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ACCELERATED COMMUNICATION

Metabotropic Glutamate Receptors Potentiate Ionotropic Glutamate Responses in the Rat Dorsal Horn

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SUMMARY

The effects of the metabotropic glutamate receptor agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD] were examined on responses mediated by the ionotropic glutamate receptor agonists *N*-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainic acid (KA), in neurons acutely isolated from the dorsal horn of the rat spinal cord. (1*S*,3*R*)-ACPD produced an increase in the intracellular Ca^{2+} concentration in 50% of acutely isolated dorsal horn neurons, which could be prevented by blockers of voltage-sensitive Ca^{2+} channels. (1*S*,3*R*)-ACPD markedly potentiated increases in the intracellular Ca^{2+} concentration induced by NMDA, AMPA, and KA but not by 10–50 mM KCl. This potentiation occurred in all cells, required the simultaneous presence of both agonists, and was rapidly reversible. In the spinal cord slice preparation, (1*S*,3*R*)-ACPD potentiated the inward currents evoked by pressure application of AMPA, NMDA, and KA, an effect that was also rapidly reversible. These short term effects of (1*S*,3*R*)-ACPD may play an important role in the regulation of ionotropic responses mediated by glutamate in the spinal cord.

Excitatory amino acid neurotransmitters produce their effects by acting on two major classes of receptors. KA/AMPA and NMDA receptors are ligand-gated ion channels. Metabotropic glutamate receptors are members of the family of heptahelical proteins that produce their effects through the participation of guanine nucleotide-binding proteins. The involvement of ionotropic glutamate receptors in fast excitatory transmission at many synapses has been clearly demonstrated. In contrast, the physiological roles of metabotropic glutamate receptors are still rather obscure. At least four types of metabotropic glutamate receptors exist (1), and they have been shown to be linked to several signal transduction systems (2), including phospholipase C (1), adenylate cyclase (1), Ca^{2+} channels (3, 4), and a variety of K^+ channels (5). In the present study, we demonstrate a novel effect of metabotropic receptor activation, a potent potentiation of the effects of agonists at ionotropic glutamate receptors. Such events could represent a key link in activity-dependent alterations in the strength of synaptic transmission observed at excitatory synapses in the central nervous system.

Materials and Methods

Experiments were performed on DH neurons from Rexed's laminae I–III, using Holtzman rats of age 9–16 days.

Acutely isolated neurons. Cells from the DH in the Rexed's laminae I–III were isolated by the method previously described (27) and were attached to poly-L-lysine-coated coverslips. For $[Ca^{2+}]_i$ measurement, cells were loaded with 5 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 10 min and then washed for 30 min. $[Ca^{2+}]_i$ was measured using fura-2-based microfluorimetry, as previously described (6). Cells were continually perfused with a solution consisting of 150 mM NaCl, 0.5 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES, 10 mM glucose, and 0.1 mg/ml bovine serum albumin, adjusted to pH 7.4 with NaOH. Tetrodotoxin (0.5 μM) was present in all solutions. Elevated KCl solutions were made up by iso-osmotic substitution of KCl for NaCl. Cell perfusion and drug application were via a pressure system. This consisted of a Y-shaped tube positioned 0.5–1.0 mm away from the cell, which was then always exposed to a rapid flow of external solution. Exchange speed of the external solution surrounding the cell ranged from 70 to 100 msec. The flow rate was approximately 1.5 ml/min. Background fluorescence was determined from a cell-free region of the coverslip, and the ratio of 340/380-nm fluorescence was calculated off-line. Ratios were converted to $[Ca^{2+}]_i$ (28) from standard

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ABBREVIATIONS: KA, kainate; NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; DH, dorsal horn; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; CNQX, 6 cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dihydroxy-2,3,dinitroquinoxaline; APV, aminophosphovalerate.

calibration curves determined for the fura-2 salt in calibration buffer [20 mM HEPES, 120 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, pH 7.1, containing 10 mM EGTA (K_d = 3.696 × 10^6 M^{-1}), with calculated amounts of Ca^{2+} to give free Ca^{2+} ranging between 0 and 2000 nM].

