^{2+} concentration in the pipette. Figure 5 compares the time course of ACPD action with pipettes containing either <1 nM $[Ca^{2+}]_i$ (Fig. 5*A*) or 100 nM $[Ca^{2+}]_i$ (Fig. 5*C*). At the low concentration of $[Ca^{2+}]_i$, ACPD modulation had both a rapid onset and recovery (Fig. 5*A*). For the neuron shown in Figure

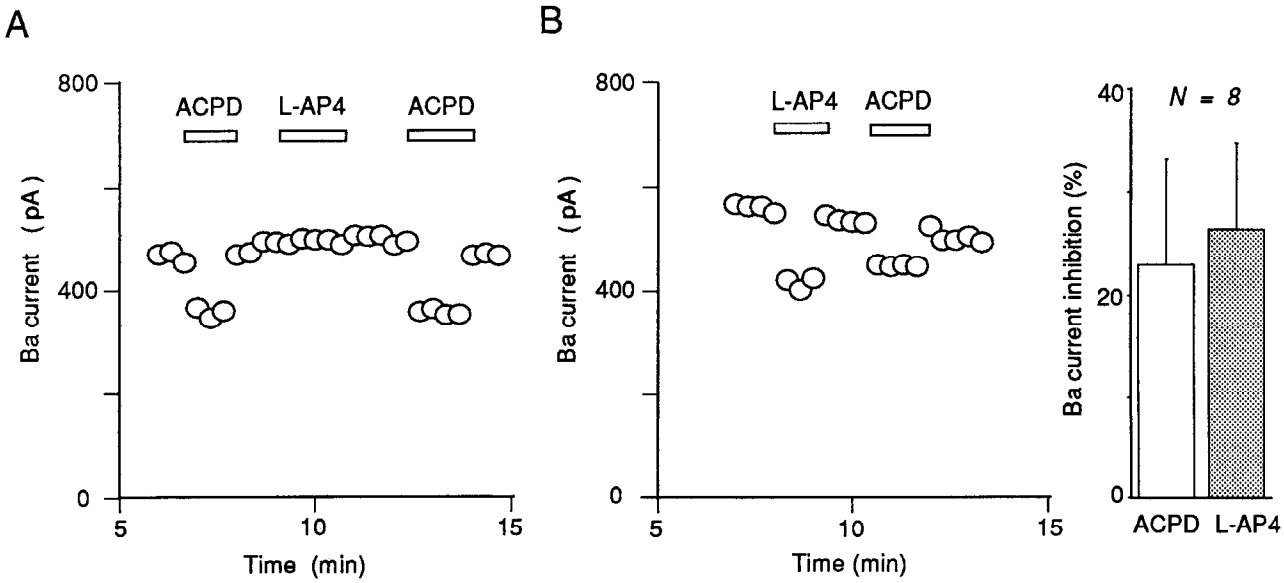

Figure 4. Inhibition of I_{Ba} by L-AP4 and ACPD involves different receptors. *A*, Both ACPD (200 μM) and L-AP4 (50 μM) were applied to 66 neurons. In the majority of the neurons (58 of 66), I_{Ba} was inhibited only by ACPD as shown for this neuron. *B*, However in the remaining eight neurons, both L-AP4 (50 μM) and ACPD were effective as shown for this neuron (*left*). The percentage inhibition of I_{Ba} by L-AP4 and ACPD was similar (*right*).

