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Wallace B. Thoreson
University of Nebraska Medical Center, wbthores@unmc.edu

R. F. Miller
University of Minnesota

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Actions of (1S,3R)-1-Aminocyclopentane-1,3-Dicarboxylic Acid (1S,3R-ACPD) in Retinal ON Bipolar Cells Indicate That It Is an Agonist at L-AP4 Receptors

WALLACE B. THORESON and ROBERT F. MILLER

From the Department of Physiology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT Metabotropic glutamate receptors (mGluRs) include receptors sensitive to L-2-amino-4-phosphonobutyrate (L-AP4) and 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD). To determine whether 1S,3R-ACPD is an agonist at retinal L-AP4 receptors, whole cell voltage clamp recordings were obtained from mudpuppy ON bipolar cells in a superfused retinal slice and L-AP4 and 1S,3R-ACPD were bath applied. Both compounds evoked similar outward currents which reversed near 0 mV and were accompanied by an increased input resistance. Responses to both agonists washed out in parallel suggesting they act through the same second messenger pathway(s). Inhibitors of cGMP-PDE activity suppressed responses to both L-AP4 and 1SR,3RS-ACPD, suggesting that both compounds activate cGMP-PDE. Responses to 1S,3R-ACPD were occluded by prior activation of L-AP4 receptors, but not blocked by the non-AP4, mGluR antagonists, L-aminophosphonopropionic acid (L-AP3) or 4-carboxy-3-hydroxyphenylglycine (4C3H-PG). These results indicate that 1S,3R-ACPD is an agonist at L-AP4 receptors. 1S,3S-ACPD and 4C3H-PG evoked outward currents similar to L-AP4 suggesting they may also be L-AP4 receptor agonists. Using the b-wave of the ERG as an assay for ON bipolar cell responses, concentration/response curves were obtained for ACPD enantiomers. The rank-order potency of ACPD enantiomers at L-AP4 receptors in ON bipolar cells is similar to their rank-order potency at non-AP4, mGluRs in brain which suggests that the receptors possess similar binding sites and may be members of a common receptor family.

INTRODUCTION

The amino acid, L-glutamate, is the major excitatory neurotransmitter in the brain, spinal cord, and retina. The effects of glutamate are mediated by two general classes of receptors: (a) ionotropic receptors which are coupled directly to ion channels and include *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methylisoxazole-4-pro-

Address correspondence to Wallace B. Thoreson at his present address: Department of Ophthalmology, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198-5540.

pionic acid (AMPA) and kainic acid (KA) receptors (for reviews see Collingridge and Lester, 1989; Monaghan, Bridges, and Cotman, 1989; and Watkins, Krogsgaard-Larsen, and Honoré, 1990) and (b) metabotropic glutamate receptors (mGluRs) which are coupled to G-proteins and act through intracellular second messengers (for review see Schoepp and Conn, 1993).

ON depolarizing bipolar cells of the vertebrate retina possess receptors sensitive to micromolar concentrations of L-2-amino-4-phosphonobutyric acid (L-AP4) (Shiells, Falk, and Naghshineh, 1981; Slaughter and Miller, 1981). L-AP4 receptors are also found in other regions of the central nervous system, including the hippocampus, spinal cord, and olfactory bulb (Koerner and Cotman, 1981; Davies and Watkins, 1982; Trombley and Westbrook, 1992). For many years, both the transduction mechanisms employed by L-AP4 receptors and the relationship of L-AP4 receptors to other glutamate receptors were unknown. However, as discussed below, recent studies have begun to clarify these issues.

The transduction mechanisms employed by L-AP4 receptors have been studied in some detail in both retinal ON bipolar cells and olfactory bulb neurons. In both cell types, L-AP4 receptors are coupled to G-proteins. In retinal ON bipolar cells, activation of the G-protein stimulates cGMP-phosphodiesterase (cGMP-PDE) activity resulting in the closing of cGMP-sensitive cation channels, similar to phototransduction (Nawy and Jahr, 1990, 1991; Shiells and Falk, 1990). In olfactory bulb neurons, G-protein activation by L-AP4 receptors results in the inhibition of Ca currents (Trombley and Westbrook, 1992).