DH neurons in the spinal cord slice preparation. Transverse slices (200 μm) of lumbar spinal cord taken from 10–12-day-old Holtzman rats were prepared as previously described (27), placed in a holding chamber containing artificial cerebrospinal fluid, which contained 126 mM NaCl, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3 mM KCl, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose, and gassed with 95% O_2/5% CO_2 for 1 hr. A single slice was then transferred to the recording chamber and continuously perfused with artificial cerebrospinal fluid supplemented with 0.5 μM tetrodotoxin, at 23°. Discontinuous, whole-cell, single-electrode, voltage-clamp recordings from visually identified substantia gelatenosa neurons were made with 2–5-MΩ patch electrodes filled with 145 mM potassium gluconate, 2 mM $MgCl_2$, 5 mM HEPES, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 5 mM K_2ATP, as previously described (22). AMPA, NMDA, and KA were delivered by pressure ejection from a pipette with a 10–20-μm tip diameter. All other drugs were delivered via the perfusate. The absence of ejection artifacts was confirmed at the conclusion of each experiment by the addition of the AMPA/KA receptor antagonist DNQX (10 μM) or the NMDA receptor antagonist APV (50 μM).

Results and Discussion

We examined the effects of various glutamate agonists on Ca^{2+} signals in acutely isolated neurons from the DH of the rat spinal cord. Fig. 1a illustrates the effects of adding the metabotropic glutamate receptor agonist (1*S*,3*R*)-ACPD to one of these neurons. It can be seen that there was a dose-dependent increase in basal $[Ca^{2+}]_i$. In 18 such experiments, similar effects were observed in half the neurons studied, with a threshold concentration of approximately 20 μM (1*S*,3*R*)-ACPD. (1*S*,3*R*)-ACPD at 50 μM produced increases in $[Ca^{2+}]_i$ in the range of 55–350 nM. The increase in $[Ca^{2+}]_i$ observed in these experiments appeared to be due to Ca^{2+} influx rather than Ca^{2+} mobilization from intracellular stores, as has been previously observed in certain other neuronal and glial preparations (2). Thus, the drug TA-3090, a nonselective blocker of high threshold Ca^{2+} currents in neurons (6), effectively inhibited the rise in $[Ca^{2+}]_i$ produced by (1*S*,3*R*)-ACPD (Fig. 1b). The same drug was also able to inhibit 50 mM K^+-induced Ca^{2+} influx into these neurons, illustrating its ability to block voltage-sensitive Ca^{2+} channels in these cells (Fig. 1c). Nimodipine (1 μM) also prevented the increase in $[Ca^{2+}]_i$ produced by 50 μM (1*S*,3*R*)-ACPD (inhibition = 88 ± 2%; mean ± standard deviation; n = 3). The (1*S*,3*R*)-ACPD-dependent increase in $[Ca^{2+}]_i$ was not blocked by a combination of the ionotropic glutamate receptor antagonists APV (50 μM) and CNQX (10 μM) (n = 2). It, therefore, appears that (1*S*,3*R*)-ACPD is able to activate Ca^{2+} influx into these neurons through voltage-sensitive Ca^{2+} channels, presumably as a result of cell depolarization. This conclusion is consistent with electrophysiological data provided below and elsewhere (2).

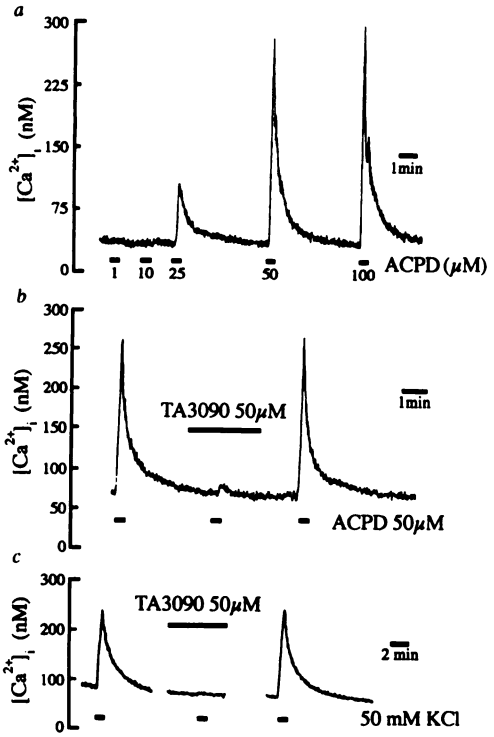

Fig. 1. a, Effects of different concentrations of (1*S*,3*R*)-ACPD on $[Ca^{2+}]_i$ were measured using fura-2-based microfluorimetry in an acutely isolated rat DH neuron. *Horizontal lines*, period of application. b, (1*S*,3*R*)-ACPD-dependent increases in $[Ca^{2+}]_i$ were prevented by the Ca^{2+} channel antagonist TA-3090 (50 μM). c, TA-3090 (50 μM) also prevented the Ca^{2+} influx due to depolarizing concentrations of extracellular K^+ (50 mM KCl).