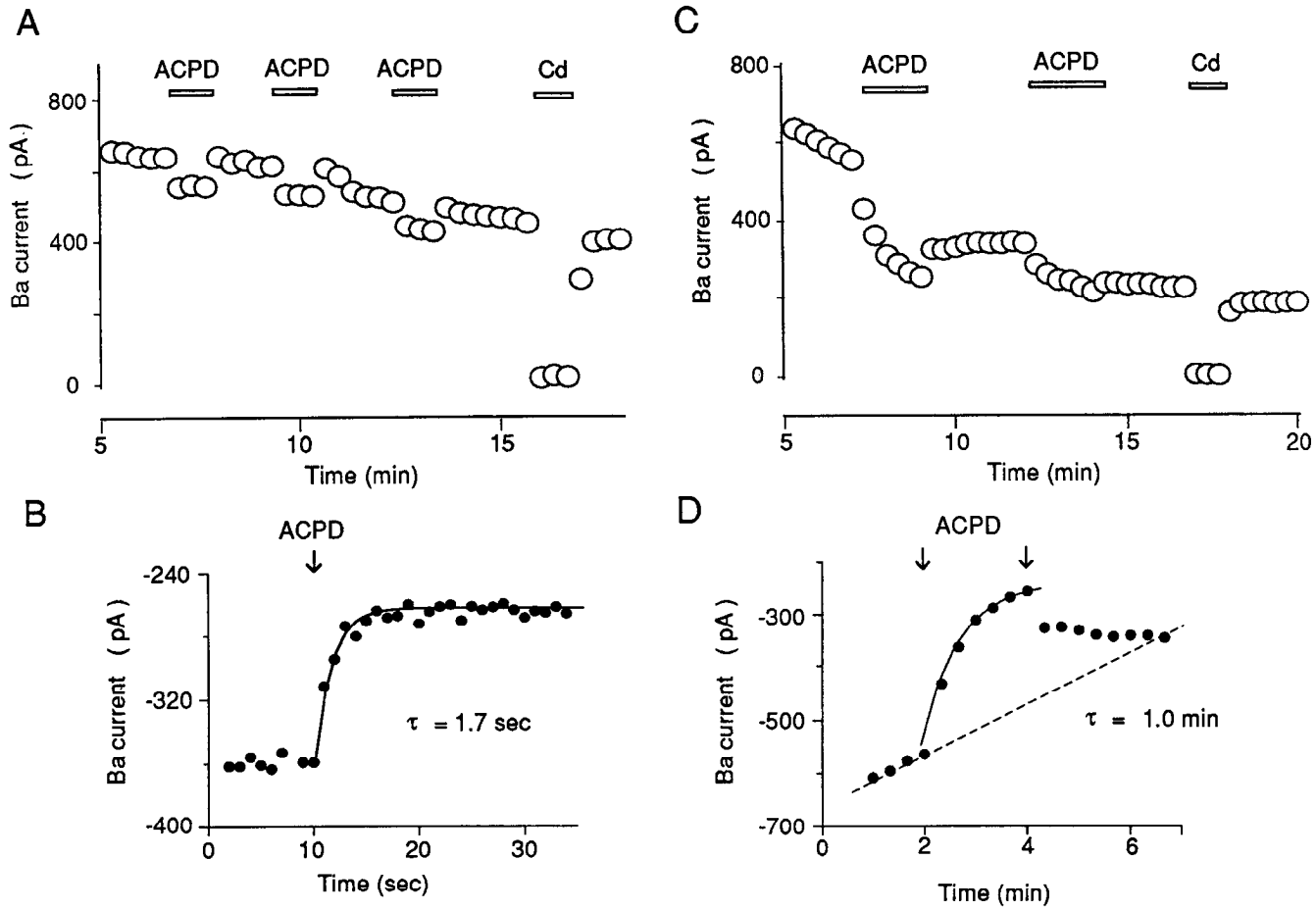

Figure 5. Increases in $[Ca^{2+}]_i$ revealed a slow component of I_{Ba} inhibition. I_{Ba} was recorded with patch pipettes buffered to either low Ca^{2+} (<1 nM) or high Ca^{2+} (100 nM) with BAPTA/Ca^{2+} mixtures. *A*, With $[Ca^{2+}]_i$ buffered to <1 nM, I_{Ba} inhibition evoked by application of ACPD (200 μM) showed rapid onset and recovery. ACPD was applied 5 min after establishment of whole-cell configuration to allow equilibration of the buffer with the cytoplasm. *B*, The rapid onset was fitted with a single exponential with a time constant of 1.7 sec. I_{Ba} was elicited every 1 sec by a 10 msec step from −60 mV to 0 mV, and leak subtracted using 100 μM Cd^{2+}. ACPD (200 μM) was applied at time as indicated by the *arrow*. *C*, In another neuron, with $[Ca^{2+}]_i$ buffered to 100 nM, the onset of inhibition was markedly slowed. Recovery following removal of ACPD showed both a fast and a slow component. *D*, The onset of the slow component was measured with I_{Ba} evoked every 20 sec during a long application (2 min) of ACPD (200 μM; *arrows*). A single exponential curve (t = 1 min) was fitted to the onset.

5*B*, the rapid onset of inhibition was fitted with a single exponential curve (t = 1.7 sec). In contrast, with the pipette buffered to 100 nM $[Ca^{2+}]_i$, I_{Ba} continued to decrease during application of ACPD (Fig. 5*C*). The time constant for the onset of the slow phase was 3.0 ± 1.7 min (n = 5; Fig. 5*D*). We were unable to resolve adequately a fast component of the onset because voltage jumps were performed only at 20 sec intervals; this was necessary to prevent rapid calcium current rundown in high-$[Ca^{2+}]_i$ solutions. However, when ACPD was rapidly washed out, both a fast and slow component of recovery were apparent (Fig. 5*C,D*). In most cases recovery of the slow component was incomplete. Subsequent ACPD application often revealed a smaller slow component, perhaps due to incomplete recovery from the prior application.

ACPD inhibits both dihydropyridine-sensitive and ω-CgTx–sensitive Ca^{2+} channels