The relationship of L-AP4 receptors to other glutamate receptors is also becoming clear. A family of mGluRs has been cloned and sequenced (Houamed, Kuijper, Gilbert, Haldeman, O'Hara, Mulvihill, Almers, and Hagen, 1991; Masu, Tanabe, Tsuchida, Shigemoto, and Nakanishi, 1991; Tanabe, Masu, Ishii, Shigemoto, and Nakanishi, 1992; Tanabe, Nomura, Masu, Shigemoto, Mizuno, and Nakanishi, 1993; Abe, Sugihara, Mizuno, and Nakanishi, 1992; Nakanishi, 1992; Thomsen, Kristensen, Mulvihill, Haldeman, and Suzdak, 1992; Duvoisin, 1993). One member of this family, mGluR4, can be stimulated by micromolar concentrations of L-AP4 and is therefore an L-AP4 receptor (Thomsen et al., 1992; Tanabe et al., 1993). mGluR6 exhibits 70% sequence homology to mGluR4 and is selectively localized in retina suggesting that it may be the L-AP4 receptor found in retinal ON bipolar cells (Nakanishi, 1992; Duvoisin, 1993). In contrast, the subtypes mGluR1-3 and mGluR5 are relatively insensitive to L-AP4 (Aramori and Nakanishi, 1992; Tanabe et al., 1992, 1993; Abe et al., 1992), as are many mGluRs in vivo (Schoepp, Bockaert, and Sladeczek, 1990a). Thus, it appears that L-AP4 receptors are members of a larger family of homologous mGluRs.

The conformationally constrained glutamate analogue, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), is a selective agonist for mGluRs (Palmer, Monaghan, and Cotman, 1989; Desai and Conn, 1990; Irving, Schofield, Watkins, Sunter, and Collingridge, 1990; Schoepp, Johnson, True, and Monn, 1991b). However, despite the apparent homology among L-AP4-sensitive and L-AP4-insensitive mGluRs, it is not clear whether 1S,3R-ACPD is an agonist for L-AP4 receptors. 1S,3R-ACPD causes a weak and inconsistent activation of mGluR4 expressed in Chinese hamster ovary cells (Tanabe et al., 1993). L-AP4 and ACPD produce similar

effects in retina (Slaughter and Miller, 1985), hippocampus (Baskys and Malenka, 1991), and spinal cord (Pook, Sunter, Udvarhelyi, and Watkins, 1992), but it is unclear from these studies whether the two agonists act at the same receptor or activate different receptors whose intracellular second messenger pathways converge. The present study assessed the similarity of L-AP4 receptor binding sites to the binding sites of L-AP4-insensitive mGluRs by determining the affinity of L-AP4 receptors for 1S,3R-ACPD and its enantiomers.

The results indicate that 1S,3R-ACPD is an agonist at L-AP4 receptors. Thus, although 1S,3R-ACPD is selective for mGluRs over ionotropic glutamate receptors, it does not select between L-AP4-sensitive and L-AP4-insensitive mGluRs. In addition, the four enantiomers of ACPD exhibit a rank-order potency at retinal L-AP4 receptors similar to their rank-order potency at non-AP4 mGluRs in brain suggesting that both classes of receptors possess similar binding sites. These results are consistent with the hypothesis that L-AP4 receptors are members of a larger family of mGluRs.

A brief abstract of these results has appeared previously (Thoreson and Miller, 1992).

MATERIALS AND METHODS

Whole Cell Voltage Clamp Recordings

Whole cell patch clamp recordings were obtained from ON bipolar cells in a superfused mudpuppy (*Necturus maculosus*) retinal slice preparation similar to that developed by Werblin (1978) and described in detail by Wu (1987).

The superfusate contained 111 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5 mM glucose, and 10 mM HEPES. For whole cell recordings, CoCl₂ (2–5 mM) was added to the superfusate to suppress synaptic transmission. The pH was adjusted to 7.8 with NaOH and bubbled continuously with 100% O₂. Experiments were done at room temperature (20–25°C). Drugs were added to the superfusate and typically applied for 30–45 s. The retina was washed with control medium for 3–5 min between drug applications.

Patch pipettes were fabricated from omega dot borosilicate glass (1.2 mm O.D., 0.95 id) on a Narashige PP-83 pipette puller. The tips were ~2 μm in diameter and had resistances of 4–8 MΩ. The access resistance was typically less than 50 MΩ while the input resistance of these cells averages 1.5 GΩ, so the access resistance introduced a voltage clamp error of less than 3% (Thoreson and Miller, 1993).