($1S,3R$)-ACPD also powerfully potentiated the effects of NMDA, AMPA, and KA at ionotropic glutamate receptors, but only when applied simultaneously with these agonists. For example, Fig. 2a shows that repetitive pulses of NMDA produced consistent increases in $[Ca^{2+}]_i$. When (1*S*,3*R*)-ACPD was added between pulses, it had no effect on subsequent applications of NMDA (n = 10). Similar results were obtained using (1*S*,3*R*)-ACPD together with repeated applications of AMPA or KA (n = 10). However, when (1*S*,3*R*)-ACPD was added concommitently with NMDA, AMPA, or KA, a powerful enhancement of the effect of the ionotropic agonist was observed (e.g., Fig. 2b for AMPA). These potentiating effects were observed when concentrations of (1*S*,3*R*)-ACPD below those that produced increases in $[Ca^{2+}]_i$ by themselves were used (Fig. 2c) Furthermore, potentiating effects of higher concentrations of (1*S*,3*R*)-ACPD were manifest both in cells in which it did and in those in which it did not increase $[Ca^{2+}]_i$ by itself. Hence, the potentiating effects of (1*S*,3*R*)-ACPD could be dissociated from its ability to increase $[Ca^{2+}]_i$ alone. Fig. 2d illustrates the ability of (1*S*,3*R*)-ACPD to potentiate responses to NMDA, AMPA, and KA in the same cell. Indeed, the potentiating effects of (1*S*,3*R*)-ACPD on $[Ca^{2+}]_i$ were observed in all cells studied (n = 24, including n = 13 for AMPA, n = 6 for NMDA, and n = 5 for KA). The degree of potentiation obtained using 20 μM (1*S*,3*R*)-ACPD ranged from approximately 20% to >500%. In order to observe the potentiating effects of (1*S*,3*R*)-ACPD, it was essential for the drug to be present during application of one of the ionotropic agonists. Furthermore, the potentiating effects reversed rapidly after its removal (e.g., Fig. 2, b–d).

Increases in $[Ca^{2+}]_i$ produced by ionotropic glutamate agonists are the result of two types of Ca^{2+} influx. Ca^{2+} can enter the cell through the ligand-gated ion channel itself (7, 8). In addition, depolarization produced by these agonists causes Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (7, 8). It was, therefore, possible that the effects of (1*S*,3*R*)-ACPD were due to effects on voltage-sensitive Ca^{2+} channels rather than on the ionotropic glutamate receptors themselves. However, this proved not to be the case, inasmuch as (1*S*,3*R*)-ACPD did not