In rat hippocampal neurons, at least three types of high-threshold Ca^{2+} channels, N-, L-, and P-type (non-L, non-N), have been described (Mogul and Fox, 1991; Regan et al., 1991). L-type current is blocked by dihydropyridine (DHP) antagonists such as nifedipine whereas N-type currents are irreversibly blocked by ω-CgTx (e.g., Hess, 1990). In our experiments, 32.3 ± 10.6% of I_{Ba}, elicited from a holding potential of −60 mV, was irreversibly blocked by ω-CgTx (0.5–1 μM), 18.1 ± 8.1% of the remaining current was sensitive to nifedipine (1 μM), and the remaining 30–50% was therefore of the non-L, non-N type (n = 20). In some cells, apparently unequal proportions of Ca^{2+} channels (<10% of L-type and 50% N-type channel) were observed.

In order to determine the Ca^{2+} channel subtype that was inhibited by ACPD, I_{Ba} was evoked before and after block of N-type current with ω-CgTx using patch pipettes buffered with EGTA (pCa = 8). As shown in Figure 6*A*, although ω-CgTx block was irreversible, subsequent application of ACPD produced a smaller, but clearly measurable, decrease in I_{Ba}. In five experiments, ACPD (200 μM) inhibited I_{Ba} by 17.4 ± 3.2 % before ω-CgTx application compared to 6.8 ± 1.2% after ω-CgTx. Thus, ACPD inhibits N-type channels as well as a residual current of either the L or the non-L, non-N type. To test whether ACPD also blocks L-type channels, ACPD was applied in the presence of nifedipine (1 μM). As shown in Figure 6*B*, nifedipine blocked part of I_{Ba} and reduced the effect of ACPD, suggesting that L-type currents are also sensitive to inhibition by an mGluR.

The reduction in I_{Ba} modulation could not be attributed to channel rundown, as ACPD was effective following washout of nifedipine. ACPD inhibited I_{Ba} by 17.0 ± 6.4% before nifedipine application, while ACPD inhibited DHP-resistant currents by 8.6 ± 0.9%, and by 14.4 ± 2.4% (n = 4) following washout. The prolongation of Ca^{2+} tail currents by DHP agonists has also been used to examine modulation of L-type channels (e.g., Plummer et al., 1991); however, the presence of dendrites on cultured neurons prevented adequate time resolution for tail current analysis.

