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Modulation of a Slowly Inactivating Potassium Current, I_D, by Metabotropic Glutamate Receptor Activation in Cultured Hippocampal Pyramidal Neurons

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I_D is a slowly inactivating 4-aminopyridine (4-AP)-sensitive potassium current of hippocampal pyramidal neurons and other CNS neurons. Although I_D exerts multifaceted influence on CNS excitability, whether I_D is subject to modulation by neurotransmitters or neurohormones has not been clear.

We report here that one prominent effect of metabotropic glutamate receptor (mGluR) activation by short (3 min) exposure to 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) (100 μM) is suppression of I_D by acceleration of its inactivation. I_D was identified as a target of mGluR-mediated modulation because inactivation of a component of outward current sensitive to 100–200 μM 4-AP was accelerated by 1*S*,3*R*-ACPD, and because 4-AP occluded any further actions of 1*S*,3*R*-ACPD. Enhancement of I_D inactivation was induced by the group I-preferring agonist *RS*-3,5-dihydroxyphenylglycine (3,5-DHPG) and the group II-preferring agonist 2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV), but not by the group III-preferring agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4); it was blocked by the broadly acting mGluR antagonist *S*-α-methyl-4-carboxyphenylglycine (S-MCPG). Furthermore, inactivation of I_D was enhanced by inclusion of GTPγS in the internal solution and blocked by inclusion of GDPβS.

Metabotropic GluR-induced suppression of I_D was manifest in three aspects of excitability previously linked to I_D by their sensitivity to 4-AP: reduction in input conductance and enhanced excitability at voltages just positive to the resting potential, reduced delay to action potential firing during depolarizing current injections, and delayed action potential repolarization. We suggest that mGluR-induced suppression of I_D could contribute to enhancement of hippocampal neuron excitability and synaptic connections.

Key words: metabotropic glutamate receptor; 4-aminopyridine; potassium current; I_D; hippocampus; pyramidal neuron; regulation of excitability

Slowly inactivating potassium currents, such as I_D, have been described in neurons from many regions of the CNS (McCormick, 1991; Surmeier et al., 1991; Ficker and Heinemann, 1992; Hammond and Crepel, 1992; Wu and Barish, 1992; Stefani et al., 1995; Bossu et al., 1996; Li and McArdle, 1997; Locke and Nerbonne, 1997a). As a class, these currents are often defined by their sensitivity to micromolar concentrations of 4-aminopyridine (4-AP), a characteristic that is striking in comparison with the millimolar concentrations required to block the more rapidly inactivating transient potassium current I_A, and in comparison with the 4-AP insensitivity of the relatively noninactivating potassium current I_K.

The higher sensitivity of I_D to 4-AP is commonly used to separate it from other voltage-gated potassium currents [e.g., I_A, I_K, or I_M (Rudy, 1988)] and has been used to identify three major influences of I_D on the electrical behavior of neurons. First, application of micromolar 4-AP reduces the time between initiation of a long depolarizing current injection and onset of firing. For this reason, Storm (1988a) named this current the "delay" potassium current, and similar observations have been made in other investigations of CNS neurons (Locke and Nerbonne, 1997b) [also see Wu and Barish (1994) for a separation not using 4-AP]. This property of I_D suggests a role in temporal integration of long epochs of excitatory input. Second, micromolar 4-AP reduces input conductance at voltages near the resting potential (Brown et al., 1990; Storm, 1990), suggesting that I_D may influence the efficacy of synaptic input in eliciting postsynaptic action potentials. Third, micromolar 4-AP also delays action potential repolarization (Storm, 1987, 1988b; Wu and Barish, 1992; Bossu et al., 1996; Locke and Nerbonne, 1997b), suggesting that by influencing action potential duration I_D may regulate Ca^{2+} entry and its sequelae.

The net effect of these influences of I_D is a multifaceted regulation of excitability in CNS neurons. This is manifest as the ability of micromolar 4-AP to increase presynaptic fiber potentials, to enhance synaptic transmission, and to act as a potent convulsant (Llinás et al., 1976; Theslef, 1980; Buckle and Haas, 1982; Kuhnt and Grinvald, 1982; Haas et al., 1983; Rutecki et al., 1987; Szente and Baranyi, 1987; Perreault and Avoli, 1989, 1991, 1992; Barish et al., 1996; Obaid and Salzberg, 1996; Wheeler et al., 1996; M. E. Barish, R. Kajiwara, and T. Iijima, unpublished observations). Yet despite these demonstrations of the importance of I_D in regulation of excitability, whether I_D is subject to physiological modulation by neurotransmitters or neurohormones has not been clear.

We hypothesized that metabotropic glutamate receptors (mGluRs) might be linked to I_D because several studies have implicated potassium currents in the pleiotropic effects of mGluR activation on hippocampal neuron excitability (Charpak et al., 1990; Lester and Jahr, 1990; Desai and Conn, 1991; Baskys, 1992;

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Swartz and Bean, 1992; Sahara and Westbrook, 1993). We report here that one prominent effect of mGluR activation by 1*S*,3*R*-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) is suppression of I_D by acceleration of its inactivation. Suppression of I_D was sensitive to group I- and group II- but not group III-preferring agonists and antagonists. It was manifest in three aspects of excitability previously linked to I_D by their sensitivity to 4-AP: reduction in input conductance and enhanced excitability at voltages just positive to the resting potential, reduced delay to action potential firing during depolarizing current injections, and delayed action potential repolarization. We suggest that mGluR-induced suppression of I_D could contribute to persistent changes in hippocampal neuron excitability (Stratton et al., 1989; Gereau and Conn, 1994) and connectivity (Bortolotto and Collingridge, 1992, 1993; Bashir et al., 1993; Breakwell et al., 1996; Cohen and Abraham, 1996).

MATERIALS AND METHODS

Preparation of cultures. The procedures for preparation of these mixed neuron-glia cultures were identical to those used previously (Wu and Barish, 1992; Wu et al., 1998). Embryonic Swiss Webster mice were removed under sterile conditions from pregnant female mice, after anesthesia (by halothane inhalation) and cervical dislocation, using procedures meeting National Institutes of Health guidelines. Hippocampi were removed from fetuses and dissociated using papain (7.2 mg/10 ml, 35 min at 35°C; Worthington, Freehold, NJ) in Ca^{2+}- and Mg^{2+}-free HBSS. Dissociated cells were plated at ~22,100 cells/cm^2 (25,000 cells per coverslip) onto poly-D-lysine- and laminin-coated 12-mm-diameter glass coverslips ("Assistent"; Carolina Biological, Burlington, NC) in a 150 μl bubble of medium (described below) supplemented to 10% total serum. After cells were allowed to settle for 2 hr, each 35-mm-diameter Petri dish containing two coverslips was flooded with 1 ml of low-serum medium.

Low-serum medium, which facilitates growth of neurons on a sparse underlying layer of astroglial cells, consisted of an 8:2 mixture of MEM and F-12, supplemented with 2 mM glutamine, B-27 additive [1:50 (Brewer et al., 1993)], 2.5% fetal bovine serum, 2.5% horse serum, 0.5% antibiotic–antimycotic solution [(Sigma, St. Louis, MO) final concentrations: 50 U/ml penicillin, 0.05 mg/ml streptomycin, 0.13 μg/ml amphotericin B], and glucose to a total concentration of 25 mM. An antimitotic, ara-C (10 μM), was added after ~48 hr to control astroglial proliferation. Except as noted, all components of tissue culture media, including sera and B-27, were purchased from Life Technologies (Gaithersburg, MD).

Procedures for electrophysiology. Procedures for whole-cell "tight seal" recordings were also standard, except that we have used an internal solution that maintains stable currents for up to 1 hr under favorable conditions. The solution consisted of the following (in mM): 125 potassium methylsulfate, 15 KCl, 2 $MgCl_2$, 0.01 $CaCl_2$, 0.11 BAPTA, 0.1 GTP (lithium salt), 4 ATP (magnesium salt), 20 HEPES, pH 7.30 (adjusted with Trizma base). Notable are the use of methylsulfate as an anion (Zhang et al., 1994; Velumian et al., 1997), low $CaCl_2$ and BAPTA concentrations, and inclusion of GTP and ATP. The standard external solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 4.2 $NaHCO_3$, 10 glucose, 15 HEPES, pH 7.30. Tetrodotoxin was added at 1 μM to block sodium currents. The bath chamber (volume 0.4 ml) was continuously perfused at a rate of 0.4 ml/min using a peristaltic pump. Channel blockers and other reagents were applied using a slightly pressurized large-bore (tip diameter ~400 μm) puffer pipette.

Currents were recorded using an Axopatch 1B (modified for phase-lag series resistance compensation; Axon Instruments, Foster City, CA) and digitized and analyzed using a Digitata 1200a interface and pCLAMP v. 6 software (Axon Instruments). Series resistance was compensated to ~80%, and currents were filtered using the amplifier's Bessel filter at 1–2 kHz (−3 dB) and digitized at 2–5 kHz. Currents linear with membrane voltage (leak currents and residual capacity transients) were subtracted using a P/−4 voltage-step protocol. Voltages were corrected for junction potentials between electrode and bath solutions. Recordings were made at 28°C.