The pipettes were filled with (in mM): CsCH₃SO₄ 98, NaCl 3.5, MgCl₂ 3, CaCl₂ 1, EGTA 11, HEPES 5, D-glucose 2, glutathione 1, ATP-Mg 1, GTP 0.5. In some experiments, KCH₃SO₄ was used instead of CsCH₃SO₄. Cs was used for all experiments unless stated otherwise. cGMP (1 mM) was sometimes added to the intracellular Ringer's solution (e.g., Fig. 2). The pH was adjusted to 7.2 with CsOH or KOH. The electrodes also contained 0.025% 5,6-carboxyfluorescein or 0.05% sulfarhodamine 101 to permit fluorescent visualization of the cell following recording.

Glutamate agonists were provided by J. C. Watkins, Tocris Neuramin. Zaprinast was provided by Rhône-Poulenc Rorer. KCH₃SO₄ was obtained from Pfaltz and Bauer. Fluorescent dyes were obtained from Molecular Probes, Inc. (Eugene, OR). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

ON bipolar cells were identified physiologically by their response to response to L-AP4 (5 μM) and morphologically by the presence of a cell body in the distal inner nuclear layer with termination of the axon in the proximal inner plexiform layer (sublamina B).

ERG Pharmacology

The b-wave of the DC-ERG was recorded intraretinally in a superfused mudpuppy eyecup preparation (see Fig. 6). A patch pipette filled with extracellular medium was used as the recording electrode. The amplitude of the b-wave was measured from its peak to the trough of the a-wave. The fraction of the b-wave remaining in the presence of the drug (fractional response) was plotted as a function of log concentration of the drug. The IC_{50} 's were determined using a weighted algorithm based on the equation: $b/b_{\text{max}} = [1 + (c/\text{IC}_{50})^h]^{-1}$ where c = agonist concentration, IC_{50} = concentration required to produce 50% inhibition of the b-wave, h = Hill coefficient, and b/b_{max} = fractional response of the b-wave. For a more complete description of the methods used for recording the ERG and calculating the IC_{50} , see Peterson, Thoreson, Johnson, Koerner, and Miller, (1991).

RESULTS

Whole Cell Voltage Clamp Recordings

Fig. 1 shows the currents in an ON bipolar cell in the mudpuppy retinal slice evoked by L-AP4 (5 μM , Fig. 1A) and 1S,3R-ACPD (160 μM , Fig. 1C). At a holding potential of -50 mV, both compounds evoked virtually identical outward membrane currents of ~ 40 pA accompanied by a reduction in membrane noise. At a concentration of 160 μM , the currents evoked by 1S,3R-ACPD averaged 35.7 ± 6.6 pA (\pm SEM, $n = 3$), while at 80 μM , the currents averaged 24.0 ± 11.3 pA ($n = 2$).

Current/voltage relationships were determined by applying 150 ms voltage steps before application of the drug and near the peak of the evoked current (Fig. 1, A, C, and E, *vertical transients*). Agonist current/voltage relationships were calculated by subtracting the control current/voltage relationships from those obtained in the presence of the agonist. As shown in the current/voltage plots in Fig. 1, B and D, the currents evoked by L-AP4 and 1S,3R-ACPD both reversed near 0 mV and were accompanied by a negative slope conductance. The similarities between the responses to L-AP4 and 1S,3R-ACPD suggest that both compounds act to close the same species of ion channels, probably nonspecific cation channels (Yamashita and Wässle, 1991; Thoreson and Miller, 1993).

Although less potent than 1S,3R-ACPD, the 1S,3S enantiomer of ACPD also evoked an outward current accompanied by a reduction in membrane noise (Fig. 1E, 640 μM). The currents evoked by 1S,3S-ACPD at 640 μM averaged 28.8 ± 4.2 pA ($n = 4$). Like L-AP4 and 1S,3R-ACPD, the currents evoked by 1S,3S-ACPD reversed near 0 mV and were accompanied by an increased input resistance (Fig. 1F). These results suggest that 1S,3S-ACPD also causes the closing of nonspecific cation channels.