Discussion

Our results demonstrate that Ca^{2+} channel modulation by G-protein–coupled glutamate receptors involves distinct receptor subtypes activated by L-AP4 and ACPD. The EC_{50} values for ACPD and antagonism by L-AP3 are consistent with activation of an mGluR. However, inhibition of I_{Ba} by ACPD had two kinetic components; the fast component was similar to the membrane-delimited effects of activated G-proteins, whereas the slow component was dependent on the steady state level of $[Ca^{2+}]_i$. Both DHP-sensitive and ω-CgTx-sensitive Ca^{2+} channels were modulated by ACPD.

The mGluR responsible for Ca^{2+} current modulation

Until recently the action of glutamate analogs on second messenger–coupled responses was considered as resulting from activation of a single mGluR. However, molecular cloning studies have revealed at least five mGluR subtypes, although the role of these subtypes in specific cellular functions is still unclear. Several glutamate agonists including quisqualate, L-AP4, ibotenate, and L-glutamate inhibit high-threshold Ca^{2+} currents (Lester and Jahr, 1990; Trombley and Westbrook, 1992). Quisqualate is a potent agonist for mGluR1 and mGluR5, but has a low affinity for mGluR2; L-glutamate, the endogenous agonist for mGluRs, is relatively nonselective. Because ACPD is a selective agonist for mGluR1, -2, and -5 (Masu et al., 1991; Abe et al., 1992; Tanabe et al., 1992), our results are consistent with modulation of Ca^{2+} channels by one of these mGluR receptor subtypes. This is also supported by recent studies in CA3 and cortical neurons (Sayer et al., 1992; Swartz and Bean, 1992a). However, L-AP4 does not activate mGluR1, -2, or -5 (Houamed et al., 1991; Abe et al., 1992; Tanabe et al., 1992), suggesting the involvement of an additional "AP4" receptor, possibly mGluR4 (Tanabe et al., 1993).

Our data also suggest that two distinct G-protein–coupled glutamate receptors modulate Ca^{2+} channels in hippocampal neurons. Only 10% of the hippocampal neurons tested were sensitive to both L-AP4 and ACPD. This is consistent with the low sensitivity of hippocampal pathways to presynaptic inhibition by L-AP4, except for the neurons projecting into the dentate gyrus via the lateral perforant pathway (reviewed in Mayer and Westbrook, 1987). This contrasts with cultured olfactory bulb neurons, where the majority of mitral/tufted cells are sensitive to L-AP4 (Trombley and Westbrook, 1992). The small number of AP4-sensitive cells precluded testing the additivity of AP4 and ACPD in the presence of GTPγS, a method useful for distinguishing separate populations of receptors. However, consistent with involvement of separate receptors, L-AP4 inhibited I_{Ba} in hippocampal neurons even after ACPD inhibition markedly declined following multiple applications.

It is not yet possible to distinguish mGluR subtypes with antagonists. In particular, prior studies with the putative mGluR antagonist L-AP3 have produced variable results. L-AP3 blocks mGluR-stimulated IP_3 production in hippocampal slices (Schoepp et al., 1990b) as well as in *Xenopus* oocytes expressing mGluR cDNAs in some experiments (Houamed et al., 1991), but not in others (Masu et al., 1991; Abe et al., 1992; Tanabe et al., 1992). Unlike the noncompetitive action previously reported (Schoepp et al., 1990b), the effect of L-AP3 in our experiments could be overcome by higher concentrations of ACPD. We have made similar observations in *Xenopus* oocytes injected with hippocampal polyA+ mRNA or mGluR1 cDNA (G. L. Westbrook, unpublished observation), suggesting that L-AP3 is a competitive, albeit weak, mGluR antagonist. Thus, L-AP3 is likely to be ineffective if high concentrations of agonist are used