Statistical tests were made using Instat for Windows (Graph Pad, San Diego, CA). Data in the figures are presented as mean ± SD, with statistical significance evaluated by repeated-measures ANOVA with Dunnett's multiple comparison post-test or by paired two-tailed *t* test, as appropriate.

All mGluR agonists and antagonists were purchased from Tocris Cookson (Ballwin, MO). Potassium methylsulfate was purchased from ICN (Aurora, OH). BAPTA was purchased from Molecular Probes (Eugene, OR). Other salts for physiological solutions were purchased from Sigma.

RESULTS

The data presented below were taken from pyramidal neurons in cultures of dissociated embryonic day 15–16 (E15–E16) hippocampal cells. Pyramidal neurons can be easily identified on the basis of the soma shape and the presence of a prominent apical dendrite. Neurons had been in culture for 4–12 d.

Under the conditions of relaxed intracellular Ca^{2+} chelation used here, both voltage-gated and Ca^{2+}-dependent currents will be activated during voltage-clamp steps to voltages positive to approximately −20 mV (Storm, 1990). The Ca^{2+}-dependent currents consist of both apamin- and charybdotoxin-sensitive components (Lancaster et al., 1991; Beck et al., 1997). These currents are considered only as they may relate to currents elicited as a consequence of mGluR activation.

Of the voltage-gated potassium currents, I_A is a rapidly activating and inactivating current sensitive to millimolar concentrations of 4-AP. The other voltage-gated currents can be separated into a slowly inactivating component and a relatively noninactivating or persistent component based on their differential sensitivity to micromolar concentrations of 4-AP (Brown et al., 1990; Storm, 1990; Ficker and Heinemann, 1992; Wu and Barish, 1992; Bossu et al., 1996; Li and McArdle, 1997). In studies of hippocampal neurons originating in various laboratories, the slowly inactivating and 4-AP-sensitive component has been termed I_D (Storm, 1988a), $I_{T,slow}$ (Ficker and Heinemann, 1992), $I_{K(AT)}$ (Bossu et al., 1996), or I_{As} (Li and McArdle, 1997), and we have referred to it as I_D in previous studies (Wu and Barish, 1992, 1994; Wu et al., 1998). Similar 4-AP-sensitive currents have been termed I_{As} in neostriatal neurons (Surmeier et al., 1991; Gabel and Nisenbaum, 1998) and lateral geniculate relay neurons (McCormick, 1991), and I_D in visual cortical neurons (Albert and Nerbonne, 1995; Locke and Nerbonne, 1997a). Whether all such currents are equivalent is not clear, because differences have been noted in absolute sensitivities to 4-AP (block by tens vs hundreds of micromolar) activation voltage ranges (negative to vs positive to I_A), sensitivities to TEA (block by low millimolar concentrations in some cases), and rates of removal of inactivation (hundreds of milliseconds vs seconds). Nevertheless, as a class they share certain functional roles, including control of the latency to first action potential generation during sustained depolarizing current injections, and control of action potential repolarization. In this study of cultured hippocampal neurons, the current sensitive to 100–200 μM 4-AP has been termed I_D, and an example of its separation is presented in Figure 3 [also see Wu and Barish (1992); Wu et al. (1998)].

In the experiments reported here, each test voltage-clamp step was preceded by a short prepulse to inactivate I_A. Therefore the slowly relaxing potassium currents elicited by test depolarizations were composed of I_D and I_K and components of $I_{K(Ca)}$, and we describe this mixture of currents as "delayed outward current" (delayed relative to initiation of the step depolarization) unless I_D or other currents were specifically isolated.

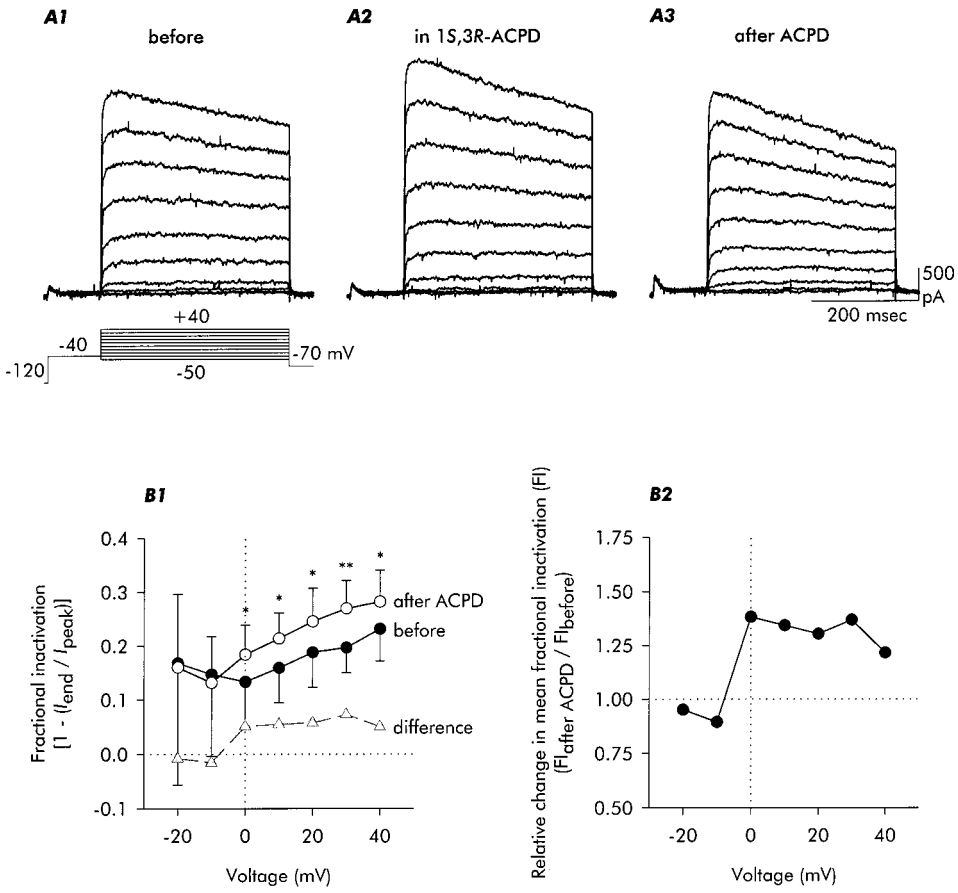

Figure 1. Delayed outward currents of mouse hippocampal neurons and patterns of changes observed during and after exposure to 100 μM 1*S*,3*R*-ACPD. These currents demonstrate the immediate increase in current amplitude seen on application of 1*S*,3*R*-ACPD, and acceleration of delayed current inactivation observed after the exposure to agonist. Note that the changes in inactivation rate and steady-state amplitude were most evident at voltages positive to approximately −10 mV. *A1*, Control currents recorded under conditions that will maximize observation of delayed outward currents, I_D, I_K, and various forms of $I_{K(Ca)}$, and minimize the contribution of I_A (see Results). Currents were recorded at voltages between −50 and +40 mV (in 10 mV increments), as illustrated in the schematic. *A2*, Currents recorded 1 min after initiating exposure to 100 μM 1*S*,3*R*-ACPD. *A3*, Currents recorded 10 min after termination of the 3-min-long exposure to 1*S*,3*R*-ACPD. *B1*, Voltage dependence of fractional inactivation of delayed outward currents $[1 - (I_{end}/I_{peak})]$, illustrating the 1*S*,3*R*-ACPD-induced increase seen at voltages positive to −10 mV and the broad bell-shape of the change in fractional inactivation with voltage (*difference*). *B2*, Ratio of mean fractional inactivation for each test voltage; 1*S*,3*R*-ACPD increased mean fractional inactivation by ~35% except at the most positive voltage. Data are mean ± SD; $n = 7$. Statistical significance is indicated in this and all subsequent figures: *ns*, not significant; $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

Modulation of delayed potassium currents by 1S,3R-ACPD: enhancement and suppression of distinct components

Figure 1*A1* illustrates potassium currents recorded from a cultured hippocampal pyramidal neuron under control conditions. In the voltage protocol illustrated, the neuron was held at −70 mV. After a conditioning hyperpolarization to −120 mV, a prepulse to −40 mV eliminated I_A, and the currents remaining were recorded during 350-msec-long steps to voltages between −50 and +40 mV (in 10 mV increments). The pattern of currents shown is typical for cultured hippocampal pyramidal neurons.

Application of the mGluR agonist 1*S*,3*R*-ACPD (100 μM) elicited an initial increase in outward current amplitude (Fig. 1*A2*) that was especially evident for peak current measured at voltages positive to approximately +20 mV. This amplitude increase was not considered in detail. After ~1 min of exposure to the agonist, outward current began to decrease in amplitude and to inactivate more rapidly. On removal of 1*S*,3*R*-ACPD the amplitude increase reversed, whereas the increase in inactivation rate persisted (Fig. 1*A3*). This change in inactivation rate was the major focus of this study.