Like the pure 1S,3R enantiomer, the racemic mixture of 1SR,3RS-ACPD (often called "*trans*-ACPD"; for a discussion of ACPD nomenclature see Schoepp et al., 1990a) evoked outward currents similar to those evoked by L-AP4 (5 μM , $I_{\text{AP4}} = 24.9 \pm 2.6$ pA, $n = 29$; 100 μM , $I_{\text{ACPD}} = 21.6 \pm 3.6$ pA, $n = 16$; 200 μM , $I_{\text{ACPD}} = 18.7 \pm 4.1$ pA, $n = 9$). The pure 1R,3S enantiomer had no effect at a

concentration of 1 mM ($n = 3$). Due to a limited availability of 1S,3R-ACPD, many of the following experiments were done using the racemic mixture of 1SR,3RS-ACPD. However, given the ineffectiveness of 1R,3S-ACPD, actions of 1SR,3RS-ACPD are likely to reflect actions of the 1S,3R enantiomer.

1SR,3RS-ACPD suppresses K channels in hippocampal neurons (Baskys, Bernstein, Barolet, and Carlin, 1990; Charpak, Gahwiler, Do, and Knopfel, 1990). In ON bipolar cells, L-AP4 and 1SR,3RS-ACPD produced virtually identical responses whether the intracellular Ringer's solution contained K ($n = 5$, data not shown) or Cs, which suggests that 1S,3R-ACPD does not act at K channels in these cells.

The finding that L-AP4 and 1S,3R-ACPD close the same type of ion channel suggests that either both compounds activate L-AP4 receptors or they activate

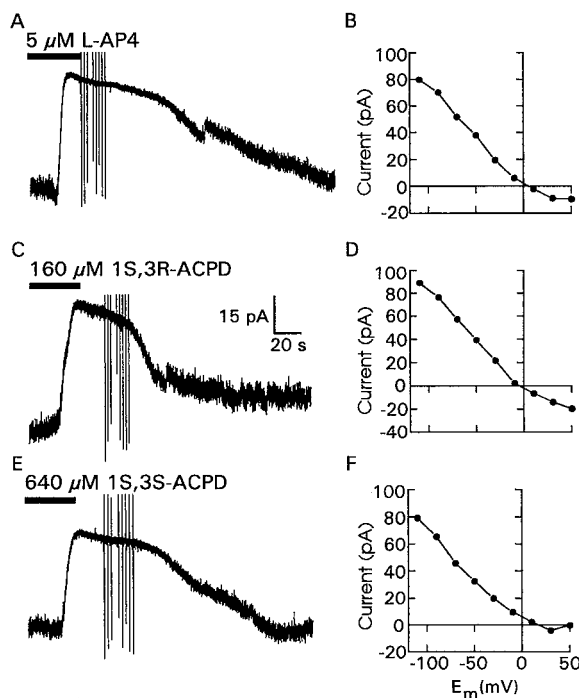


FIGURE 1. Membrane currents of an ON bipolar cell in response to L-AP4 (5 μ M; *A* and *B*), 1S,3R-ACPD (160 μ M; *C* and *D*), and 1S,3S-ACPD (640 μ M; *E* and *F*). The currents evoked by the agonists at a holding potential of -50 mV are shown at the left (*A*, *C*, and *E*). Current/voltage relationships were determined by applying 150 ms voltage steps (vertical transients). The agonist current/voltage relationships shown at the right (*B*, *D*, and *F*) were calculated by subtracting the control current/voltage relationship from that obtained in the presence of the agonist.

separate receptors whose intracellular second messenger cascades converge onto the same class of ion channels. The following experiments were undertaken to distinguish between these two possibilities.

In ON bipolar cells, L-AP4 receptors are coupled to G-proteins which stimulate a cGMP-PDE to cleave cGMP, resulting in the closure of cGMP-sensitive, cation channels (Nawy and Jahr, 1990, 1991; Shiells and Falks, 1990). Despite the presence of GTP (0.5 mM), L-AP4-evoked responses steadily diminished during the course of our recordings, disappearing altogether 30 min or more into the recording. A similar, slow "washout" was also observed when cGMP (1.0 mM) was present in the pipette (e.g., see Fig. 2). The observation that a slow washout was observed in both the presence and absence of cGMP in the pipette suggests that the washout reflects

loss of cGMP-PDE activity, rather than saturation of the cation channels by cGMP or loss of guanylate cyclase activity.