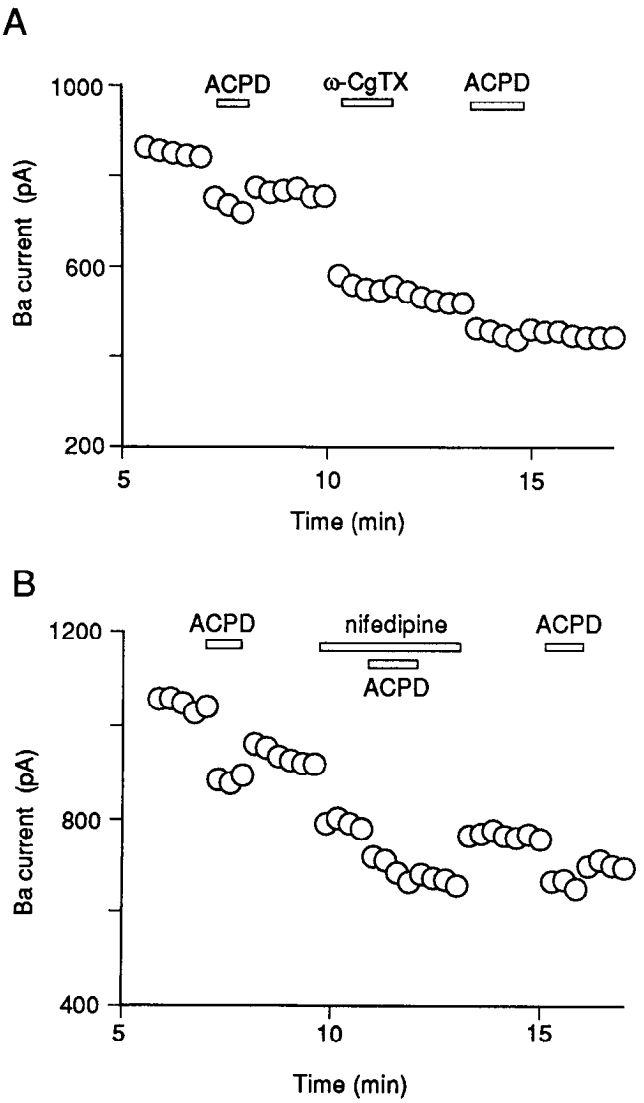

Figure 6. ACPD acts on both ω-CgTx–sensitive and DHP-sensitive Ba^{2+} currents. *A*, ACPD (200 μM) was applied before and after irreversible block of N-type channels by ω-CgTx (1 μM). ω-CgTx blocked 25% of I_{Ba} in this neuron and reduced the ACPD-sensitive current from 110 pA to 65 pA, suggesting that both ω-CgTx–sensitive and –insensitive currents are affected by mGluRs. *B*, In another neuron, ACPD (200 μM) inhibition was partially blocked during simultaneous application of nifedipine (1 μM), consistent with inhibition of L-type channels by ACPD. The I_{Ba} inhibition returned to control levels following washout of nifedipine. Patch pipettes contained 11 mM EGTA and 1 mM Ca^{2+}. I_{Ba} was evoked by voltage jumps to 0 mV from a holding potential of −60 mV.

or in the presence of "spare" receptors, that is, if less than full receptor occupancy results in a maximal response.