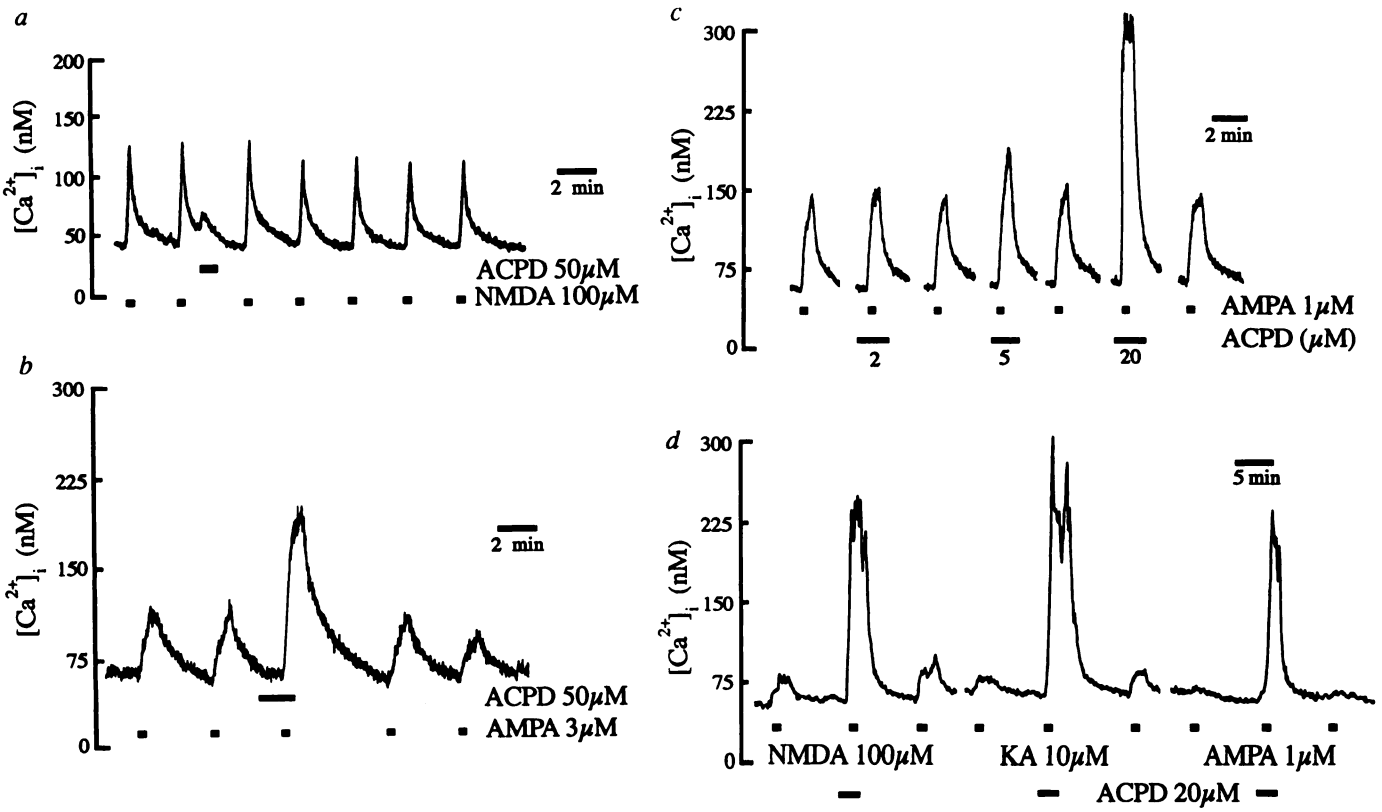

Fig. 2. a, Increases in $[Ca^{2+}]_i$ in an acutely isolated DH neuron were produced by fast application of NMDA (100 μM, 15 sec). These regular increases were unaffected by the application of (1*S*,3*R*)-ACPD (50 μM) between the NMDA responses. b, Increases in $[Ca^{2+}]_i$ were produced by applications of AMPA (3 μM, 15 sec). Upon application of (1*S*,3*R*)-ACPD (50 μM) to this neuron, there was no observed increase in $[Ca^{2+}]_i$. However, the AMPA response was potentiated when AMPA and (1*S*,3*R*)-ACPD were added together. Upon washout of the (1*S*,3*R*)-ACPD, AMPA responses were no longer potentiated. c, (1*S*,3*R*)-ACPD (2–20 μM) concentration-dependently potentiated the magnitude of the AMPA response. d, (1*S*,3*R*)-ACPD (50 μM) potentiated the increases in $[Ca^{2+}]_i$ evoked by NMDA (100 μM), KA (10 μM), and AMPA (1 μM) in the same neuron.