We defined fractional inactivation, an index proportional to the extent of inactivation during a voltage step, as $[1 - (I_{end}/I_{peak})]$, where I_{peak} was the maximum current amplitude during the test depolarization and I_{end} was the current amplitude at the end of the test pulse just before repolarization. Thus a fractional inactivation of 0 indicates no relaxation during the test depolarization, and a fractional inactivation of 1 indicates complete inactivation. As illustrated in Figure 1*B1*, exposure to 1*S*,3*R*-ACPD increased fractional inactivation at voltages positive to approximately −10 mV, and the magnitude of the increase (shown by the *difference* plot) had a broad bell-like shape. The relative increase in fractional inactivation, as defined by the ratio of fractional inactivations before and after exposure to 1*S*,3*R*-ACPD, was ~35% at all but the most positive voltage examined (Fig. 1*B2*).

A more detailed analysis of changes in delayed outward currents is presented in Figure 2. Currents recorded under control conditions are shown in Figure 2*A*. In most of our analyses, measurements were made at a test voltage of +40 mV, after a conditioning hyperpolarization to −120 mV and a prepulse to −40 mV. The records of Figure 2*A* demonstrate stable recordings maintained during 16 test depolarizations applied at two per minute under control conditions for the 8-min-long standard test interval.

The traces in Figure 2*B1* illustrate the changes induced by exposure to 100 μM 1*S*,3*R*-ACPD. In the standard protocol, agonist was applied for ~3 min, added between the 3rd and 4th depolarizations and removed between the 9th and 10th. The typical pattern, which consisted of an initial increase in current amplitude followed by an acceleration of its inactivation, is evident in the traces and in the plots of peak current amplitude and current amplitude at the time of repolarization in Figure 2*B2*. In the selected traces shown in Figure 2*B3*, the current to the first test depolarization (*before*) is compared with the first current recorded in the presence of agonist (*first ACPD*) and the final current recorded 3.5 min after removal of ACPD (*after ACPD*). Mean ± SD current amplitudes at the peak and at the time of

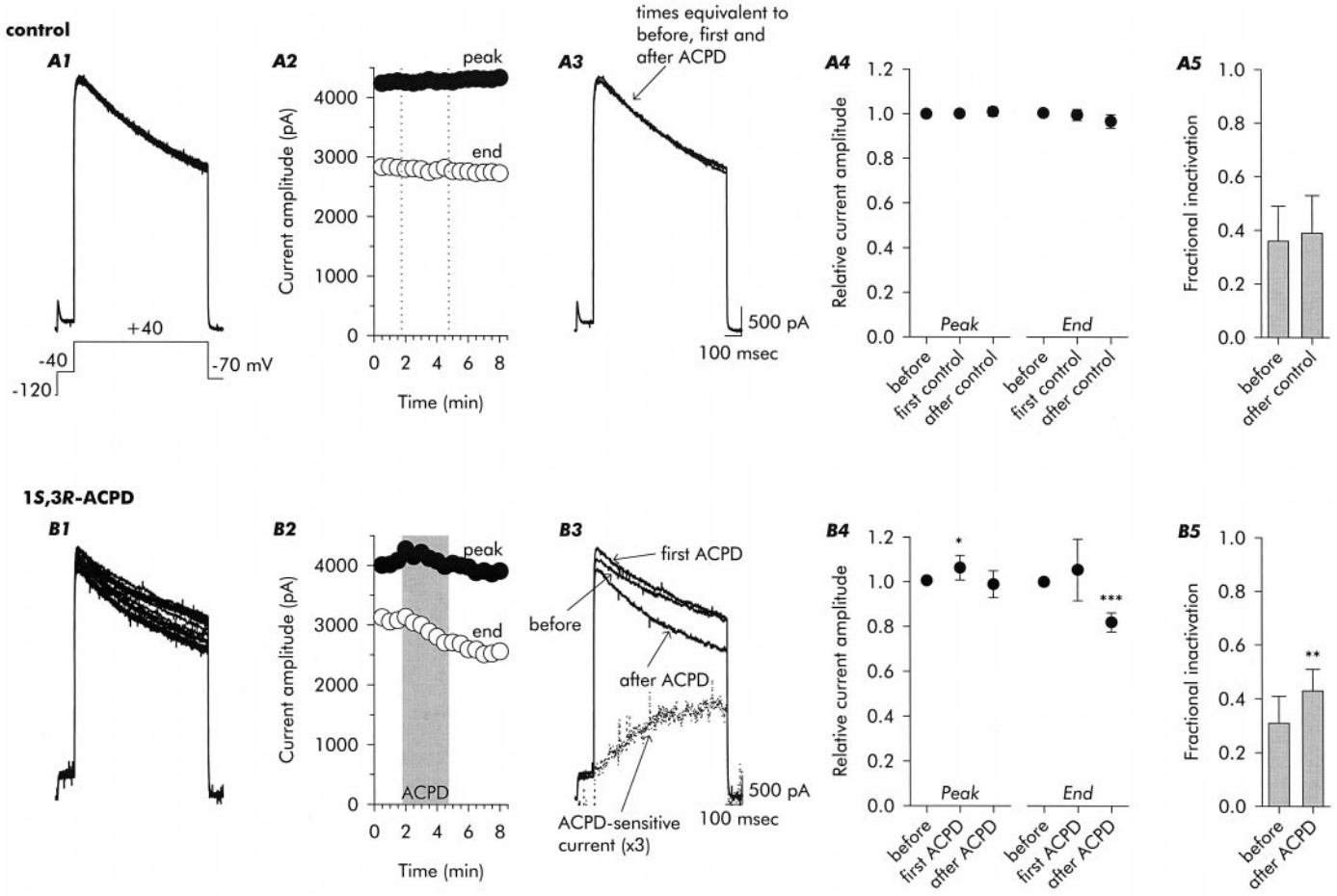

Figure 2. Time course of increase in delayed outward current amplitude followed by acceleration of inactivation, during and after 3-min-long exposures to 1*S*,3*R*-ACPD (100 μM). Control records in *A*, taken during test depolarizations to +40 mV, demonstrate the stability of currents when the standard internal solution was used (see Materials and Methods). Sixteen traces taken over an 8-min-long interval are shown in *A1*, and their peak and end amplitudes (end amplitude is the amplitude just before repolarization) are plotted in *A2*. The *vertical dotted lines* in *A2* refer to the times at which mGluR agonist would be applied to and removed from experimental neurons, and the selected traces in *A3*, and the aggregate data presented in *A4* and *A5*, are all from the times at which data were taken from experimental cells. Experimental records in *B* illustrate the increase in peak current amplitude seen immediately after application of 1*S*,3*R*-ACPD (compare *before* and *first ACPD* in *B3*) and the amplitude reduction and acceleration of inactivation that became evident after a few minutes and was maintained after removal of agonist (*after ACPD* in *B3*). Also shown in *B3* is the waveform of the ACPD-sensitive current (computed by point-by-point subtraction as the difference between *before* and *after ACPD* traces and multiplied by 3 for clarity), illustrating its progressive increase throughout the duration of the 800-msec-long test pulse. *B4*, Under normal recording conditions 1*S*,3*R*-ACPD increased peak current amplitude to ~106% of control and reduced current amplitude at the time of repolarization to ~83% of control. The decrease in current amplitude at the time of repolarization was reflected in an increase in fractional inactivation after exposure to 1*S*,3*R*-ACPD (*B5*). Data are mean ± SD; $n = 9$ for control, $n = 6$ for 1*S*,3*R*-ACPD.

repolarization are shown in Figure 2*B4*. In the aggregate, peak current amplitude was increased by ~6% during the period that agonist was present in the external solution, and after exposure to 1*S*,3*R*-ACPD, current amplitude at the end of the test depolarization was decreased by ~17%. We did not observe reversal of enhanced inactivation after removal of 1*S*,3*R*-ACPD during observations that lasted for up to ~30 min.

The change in inactivation rate was manifest as an increase in fractional inactivation (Fig. 2*B5*). The inactivation time constant decreased from 850 msec before to 508 msec after exposure to ACPD in the example shown in Figure 2*B3*, which was typical.

Separation and identification of 1S,3R-ACPD-sensitive potassium currents

We did not study the conductances underlying the increase in current amplitude in detail; possible candidates include nonselective cation conductances (Crépel et al., 1994; Guérineau et al., 1995; Congar et al., 1997) and Ca^{2+}-dependent potassium conductances (Shirasaki et al., 1994).