Coupling of mGluRs to Ca^{2+} channels

It has been proposed that mGluR and AP4 receptors inhibit Ca^{2+} channels via a membrane-delimited pathway (Lester and Jahr, 1990; Swartz and Bean, 1992a; Trombley and Westbrook, 1992). Membrane-delimited coupling to high-threshold Ca^{2+} channels appears to be a common general mechanism for G-protein–coupled receptors (reviewed in Hille, 1992). This coupling mechanism has several characteristics features including prominent slowing of Ca^{2+} current activation kinetics (Bean, 1989; Elmslie et al., 1990) and kinetics faster than expected for a diffusible second messenger system (Jones, 1991; Beech et al., 1992). For example, muscarinic receptors in cardiac myocytes open K(ACh) channels via PTX-sensitive pathway with a latency of 30–150 msec (Yatani and Brown, 1989), and the fast muscarinic inhibition of Ca^{2+} currents on superior cervical ganglion neurons occurs in less than 1 sec (Bernheim et al., 1991; Beech et al., 1992). ACPD inhibition of Ca^{2+} currents in our experiments shared these features when $[Ca^{2+}]_i$ was tightly buffered, consistent with a membrane-delimited action Ca^{2+} channel activation.

Although the G-protein involved in the fast component of inhibition was PTX sensitive in our experiments, others have been unable to block mGluR inhibition with PTX (Lester and Jahr, 1990; Swartz and Bean, 1992a). PTX does block mGluR2-induced inhibition of cAMP formation as well as 40% of IP_3 synthesis by mGluR1, but has only a small effect on IP_3 synthesis by mGluR5 (Masu et al., 1991; Abe et al., 1992; Tanabe et al., 1992). Thus, it is possible that more than one G-protein is involved in Ca^{2+} channel modulation by mGluRs, as has been reported for muscarinic receptors (see, e.g., Beech et al., 1992). The stimulation of independent second messenger pathways by mGluR1 in CHO cells provides further evidence for such coupling diversity (Aramori and Nakanishi, 1992).

Dependence of Ca^{2+} channel modulation on $[Ca^{2+}]_i$

In addition to the fast component of Ca^{2+} channel inhibition, increases in the steady state $[Ca^{2+}]_i$ revealed a slow component of inhibition by ACPD that had an onset time constant of $\approx$3 min and a equally slow recovery rate. Because we used Ba^{2+} as the charge carrier, transmembrane Ca^{2+} influx could not be responsible for the slow inhibitory component. Although mGluR1 and mGluR5 stimulate release of intracellular Ca^{2+} stores, the rise time of the slow component was much longer than the time course of cytoplasmic Ca^{2+} transients evoked by quisqualate in Ca^{2+}-free solutions (ca. 3 sec; Furuya et al., 1989). Likewise, in the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ transients disappear with multiple agonist applications, presumably due to depletion of stores (Murphy and Miller, 1988; Furuya et al., 1989). Thus, the Ca^{2+} dependence would not appear to be simply due to facilitation of IP_3-mediated release of intracellular Ca^{2+}. The $[Ca^{2+}]_i$ dependence of the slow component of inhibition is likely to explain the Ca^{2+} dependence observed with quisqualate-induced depression of Ca^{2+} currents (Lester and Jahr, 1990). However, the mechanism of action of Ca^{2+} remains unclear. Both mGluR1 and mGluR5 would be expected to activate protein kinase C (PKC), and PKC activators have been shown to decrease whole-cell Ca^{2+} currents (Doerner et al., 1988; Rane et al., 1989; Plummer et al., 1991). However, this does not appear to explain ACPD modulation of Ca^{2+} currents, as PKC enhances Ca^{2+} currents while occluding mGluR-mediated inhibition of N-type Ca^{2+} currents in CA3 neurons (Swartz et al., 1993).

The distinct kinetic components of ACPD modulation in our experiments are strikingly similar to modulation of Ca^{2+} channels in sympathetic neurons (Bernheim et al., 1991; Beech et al., 1992). In sympathetic neurons, muscarinic receptors mediate a fast membrane-delimited inhibition and a slow inhibition dependent on the steady state $[Ca^{2+}]_i$, whereas noradrenaline activates only the fast component. In our experiments the increase in percentage inhibition in high $[Ca^{2+}]_i$ also suggests that both fast and slow mechanisms are involved, although we were unable to resolve the fast component of the onset in high $[Ca^{2+}]_i$ due to the limited sampling rate. However two components were apparent in the recovery. An alternative possibility, that Ca slows a single kinetic process, is thus unlikely. The second messenger mediating the slow component is unknown, but does not appear to involve release of intracellular Ca^{2+}, PKC, or cyclic nucleotides (Bernheim et al., 1991). It has been suggested that a Ca^{2+}-dependent phosphatase could be this messenger (Armstrong and White, 1992).