Responses to 1SR,3RS-ACPD diminished in parallel with the decrease in amplitude of the responses to L-AP4. This parallel washout is illustrated in Fig. 2. In this experiment, L-AP4 (5 μM) and 1SR,3RS-ACPD (200 μM) were alternately bath applied. The filled triangles plot the amplitude of the current evoked by L-AP4 (5 μM) as a function of the time of recording. The solid curve is a single exponential which provided the best fit to the AP4 data. The open triangles plot the amplitude of responses to 1SR,3RS-ACPD (200 μM). The strong parallel between the washout of L-AP4- and ACPD-evoked responses suggests that both agents act through the same second messenger cascade.

Inhibition of cGMP-PDE by dipyrindamole suppresses the current evoked by L-AP4 (Nawy and Jahr, 1991). Similarly, we found that the cGMP-PDE inhibitors, dipyrindamole (200 μM , $n = 3$) or zaprinast (100 μM , $n = 2$) (Gillespie and Beavo, 1989) increased the rate of washout of the L-AP4-evoked current. The filled circles in Fig. 3 A represent responses to L-AP4 (5 μM) recorded with zaprinast or dipyrindamole in

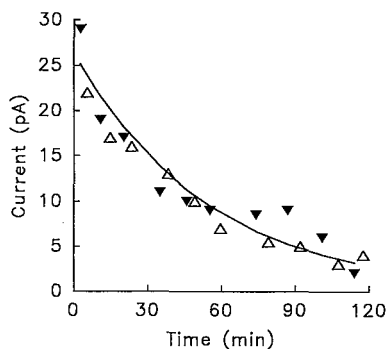


FIGURE 2. Amplitude of the currents evoked by L-AP4 (5 μM , filled triangles) and 1SR,3RS-ACPD (200 μM , open triangles) in an ON bipolar cell as a function of time of recording. The responses to L-AP4 were fit by the equation $y = ae^{-bx}$ where $a = 26.38$ pA and $b = 0.02$ s (solid curve). The drugs were alternately applied for 30–40 s apiece. In addition to 0.5 mM GTP, the patch pipette in this experiment contained 1 mM cGMP.

the pipette ($n = 5$ cells) whereas the open triangles represent control responses to L-AP4 recorded without the PDE inhibitors ($n = 20$ cells). The solid line is the linear regression on the cGMP-PDE inhibitor data; the dashed line is the regression on the control data. The slope of the cGMP-PDE regression line is significantly steeper than the control regression line ($P < 0.05$) indicating that the cGMP-PDE inhibitors diffused into the cells from the pipettes and suppressed responses to L-AP4.

Fig. 3 B shows that in the presence of zaprinast and dipyrindamole, responses to 1SR,3RS-ACPD (100 μM) continue to parallel responses to L-AP4. The filled circles in Fig. 3 B represent responses to L-AP4 in the presence of cGMP-PDE inhibitors, whereas the open circles represent responses to 1SR,3RS-ACPD in the presence of these same inhibitors. Note that the concentration of ACPD in Fig. 3, B and C, is 100 μM , not 200 μM as used in Fig. 2. The solid line is the linear regression on the L-AP4 data and the dotted line is the linear regression on the 1SR,3RS-ACPD data. As with L-AP4, the slope of the regression line for responses to ACPD in the presence of the inhibitors is significantly steeper than the control regression (dashed line, Fig. 3 A; $P < 0.05$). In contrast, the ACPD and AP4 regression lines in the presence of the

inhibitors do not differ significantly. These results suggest that, like responses to L-AP4, responses to 1SR,3RS-ACPD are suppressed by the cGMP-PDE inhibitors, zaprinast and dipyrindamole.