Our data indicate that mGluR activation accelerated inactivation of a 4-AP-sensitive current that is commonly noted as I_D (see introductory remarks). Of the various components of delayed outward current in hippocampal neurons, I_D is differentially sensitive to micromolar concentrations of 4-AP. As illustrated in Figure 3*A1*, subtraction of trace *(2) in 4-AP* from trace *(1) before 4-AP* yielded I_D before exposure to 1*S*,3*R*-ACPD (Fig. 3*A2*). Then (Fig. 3*B1*), after removal of 4-AP [trace *(3) wash*], application and removal of 1*S*,3*R*-ACPD resulted in accelerated inactivation of outward current [trace *(4) after ACPD*]. Subsequent addition of 4-AP [trace *(5) after ACPD in 4-AP*] and subtraction yielded I_D after exposure to ACPD (Fig. 3*B2*). At this time I_D inactivation was substantially more rapid; in the aggregate, 1*S*,3*R*-

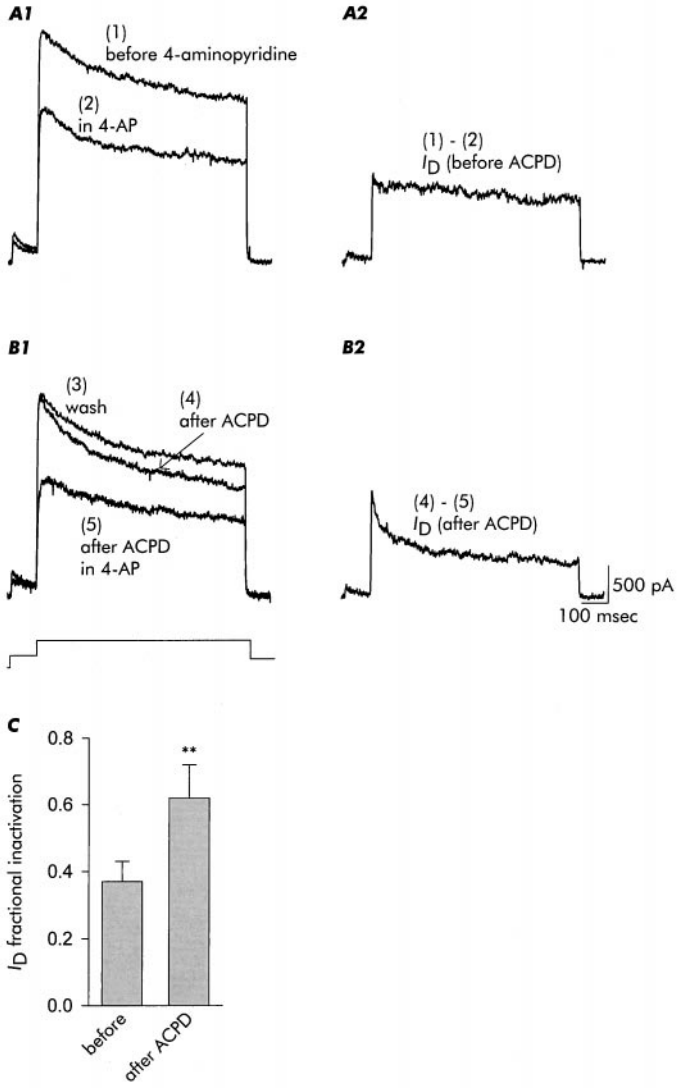

Figure 3. Inactivation of I_D, as isolated by its sensitivity to 4-AP (100 μM), is accelerated after exposure to 1*S*,3*R*-ACPD. The five traces shown in this figure were acquired in the order indicated from the same neuron. *A1*, Currents recorded before (*trace 1*) and in the presence of 4-AP (*trace 2*). *A2*, I_D isolated by subtraction as the difference between the two traces in *A1*. *B1*, After removal of 4-AP and recovery of outward current (*trace 3*), the neuron was exposed to 1*S*,3*R*-ACPD for 3 min as in the previous figure. After removal of 1*S*,3*R*-ACPD, inactivation of outward current was enhanced (*trace 4*). Outward current was then recorded in the presence of 4-AP (*trace 5*). *B2*, I_D, again isolated as the 4-AP-sensitive current [*trace (4) - (5)*], inactivates more rapidly after exposure to 1*S*,3*R*-ACPD. *C*, Increase in fractional inactivation of I_D, isolated by subtraction in each case, after exposure to 1*S*,3*R*-ACPD. Data are mean ± SD; $n = 4$.

ACPD increased I_D fractional inactivation by ~68%, from ~0.37 to ~0.62 (Fig. 3*C*).

The increase in inactivation induced by mGluR activation was also analyzed using an occlusion paradigm in which we determined the ability of various agents to block effects of subsequent application of 1*S*,3*R*-ACPD. Most significantly, in the presence of 4-AP, the effects of 1*S*,3*R*-ACPD on inactivation observed after agonist removal were completely occluded (Fig. 4*A*), indicating that although I_D was not the only current showing inactivation, acceleration of inactivation was restricted to 4-AP-sensitive current. At the same time, the increase in outward current seen in

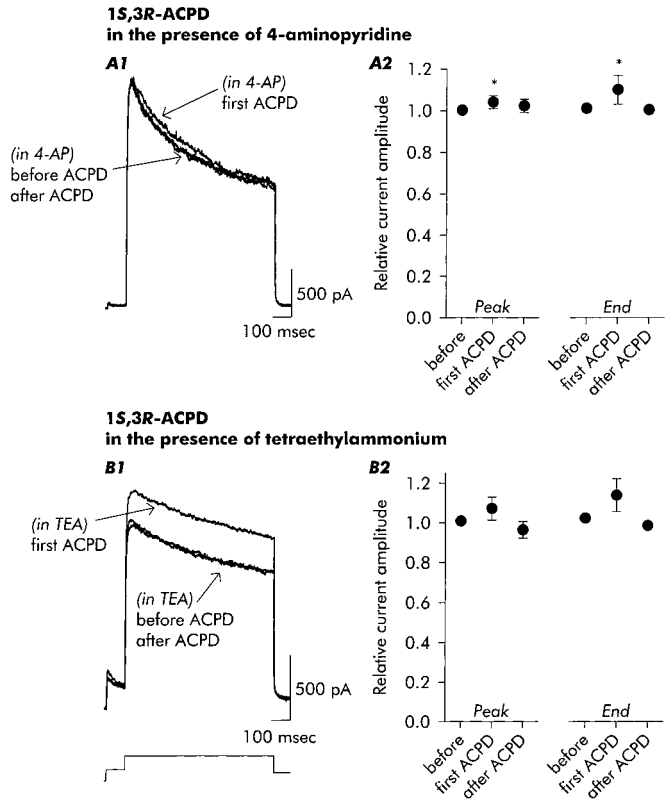

Figure 4. After block of I_D, 1*S*,3*R*-ACPD-induced changes in outward current inactivation were occluded. *A1, A2*, In the presence of 4-AP (200 μM), an increase in outward current was observed in the presence of 1*S*,3*R*-ACPD, but no acceleration of delayed current inactivation or reduction of current amplitude at the time of repolarization was observed. *B1, B2*, TEA (1.5 mM) also occluded 1*S*,3*R*-ACPD-induced changes in delayed current inactivation but spared the initial current increase. Data are mean ± SD; $n = 5$ for 4-AP, $n = 3$ for TEA.

the presence of agonist was not affected (compare Fig. 4*A2* with Fig. 2*B4*).

Exposure to TEA (1.5 mM) also occluded the changes in inactivation rate elicited by 1*S*,3*R*-ACPD (Fig. 4*B*). This observation is consistent with an action on I_D and other slowly inactivating potassium currents, which in some studies are reported to be sensitive to TEA (Ficker and Heinemann, 1992; Li and McArdle, 1997), although TEA does not preferentially block I_D.

Recovery of I_D from inactivation

Recovery of I_D from inactivation was not affected by exposure to 1*S*,3*R*-ACPD. The records in Figure 5*A* show currents recorded using a two-pulse protocol, with a long depolarization to induce maximal inactivation followed by a shorter test pulse delivered at varying intervals (see legend). Despite the clear acceleration of inactivation induced by application of 1*S*,3*R*-ACPD (the *dotted trace* in the experimental records of Fig. 5*A2* is the first control record, presented for reference), when recovery was assessed by normalizing test current amplitude to the initial peak of the initial current, its time course was not affected. As illustrated in Figure 5*B*, the time constant of recovery was ~82–83 msec in each case.

Involvement of GTP binding proteins

Metabotropic GluRs are coupled by GTP binding proteins to various second messengers, including phospholipases C and D, adenyl cyclase, and arachidonic acid. Experiments with GTP

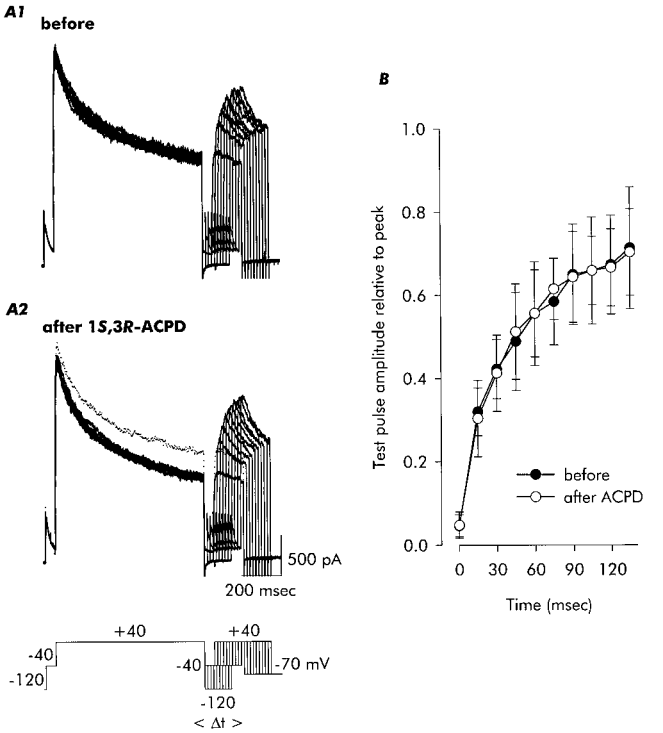

Figure 5. Recovery from inactivation, in contrast to onset of inactivation, was not affected by 1*S*,3*R*-ACPD. A two-pulse protocol was used in which an initial 750-msec-long conditioning depolarization to +40 mV (to induce inactivation) was followed, at intervals incremented by 15 msec during which the cell was held at −120 mV, by a 150-sec-long test depolarization (see pulse schematic). Although inactivation was clearly accelerated by exposure to 1*S*,3*R*-ACPD (the *dotted trace* in *A2* is a control trace from *A1* for reference), the time course of recovery from inactivation was not altered (*B*). Data are mean ± SD; $n = 4$ for control, $n = 4$ for 1*S*,3*R*-ACPD.