Ca^{2+} channel subtypes

Both ω-CgTx–sensitive as well as DHP-sensitive Ca^{2+} channels were inhibited by ACPD. Many neurotransmitters and neuropeptides can inhibit Ca^{2+} channels, although in most cases N-type channels appear to be the target (Tsien et al., 1988; Hess, 1990). DHP-sensitive Ca^{2+} channels are also modulated by neurotransmitters. The best studied is the enhancement of L-type channels in cardiac myocytes by cAMP-dependent protein kinase (for a review, see Birnbaumer et al., 1990). Activation of PKC has been reported to increase or decrease L-type Ca^{2+} current in cardiac cells (Lacerda et al., 1988), and leutinizing hormone–releasing hormone inhibits L- as well as N-type Ca^{2+} channels in frog sympathetic neurons (Bley and Tsien, 1990). Sayer et al. (1992) reported that *trans*-ACPD selectively inhibits L-type channels in cortical neurons, while Swartz and Bean (1992a) reported that ACPD selectively inhibits N-type channels in CA3 hippocampal neurons. The kinetics of N-type channel inhibition were similar to the fast component seen in our experiments. As these experiments were done with tightly buffered $[Ca^{2+}]_i$, this may explain the lack of a slow component of inhibition. Taken together, these results are consistent with inhibition of N-type channels primarily by the fast mechanism whereas L-type channels are only affected by the slow inhibitory process. This would also be analogous to muscarinic receptors in sympathetic neurons (Mathie et al., 1992).

Functional significance

Inhibition of N-type Ca^{2+} channels is considered a likely mechanism for regulation of transmitter release by presynaptic autoreceptors (e.g., Hirning et al., 1988; Holz et al., 1988; Lipscombe et al., 1989; Bley and Tsien, 1990). The role of L-type currents in transmitter release is less clear. L-AP4 and *trans*-ACPD can inhibit transmitter release (e.g., Forsythe and Clements, 1990; Baskys and Malenka, 1991; Lovinger, 1991; Trombley and Westbrook, 1992). We have confirmed that ω-CgTx blocks spontaneous EPSCs in cultured hippocampal neurons while nifedipine has little effect. However, in the presence of TTX (1 μM), miniature EPSCs are more effectively blocked by nifedipine than by ω-CgTx (Y. Sahara, unpublished observation). Depression of transmitter release also results from

an increase in K^+ conductance in presynaptic terminals as suggested by Cox and Dunlap (1992), and ACPD also modulates at least two separate K^+ conductances, Ca^{2+}-activated K^+ (I_{AHP}) and I_M (Charpak et al., 1990).

Our results add further to this complexity in that Ca^{2+} channel modulation by mGluRs appears to involve both multiple receptors and distinct kinetic components. This remarkable diversity provides an array of possible cellular effects as has been suggested for other G-protein–coupled receptors (e.g., Hille, 1992). A critical variable in sorting out this complexity is the time course of transmitter during normal synaptic transmission and the cellular location of mGluRs. Glutamate is present at high concentrations in the synaptic cleft for only brief periods (e.g., ≈1 mM for 1 msec; Clements et al., 1992). As glutamate has a relatively low affinity for mGluRs (Schoepp et al., 1990a), it seems unlikely these receptors will be significantly activated during normal synaptic activity unless they are located within the synaptic cleft. If mGluRs are more uniformly distributed on the membrane surface, activation may occur only during intense or repetitive synaptic activity.

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