ACPD-sensitive mGluRs can induce phosphoinositide (PI) hydrolysis (Sladeczek, Pin, Recasens, Bockaert, and Weiss, 1985; Sugiyama, Ito, and Hirono, 1987; Schoepp and Conn, 1993). An inhibitor of PI hydrolysis, neomycin (100 μ M, $n = 5$) (Schacht,

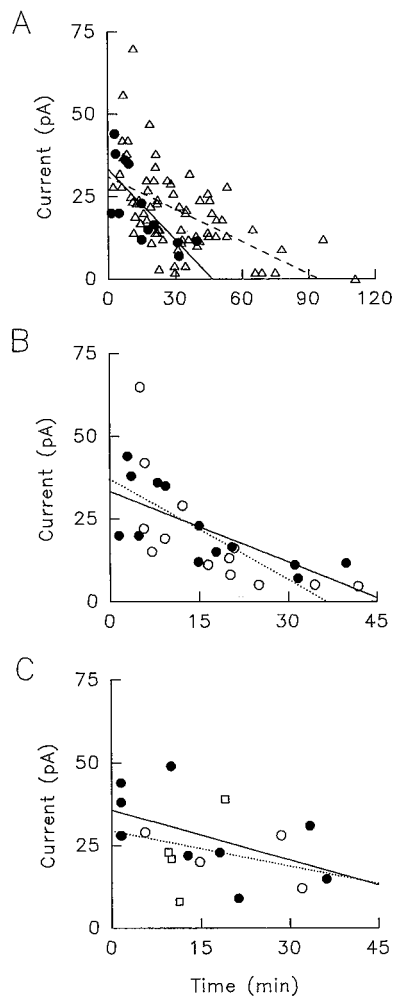


FIGURE 3. (A) Responses to L-AP4 (5 μ M) recorded with cGMP-PDE inhibitors, dipyrindamole (200 μ M, $n = 3$ cells) or zaprinast (100 μ M, $n = 2$ cells), in the pipette (filled circles) and control responses recorded without cGMP-PDE inhibitors ($n = 20$ cells, open triangles). The solid line is a linear regression on the cGMP-PDE data, whereas the dashed line is a regression on the control data. The slope of the regression line on the cGMP-PDE data ($b = -0.715 \pm 0.200$) is significantly greater ($P < 0.05$) than the slope of the control regression ($b = -0.328 \pm 0.061$). (B) Responses to L-AP4 (5 μ M, filled circles, same data as in A) and 1SR,3RS-ACPD (100 μ M, open circles) in the presence of dipyrindamole and zaprinast. Regression lines on responses to L-AP4 (solid line; $b = -1.016 \pm 0.280$) and ACPD (dotted line; $b = -1.016 \pm 0.280$) do not differ significantly in slope. (C) Responses to L-AP4 (filled circles), 1SR,3RS-ACPD (100 μ M, open circles), and 1S,3R-ACPD (80 μ M, open squares) in the presence of a PI hydrolysis inhibitor, neomycin (100 μ M, $n = 5$ cells). Regression lines on responses to L-AP4 (solid line; $b = -0.500 \pm 0.489$) and 1SR,3RS-ACPD (dotted line; $b = -0.352 \pm 0.449$) do not differ significantly in slope.

1976; Wang, Wiener, Takada, and Schacht, 1984), did not appear to affect the rate of washout of either L-AP4 or ACPD-evoked responses (Fig. 3 C). The absence of a selective suppression of responses to ACPD by neomycin suggests that the ACPD responses are not mediated by an mGluR coupled to PI hydrolysis and is consistent with the hypothesis that the effects of ACPD are mediated by its actions at L-AP4 receptors.

If L-AP4 and ACPD activate different receptors, messengers, and ion channels, currents evoked by the two agents should add linearly under voltage clamp conditions. Conversely, if the two activate the same receptors, second messengers, or ion channels, nonadditive interactions should occur. We found that strong activation of L-AP4 receptors occluded the current evoked by 1SR,3RS-ACPD. Fig. 4 *A* shows an outward current of 60 pA evoked by a near saturating concentration of L-AP4 (5 μ M). Saturation of the response to L-AP4 could reflect saturation either of the receptor