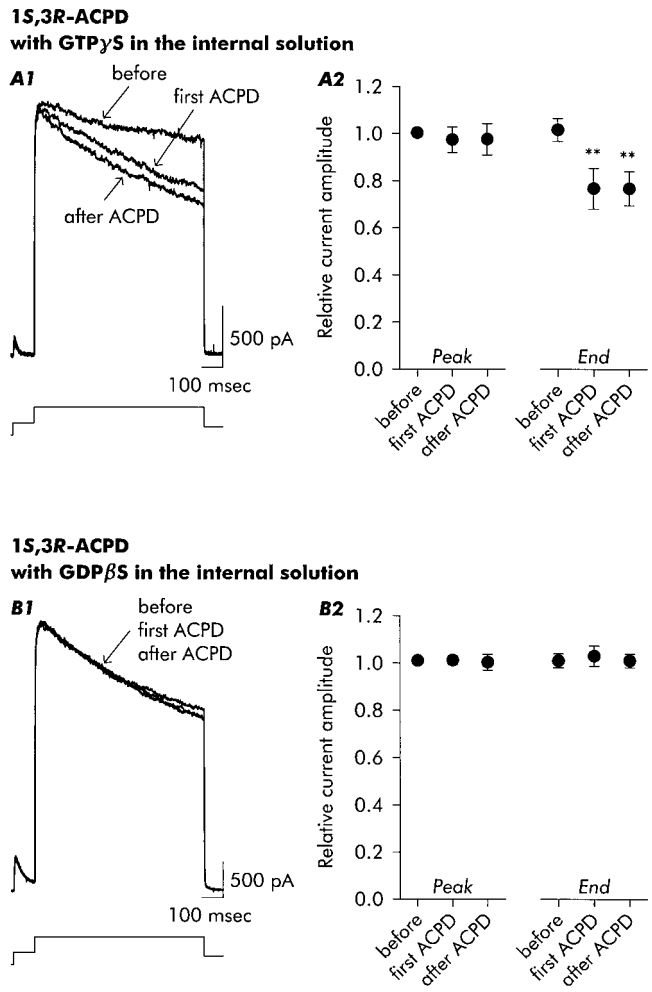

Figure 6. Evaluation of G-protein analogs on the actions of 1*S*,3*R*-ACPD. *A1, A2*, Inclusion of the nonhydrolyzable analog GTPγS (250 μM) in the internal solution resulted in almost maximal acceleration of delayed current inactivation immediately on application of 1*S*,3*R*-ACPD. *B1, B2*, In contrast, inclusion of the nondisplaceable analog GDPβS (500 μM) in the internal solution blocked any actions of 1*S*,3*R*-ACPD. Data are mean ± SD; $n = 5$ for GTPγS, $n = 5$ for GDPβS.

analogs indicated that acceleration of I_D inactivation was a G-protein-mediated process. Inclusion of GTPγS, a nonhydrolyzable GTP analog (Gilman, 1984), in the internal solution resulted in maximal acceleration of I_D inactivation (Fig. 6*A*). Current amplitude at the time of repolarization was reduced by ~23%, and the maximal effect was seen on the first test depolarization after application of 1*S*,3*R*-ACPD. Curiously, the increase in outward current in the presence of agonist was not evident. This could reflect masking by the large changes in I_D, or maximal desensitization of the signaling pathway leading to outward current activation (Guérineau et al., 1997).

Conversely, inclusion of the nondisplaceable GDP analog GDPβS (Eckstein et al., 1979) blocked both the increase in outward current in the presence of agonist and the acceleration of I_D inactivation (Fig. 6*B*).

Effects of mGluR subtype-preferring antagonists and agonists

We performed a series of experiments comparing the actions of 1*S*,3*R*-ACPD with those of agonists preferring various mGluR subtypes. Metabotropic GluRs may be divided into groups I, II, or III on the basis of amino acid sequence and pharmacology, and additional mGluRs not conforming to this scheme have been proposed (Pin and Duvoisin, 1995; Conn and Pin, 1997; Albani-Torregrossa et al., 1998). 1*S*,3*R*-ACPD is an agonist preferring group I, group II, and a few group III mGluRs, as well as mGluRs coupled to phospholipase D (Pellegrini-Giampietro et al., 1996; Conn and Pin, 1997).

S-α-methyl-4-carboxyphenylglycine (*S*-MCPG) is a blocker preferring group I and group II mGluRs, is ineffective on most group III mGluRs or on ionotropic glutamate receptors, and is an agonist for phospholipase D-coupled mGluRs (Watkins and Collingridge, 1994; Pellegrini-Giampietro et al., 1996). As illustrated in Figure 7, application of *S*-MCPG (1 mM) along with 1*S*,3*R*-ACPD virtually completely blocked both the increase in outward current in the presence of agonist and the subsequent acceleration of I_D inactivation.

Exposure to *RS*-3,5-dihydroxyphenylglycine (3,5-DHPG; 100 μM), an agonist for group I mGluRs (Schoepp et al., 1994) and an antagonist for phospholipase D-coupled mGluRs (Pellegrini-Giampietro et al., 1996), failed to elicit the increase in outward current but did cause a significant decrease in steady-state current amplitude and increase in I_D inactivation (Fig. 8*A*).

Application of the group II-preferring agonist (DCG-IV; 1–100 μM (Hayashi et al., 1993) (Fig. 8*B*) resulted in a small transient increase in outward current, followed by an enhancement of

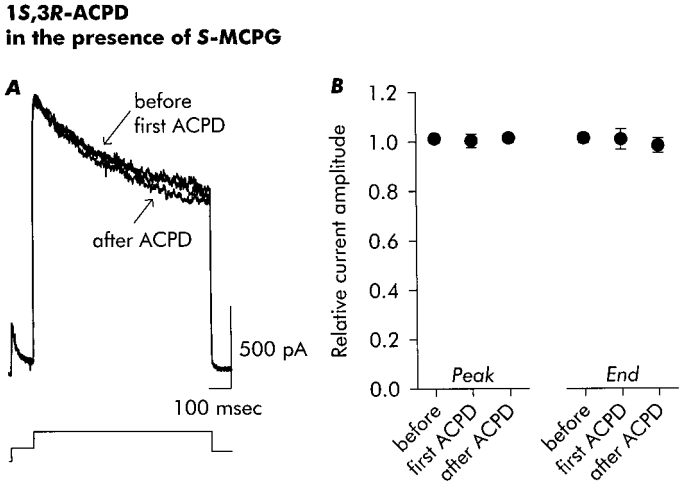

Figure 7. Effects of 1*S*,3*R*-ACPD were not observed when agonist was applied in the presence of the broadly acting mGluR antagonist *S*-MCPG (1 mM). The small reduction in steady-state current (*after ACPD*; *A*), although seen consistently (*B*), was not statistically significant. Data are mean ± SD; $n = 4$.

inactivation comparable to that seen with the group I agonist 3,5-DHPG (both showed a ~14% reduction in steady state current amplitude).

Exposure to L(+)-2-amino-4-phosphonobutyric acid (L-AP4; 100 μM), an agonist preferring group III mGluRs and not active on phospholipase D-coupled mGluRs (Pellegrini-Giampietro et al., 1996), was without noticeable effect on delayed outward current (Fig. 8*C*).

1S,3R-ACPD-sensitive conductances active near the resting potential

In addition to modulation of currents activated at positive potentials, mGluR activation also reduces conductances active near the resting potential (Charpak et al., 1990; Guérineau et al., 1994). We assessed possible overlap between these conductances and I_D by examining the 4-AP sensitivity of currents suppressed by 1*S*,3*R*-ACPD during steps to voltages between −120 and −40 mV from the holding potential of −70 mV.

We determined slope conductance near the resting potential from the amplitudes of nonleak-subtracted steady-state currents measured between −60 and −40 mV. As shown in Figure 9*A*, exposure to 1*S*,3*R*-ACPD reduced the slope conductance to ~47% of control, and in most cells this decrease was reversible. 1*S*,3*R*-ACPD did not affect the slope conductance at voltages negative to −60 mV (data not shown).