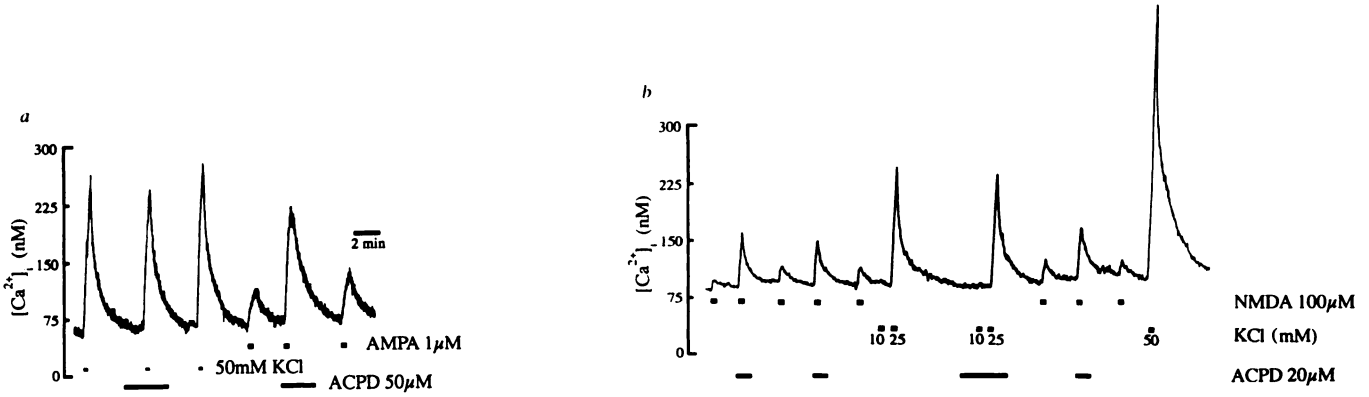

Fig. 3. a, Potentiation of AMPA responses by (1*S*,3*R*)-ACPD (50 μM) was observed in a DH neuron in which there was no effect of (1*S*,3*R*)-ACPD on the increase in $[Ca^{2+}]_i$ produced by 50 mM KCl. b, Potentiation of a NMDA (100 μM) response by (1*S*,3*R*)-ACPD (50 μM) was observed in a DH neuron in which there was no effect of (1*S*,3*R*)-ACPD on the increase in $[Ca^{2+}]_i$ produced by 10 or 25 mM KCl (data are representative of four similar experiments in which submaximal concentrations of KCl were used).

potentiate the increase in $[Ca^{2+}]_i$ produced by a 50 mM K^+ depolarization, even though it was effective in enhancing ionotropic agonist effects in the same cells (Fig. 3a). Fig. 3b also illustrates potentiation of an NMDA response in a cell in which there was no potentiation of submaximal concentrations of KCl (10 or 25 mM) by (1*S*,3*R*)-ACPD. Similar results were observed in four other experiments. Such results also rule out the possibility that the effects of (1*S*,3*R*)-ACPD are produced at the level of Ca^{2+} buffering, because if this were the case potentiation should have been observed regardless of the route of Ca^{2+} influx.

The results described above, obtained in acutely isolated DH neurons, suggest that activation of metabotropic receptors simultaneously potentiates the effects of agonists at two types of ionotropic glutamate receptors. In order to investigate this phenomenon further, in a different context, we examined the interaction between (1*S*,3*R*)-ACPD and AMPA, KA, and NMDA in spinal cord slices from the rat DH. In these experiments, neurons in the substantia gelatinosa were voltage-clamped by using the whole-cell voltage-clamp procedure, and the effect of (1*S*,3*R*)-ACPD on membrane currents obtained by puffing AMPA, KA, or NMDA onto the cells was examined. Small puffs of the agonists were used in order to produce