This reduction in slope conductance was occluded by 4-AP. As illustrated in Figure 9*B*, 4-AP caused a clear reduction in slope conductance near the resting potential [see also Brown et al. (1990); Storm (1990)]. When 1*S*,3*R*-ACPD was applied in the presence of 4-AP, there was no further change in slope conductance, indicating that mGluR activation was affecting a 4-AP-sensitive conductance. Identification of this current as I_D is consistent with its properties in cultured pyramidal neurons, because the foot of the I_D activation curve is near −60 mV and a window of current showing partial inactivation is seen between approximately −60 and −20 mV (Ficker and Heinemann, 1992; Wu and Barish, 1992). However, these observations may also reflect modulation of a different 4-AP-sensitive current.

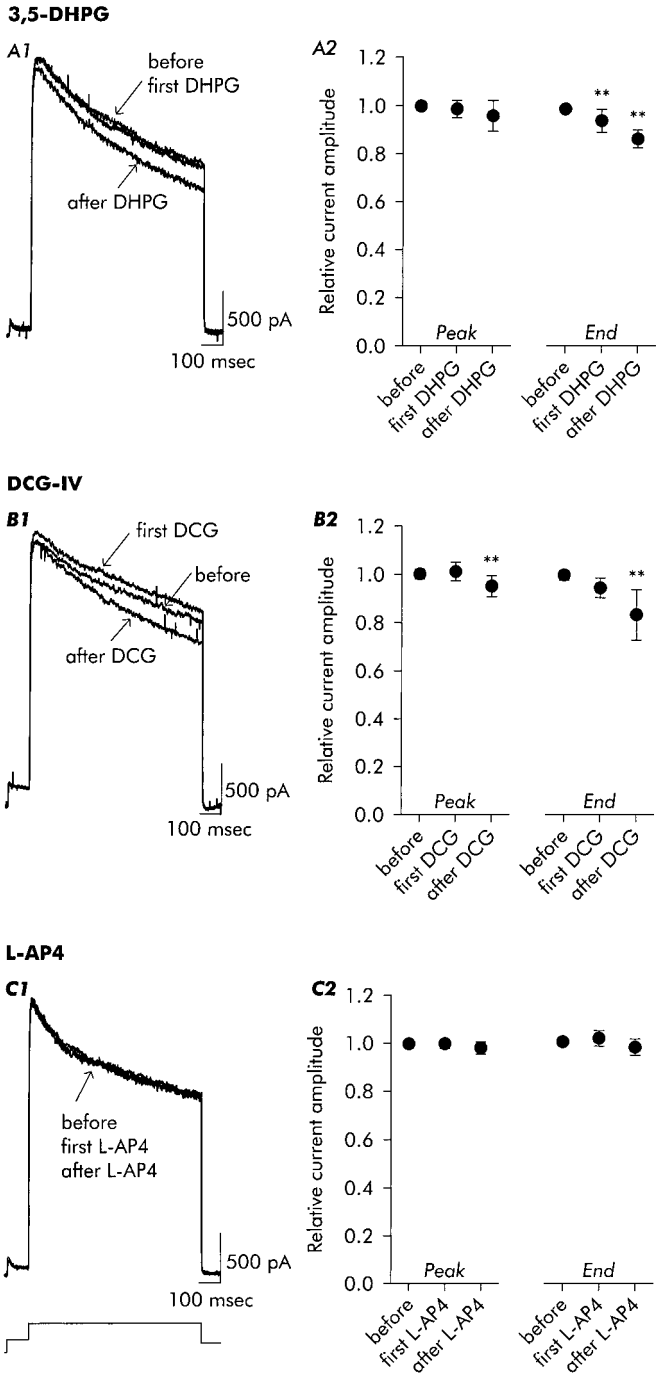

Figure 8. Evaluation of agonists acting preferentially on group I (3,5-DHPG), group II (DCG-IV), or group III (L-AP4) mGluRs. *A1, A2*, Application of 3,5-DHPG (100 μM) resulted in acceleration of inactivation in the presence of agonist and after its removal. *B1, B2*, Application of DCG-IV (100 μM) elicited a small increase in delayed current amplitude as well as acceleration of inactivation. In contrast, application of L-AP4 (100 μM) (*C1, C2*) failed to elicit change either in peak current amplitude or in inactivation rate and current amplitude at the time of repolarization. Data are mean ± SD; $n = 9$ for 3,5-DHPG, $n = 11$ for DCG-IV, $n = 5$ for L-AP4.

Effects on electrogenesis

We specifically examined three aspects of excitability linked by other investigations to I_D by the actions of micromolar concentrations of 4-AP: excitability near the resting potential, latency to

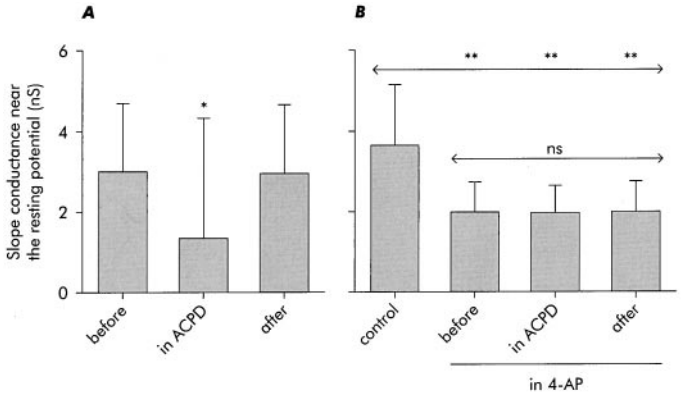

Figure 9. Reduction of whole-cell conductance near the resting potential by 1*S*,3*R*-ACPD, and occlusion by 4-AP. *A*, Slope conductance, measured between −60 and −40 mV, before, during, and (3 min) after exposure to 1*S*,3*R*-ACPD. *B*, Occlusion of 1*S*,3*R*-ACPD-induced suppression of resting conductance by addition of 4-AP to the external solution. 4-AP reduced whole-cell conductance at voltages positive to approximately −60 mV, and no further change was seen on application of 1*S*,3*R*-ACPD. Note that neither 4-AP nor 1*S*,3*R*-ACPD induced changes in conductance at voltages negative to −60 mV. Data are mean ± SD; $n = 8$ for 1*S*,3*R*-ACPD, $n = 3$ for 1*S*,3*R*-ACPD in 4-AP.

action potential generation during sustained depolarizations, and waveform of action potential repolarization. In these current-clamp experiments, changes in excitability reflected the pleiotropic actions of mGluR activation, and the effects of 1*S*,3*R*-ACPD on excitability followed the biphasic pattern predicted from the voltage-clamp data (initial increase of outward current followed by enhancement of I_D inactivation).

Excitability

The changes in excitability elicited by 1*S*,3*R*-ACPD are illustrated in Figure 10. The initial resting potential, −62 mV, is indicated by the dotted line. At each time relative to application of 1*S*,3*R*-ACPD, a series of short current injections of increasing intensity were delivered through the patch pipette. 1*S*,3*R*-ACPD caused an immediate hyperpolarization and reduction in excitability (1.5 min in ACPD), which was followed by a return to the original resting potential and an enduring reduction in excitability (2 min in ACPD). Subsequently, after removal of 1*S*,3*R*-ACPD, persistent enhancement of excitability (5.5 min after ACPD) was observed.

Response to sustained depolarization

A reduction in first action potential latency induced by 1*S*,3*R*-ACPD is illustrated in Figure 11. In the control case, injection of positive-going current resulted in a rapid depolarization followed by a slower positive-going shift in membrane voltage that eventually reached threshold. During application of 1*S*,3*R*-ACPD, the resting potential shifted slightly positive (the original −74 mV resting potential is indicated), and the current injection evoked a single action potential followed by another after a long delay. Most significantly, after exposure to 1*S*,3*R*-ACPD was terminated, the cell returned to the original resting potential, the slow rise toward threshold was eliminated, and repetitive action potentials were generated immediately as the rapidly depolarizing membrane voltage reached threshold [also see Bashir et al. (1993)]. Note that at all three time points only minimal alterations were observed in the response to hyperpolarization (the dotted line at −80 mV marks the control response). Effects similar to