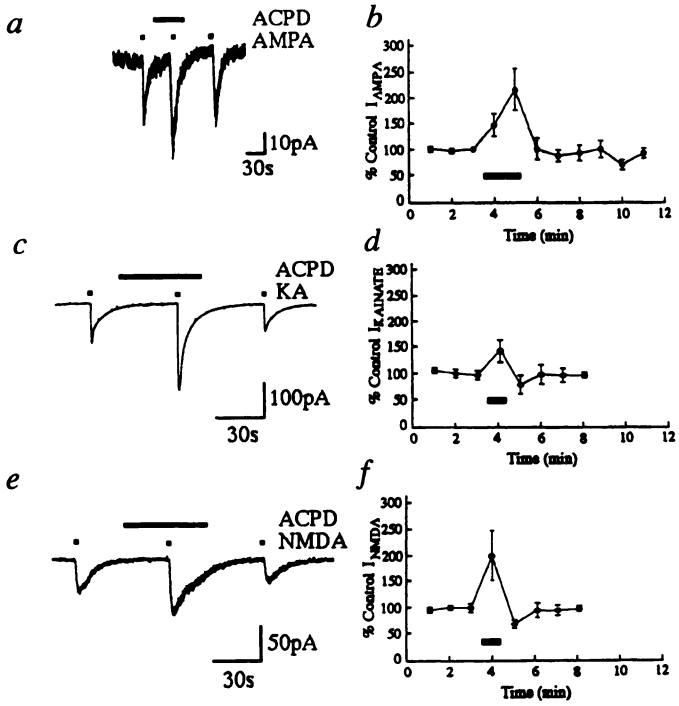

Fig. 4. Currents I_{AMPA}, I_{KA} and I_{NMDA}, evoked by pressure ejection of AMPA (a and b), KA (c and d), or NMDA (e and f), respectively, onto substantia gelatinosa neurons in transverse spinal cord slices were potentiated in the presence of the metabotropic glutamate agonist (1*S*,3*R*)-ACPD (20 μM). No change in the resting holding current was produced by (1*S*,3*R*)-ACPD at this concentration. Continuous chart records (a, c, and f) illustrate that the potentiating effect of (1*S*,3*R*)-ACPD reversed rapidly after washout. The time course of (1*S*,3*R*)-ACPD (20 μM) potentiation of AMPA- (b, $n = 7$), KA- (d, $n = 4$), or NMDA- (f, $n = 4$) evoked currents was recorded in separate cells. *Bar*, period of (1*S*,3*R*)-ACPD application. The ionotropic agonist-induced current amplitude was normalized to 100% measured immediately before the application of (1*S*,3*R*)-ACPD. Data are expressed as mean ± standard error. The holding potential for a–d was −50 mV and for e and f was −30 mV.

responses approximating in size those obtained physiologically by activation of the synaptic inputs to these neurons. Perfusion of slices with (1*S*,3*R*)-ACPD by itself produced a small inward current, in the same concentration range in which it also produced Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in acutely isolated cells (>20 μM) (see Fig. 1). A similar effect has been reported in several other tissues (2). As with the Ca^{2+} signals observed, simultaneous addition of (1*S*,3*R*)-ACPD produced a consistent potentiation of the effects of AMPA, KA, and NMDA (Fig. 4). This occurred even at concentrations of (1*S*,3*R*)-ACPD below those at which it produced an inward current (<20 μM). Examination of the AMPA I-V curve showed that (1*S*,3*R*)-ACPD increased the slope conductance without altering the reversal potential of the AMPA-induced current. (1*S*,3*R*)-ACPD at 10 μM enhanced the amplitude of the AMPA-induced current to 143.6 ± 7.4% of control ($n = 3$), whereas 20 μM (1*S*,3*R*)-ACPD enhanced the current to 218.9 ± 46.4% of control ($n = 7$). As in the experiments using acutely isolated neurons, the effects of (1*S*,3*R*)-ACPD reversed rapidly after removal of the metabotropic agonist, although in the slice complete reversal sometimes took several minutes.

These results clearly illustrate the ability of a metabotropic glutamate receptor agonist to potentiate powerfully the effects of agonists at ionotropic glutamate receptors. Several other recent studies have illustrated the ability of a variety of neurotransmitters to potentiate the effects of NMDA in different preparations (9–14), although neurotransmitter modulation of KA/AMPA responses has not been widely reported. Examination of the structure of the recently cloned rat brain NMDA receptor (15), as well as the various KA/AMPA receptors (16), reveals the presence of a large number of consensus phosphorylation sites, suggesting the possibility that these receptors may be modulated through second messenger-mediated mechanisms. Indeed, potentiation of NMDA and KA/AMPA responses after activation of protein kinase C (17) and cAMP-dependent protein kinases (18–20), respectively, has been demonstrated previously. However, the effects of (1*S*,3*R*)-ACPD appear to be dissociated from its ability to increase $[Ca^{2+}]_i$. Moreover, potentiation also appears to be rather short lived, whereas previous reports on the potentiation of NMDA responses showed it to be long lasting, as might be expected for a phosphorylation-mediated effect (9–13).

From the physiological perspective, the present results have important implications. Previous reports concerning the hippocampus (20) and nucleus of the solitary tract (22) have indicated that synaptic activation of metabotropic glutamate receptors seems to occur during high frequency stimulation. Such activation could lead to the potentiation of postsynaptic ionotropic responses, leading to the increased production of retrograde messengers, increased glutamate release, and additional postsynaptic effects. Thus, the metabotropic receptor would be part of a positive feedback loop, resulting in an enhanced synaptic response (23, 24). It is interesting to note that increased sensitivity of neurons to both NMDA (25) and KA/AMPA (26) receptor agonists has been reported during long term potentiation at hippocampal synapses. It is possible that metabotropic receptors play a critical role in such events in different parts of the central nervous system.

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