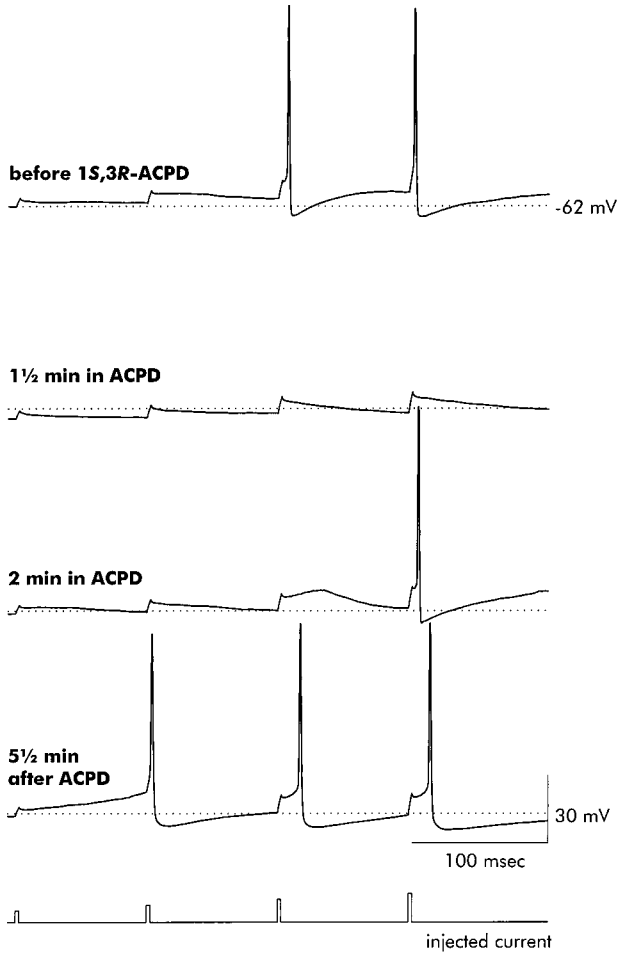

Figure 10. Exposure to 1*S*,3*R*-ACPD ultimately increases pyramidal neuron excitability. A series of short (duration 2.5 msec) depolarizing current injections of increasing amplitude (2, 4, 6, and 8 nA) were delivered, separated by 100 msec. The initial resting potential, −62 mV, is indicated by the *dotted line*. Before application of 1*S*,3*R*-ACPD, only the two largest current injections were sufficient to elicit action potentials. Exposure to 1*S*,3*R*-ACPD resulted in a transient hyperpolarization (1.5 min in ACPD), but excitability was reduced even after the return of the resting potential to near its initial value (2 min in ACPD). Note also the enhanced repolarization seen in the action potential recorded after 2 min in ACPD. In the *bottom trace*, recorded 5.5 min after washing off 1*S*,3*R*-ACPD, excitability was enhanced, as judged by the ability of the second current injection to elicit an action potential. The durations of these action potentials were also affected, as illustrated in Figure 12. Records are representative of six neurons that were similarly examined.

those of 1*S*,3*R*-ACPD were also seen with 3,5-DHPG (100 μM; $n = 3$) and DCG-IV (100 μM; $n = 3$).

Action potential waveform

Individual action potentials showed the pattern of change illustrated in Figure 12: initial acceleration of repolarization followed by a sustained increase in duration. Shown in Figure 12*C* are superimposed action potentials by elicited current injections before, during, and after exposure to 1*S*,3*R*-ACPD. These action potentials illustrate that in the presence of ACPD the resting potential shifted negatively (a more typical finding than the positive shift shown in the previous figure) and that action potential repolarization was accelerated. Shortly after removal of 1*S*,3*R*-ACPD, the resting potential returned to control levels, and yet action potential repolarization was prolonged [also see Hu

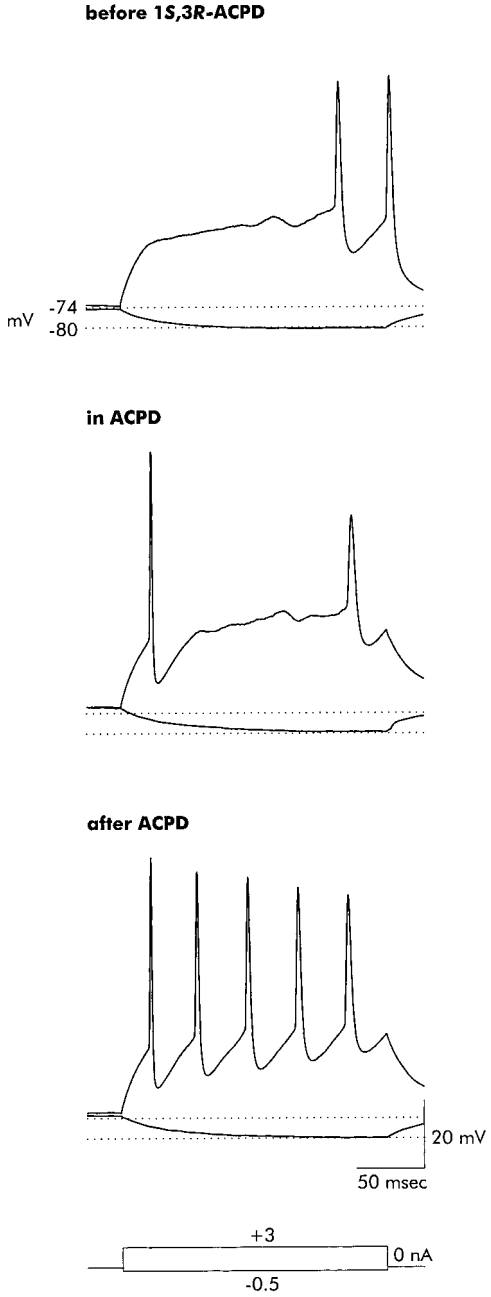

Figure 11. Exposure to 1*S*,3*R*-ACPD ultimately reduces the delay to onset of repetitive firing in response to sustained depolarizing current injection. Before application of 1*S*,3*R*-ACPD, a depolarizing current injection rapidly shifted voltage from the resting potential (−74 mV, indicated by the *dotted line*) to a slowly rising plateau from which an action potential was eventually generated. In the presence of 1*S*,3*R*-ACPD, a small positive shift in the resting potential resulted in initial generation of an action potential during the rapid depolarizing phase, but there was a long delay until the next action potential. After removal of ACPD (2 min wash) the delay to firing was completely eliminated, and the neuron fired steadily during the depolarizing current injection. Note that the response to the hyperpolarizing current injection (−80 mV, indicated by the *second dotted line*) was not altered during or after exposure to 1*S*,3*R*-ACPD. Records are representative of seven neurons that were similarly examined.

and Storm (1991)]. The progressive increase in action potential duration after removal of 1*S*,3*R*-ACPD, despite stabilization of the resting potential near its initial level, is shown graphically in Figure 12*D*. After ~20 min, action potential duration (measured at half amplitude) increased by ~27%, from ~3.75 to ~4.75 msec. In the aggregate, 1*S*,3*R*-ACPD increased action potential duration at half amplitude by 31.0 ± 8.4%, from 2.3 ± 1.0 msec to 3.1 ± 1.4 msec (mean ± SD, $p = < 0.05$; $n = 4$). No changes in these parameters were observed in control recordings (Fig. 12*A,B*).

Applications of 4-AP elicited the responses expected for block of I_D (increased excitability, reduction of latency to first action potential, delayed action potential repolarization). However, parameters of interest changed continuously and progressively over the duration of our measurements and did not reach a stable steady state from which to test occlusion of the actions of 1*S*,3*R*-ACPD.

DISCUSSION

In the experiments presented here, we have shown that activation of mGluR in cultured hippocampal pyramidal neurons by 1*S*,3*R*-ACPD results in a suppression of I_D that persists after agonist removal. I_D was isolated on the basis of its sensitivity to 100–200 μM 4-AP and in isolation showed accelerated inactivation after exposure to 1*S*,3*R*-ACPD. Furthermore, previous block of I_D by 4-AP precluded subsequent effects of mGluR activation, indicating that additional 4-AP-insensitive conductances were not involved.

Application of 4-AP also occluded a reduction in resting conductance induced by 1*S*,3*R*-ACPD. This component of whole-cell resting conductance may be a manifestation of tonic I_D activation in a voltage region in which its activation and inactivation curves overlap (a "window current"), or an indication of another 4-AP-sensitive current, or both.

We further demonstrated that effects on excitability attributed to I_D by their sensitivity to 4-AP—increased excitability to short depolarizations, reduced latency to first action potential during sustained depolarizations, and increased action potential duration—are all also produced by exposure to 1*S*,3*R*-ACPD.

I_D is a novel target for mGluR-initiated signaling

Although there are a number of other reports of potassium current modulation in hippocampal neurons by mGluR activation (Gerber and Gähwiler, 1994), in most cases the currents affected, I_{AHP}, I_M, and $I_{K(slow)}$, do not appear to overlap with I_D as described here. An inhibition of I_{AHP} and I_M after mGluR activation by quisqualate was described by Charpak et al. (1990), but neither I_{AHP} nor I_M is blocked by 4-AP or TEA (Brown and Adams, 1980; Lancaster and Adams, 1986), both of which affect I_D, and the kinetics of I_M is quite different from that of I_D (Brown and Adams, 1980). Somewhat closer is the reduction in $I_{K(slow)}$ by 1*S*,3*R*-ACPD described by Lüthi et al. (1996), but this study separated slow outward currents in rat hippocampal neurons into a 4-AP-sensitive but 1*S,3R*-ACPD-resistant I_D (with inactivation kinetics similar to the I_D considered here), and a much more slowly inactivating 4-AP-resistant $I_{K(slow)}$ that was sensitive to 1*S*,3*R*-ACPD. These observations are reminiscent of those presented here, and the source of the differences is not obvious. Lüthi et al. (1996) studied rat hippocampal neurons in organotypic slice cultures, but differences between rat and mouse are not likely to be relevant because the properties of I_D of pyramidal neurons from the two species grown in dissociated cell cultures

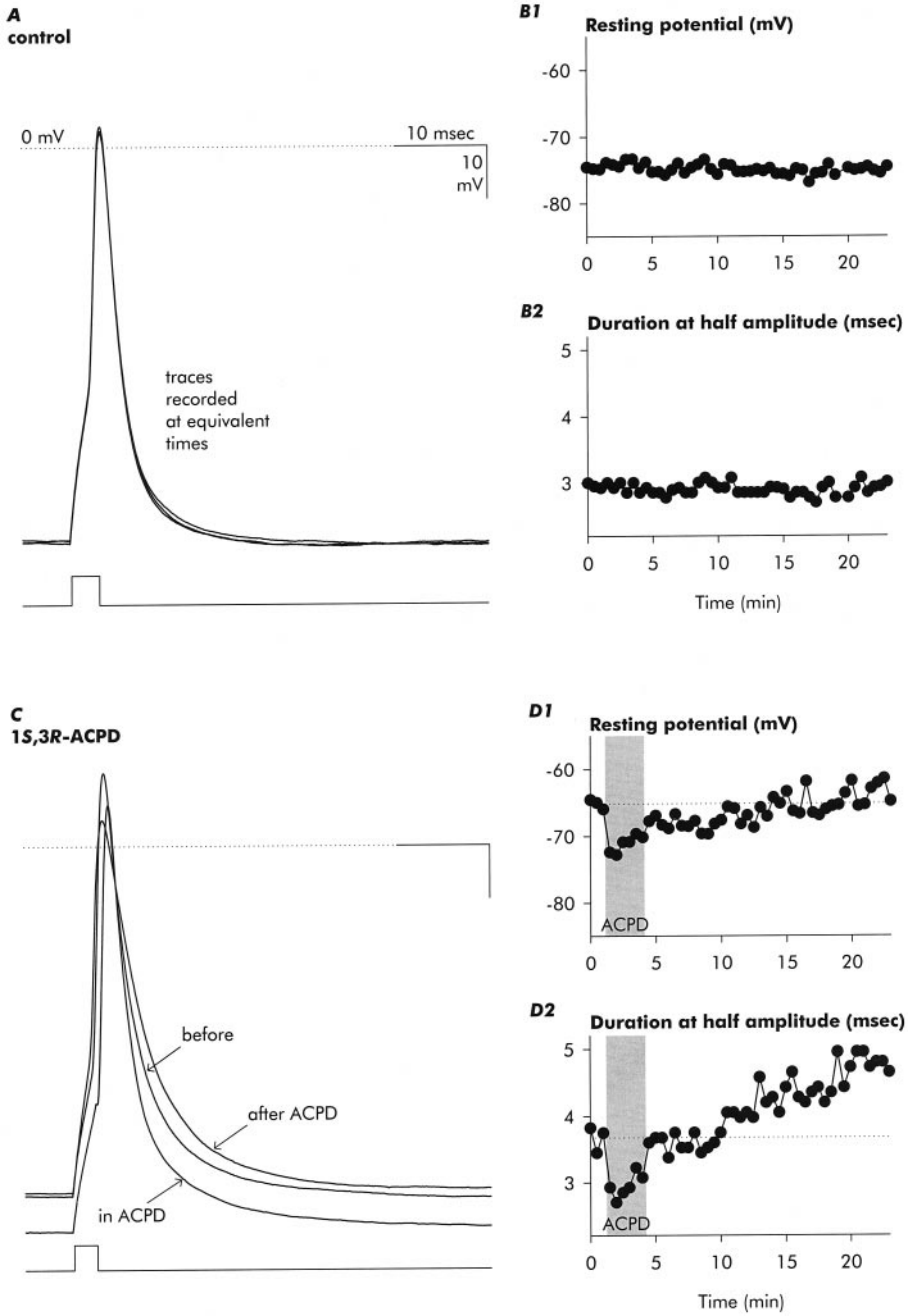

Figure 12. Exposure to 1*S*,3*R*-ACPD ultimately increases action potential duration. *A*, Action potentials recorded in response to short (duration 2.5 msec) depolarizing current injections at times equivalent to those presented for the experimental neuron in *C*. In control neurons action potential waveforms remained stable, and neither resting potential (*B1*) nor action potential duration (*B2*) showed spontaneous changes. *C*, Action potentials recorded before, during, and after exposure to 1*S*,3*R*-ACPD, illustrating initial enhancement of repolarization, followed by increase in action potential duration (the *trace* shown was recorded 18 min after removal of agonist). A transient negative shift in the resting potential was followed by eventual recovery (*D1*), whereas action potential duration (measured at half amplitude) showed an initial decrease followed by progressive and sustained increase after wash off of 1*S*,3*R*-ACPD (*D2*). Records are representative of three control and four 1*S*,3*R*-ACPD-exposed neurons.

are similar [compare Ficker and Heinemann (1992) for rat with Wu and Barish (1992) for mouse].

A possibly related observation from a study on neostriatal neurons was reported by Nisenbaum et al. (1996), who observed enhanced inactivation of outward current when GTP and KF were present in the internal solution. This effect was not seen when GTPβS was substituted for GTP, and these authors concluded that inactivation was accelerated as a consequence of G-protein stimulation. One component of outward current in neostriatal neurons is I_{As}, a slowly inactivating potassium current very similar to I_D (Surmeier et al., 1991, 1994; Gabel and Nisenbaum, 1998). This current could be subject to G-protein-mediated regulation.

1*S*,3*R*-ACPD-induced reduction in conductance at voltages near or just positive to the resting potential, as described by Guérineau et al. (1994), who termed the current affected $I_{K,leak}$, and by Lüthi et al. (1997), does appear similar to the inhibition of resting conductance seen here. Shared properties include induction by 1*S*,3*R*-ACPD, block by *S*-MCPG, enhancement by GTPγS, and block by GDPβS. Neither Guérineau et al. (1994) nor Lüthi et al. (1997) examined the 4-AP sensitivity of their currents.

Mechanisms linking specific mGluR to particular potassium channel subunits

There is no clear answer to the question of which mGluR subtypes, and by implication second messenger systems, may be linked to modulation of I_D. The group I-preferring agonist 3,5-DHPG (Schoepp et al., 1994) and the group II-preferring agonist DCG-IV (Hayashi et al., 1993) both enhanced I_D inactivation.

This pharmacological profile was unexpected because of the divergent signaling characteristics of group I and group II mGluRs, but interactions between phosphoinositide- and cAMP-linked intracellular signaling pathways have been described (Gereau and Conn, 1994; Gereau et al., 1995; Nouranifar et al., 1998), and delayed potassium currents may be regulated by cAMP-sensitive phosphorylation reactions (Mu et al., 1997). An additional possibility is mGluR activation of phospholipase D in hippocampal neurons (Boss and Conn, 1992; Holler et al., 1993), which may be stimulated by both 3,5-DHPG and DCG-IV but is mediated by group I mGluRs (Klein et al., 1997) or, alternatively, by a novel mGluR subtype (Pellegrini-Giampietro et al., 1996).

A related issue is the molecular identities of the targets of mGluR modulation. Multiple cloned potassium channel subunits are sensitive to low concentrations of 4-AP (Grissmer et al., 1994), and one or more of these subunits may contribute to the channels carrying I_D in pyramidal neurons and be a target of the second messenger system(s) stimulated by mGluR activation. However, it is not clear at this time which of these subunits are actually involved.

Both of these issues will be investigated further.

Significance of I_D modulation

The distinguishing property of I_D is high sensitivity to 4-AP (see references herein). In his initial description of I_D in CA1 pyramidal neurons, Storm (1988a) used 4-AP to perturb I_D and determined that the current influenced the time to first action potential generation during long depolarizing current injections (see introductory remarks). Subsequent investigations, also using 4-AP, have assigned it an additional role in repolarization of pyramidal neuron action potentials (see introductory remarks). Thus activation of I_D will tend to both retard approach of the action potential to threshold and hasten repolarization of the action potential once it has been generated. Its importance in regulating excitability is demonstrated by the potent convulsant activity of 4-AP when used at concentrations selective for inhibition of I_D (see introductory remarks). Because mGluR activation and 4-AP appear to target the same potassium current, one may predict that the more moderate inhibition of I_D seen with mGluR activation could affect, in a less catastrophic manner, the same processes altered by 4-AP.

What might these changes be? First, reduction in I_D at voltages just positive to the resting potential will amplify any excitatory input, as has been seen after mGluR activation (Desai and Conn, 1991), and this inhibition could contribute to EPSP-spike potentiation (Breakwell et al., 1996). Reduction in I_D near the resting potential could also contribute to the slow conductance decrease EPSP elicited by mGluR activation in hippocampal neurons (Charpak and Gähwiler, 1991; Gerber et al., 1993). Second, because suppression of I_D reduces the delay to action potential firing, excitability during long trains of excitatory input may be increased. Third, because changes in the action potential waveform will affect Ca^{2+} entry (McCobb and Beam, 1991; Scroggs and Fox, 1992; Wheeler et al., 1996; Sabatini and Regehr, 1997), modulation of I_D may influence Ca^{2+}-dependent neurotransmitter release (see introductory remarks). All of these actions of mGluR on I_D could contribute to, among other things, the facilitating actions of mGluR activation on long-term potentiation (LTP) induction (Bashir et al., 1993; Cohen and Abraham, 1996), EPSP-spike potentiation associated with mGluR activation (Breakwell et al., 1996), and mGluR-induced slow-onset LTP (Bortolotto and Collingridge, 1992, 1993; Chinestra et al., 1994; Manahan-Vaughan and Reymann, 1995).

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