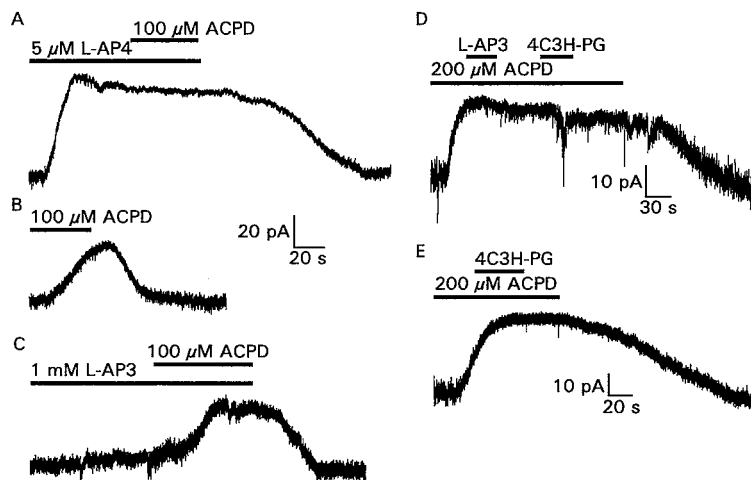


FIGURE 4. Effects of L-AP4 and the mGluR antagonists, L-AP3 and 4C3H-PG, on responses to 1SR,3RS-ACPD. (*A*) L-AP4 (5 μ M) was applied for 40 s before applying 1SR,3RS-ACPD (100 μ M). At the holding potential of -50 mV, L-AP4 evoked an outward current which was unchanged by the concurrent application of 1SR,3RS-ACPD. The responses illustrated in *A*–*C* are all from the same cell. (*B*) Following washout of L-AP4 from the bath, the application of 1SR,3RS-ACPD evoked an outward current. (*C*) The ACPD receptor antagonist, L-AP3 (1 mM), was applied for 70 s before beginning 1SR,3RS-ACPD (100 μ M). The response to 1SR,3RS-ACPD was almost identical to that in *B* and therefore appeared to be unaffected by L-AP3. (*D*) 1SR,3RS-ACPD (200 μ M) evoked an outward current which was not appreciably reduced by the application of either L-AP3 (1 mM) or 4C3H-PG (0.5 mM). The rapid inward transient observed after the application of 4C3H-PG had a much faster time course than the responses to bath applied drugs and was probably an artifact. (*E*) Another trial from a different ON bipolar cell showing that the outward current evoked by 1SR,3RS-ACPD (200 μ M) was not detectably reduced by the application of 4C3H-PG (0.5 mM).

binding site or of some subsequent process, such as the closure of cation channels. In the presence of L-AP4, 1SR,3RS-ACPD (100 μ M) failed to induce any detectable, additional current. Following recovery from L-AP4, 1SR,3RS-ACPD evoked an outward current of 35 pA (Fig. 4 *B*). Similar results were obtained in four cells. The ability of L-AP4 to occlude the ACPD current strongly indicates that at some point along the pathway, either at the receptor level or subsequent cascade of events, both ACPD and AP4 activate the same post-synaptic mechanism. There is currently no

known antagonist to L-AP4 receptors (Koerner and Johnson, 1992) so it was not possible to test the effects of an L-AP4 antagonist.

L-2-amino-3-phosphonopropionic acid (L-AP3) is an antagonist at some mGluRs (Schoepp, Johnson, Smith, and McQuaid, 1990b; Sahara and Westbrook, 1993; Schoepp and Conn, 1993). Fig. 4 C illustrates responses evoked by 1SR,3RS-ACPD (100 μ M) and L-AP3 (1 mM) in the same cell as the responses illustrated in Fig. 4, A and B. Application of L-AP3 alone evoked no response. In the presence of L-AP3, 1SR,3RS-ACPD evoked an outward current of 35 pA, the same amplitude as the earlier control response shown in Fig. 4 B. The responses to 1SR,3RS-ACPD in L-AP3 averaged 17.8 ± 4.9 pA whereas the control responses to ACPD in these trials averaged 17.3 ± 5.0 pA ($n = 4$). This indicates that L-AP3 failed to antagonize significantly the responses to ACPD in ON bipolar cells.

Because of the washout of responses to L-AP4 and ACPD (Figs. 2 and 3), assessing the degree of antagonism by comparing the amplitudes of control and test responses is problematic. Therefore the possible antagonism of L-AP3 was also tested applying L-AP3 in the presence of 1SR,3RS-ACPD. As shown in Fig. 4 D, the outward current evoked by 1SR,3RS-ACPD (200 μ M) appeared to be unaffected by L-AP3 (1 mM) indicating that L-AP3 does not block the effects of ACPD in this cell. A similar absence of antagonism was seen in all three ON bipolar cells tested with this paradigm.

Electrophysiological responses to 1S,3R-ACPD, but not PI hydrolysis, are antagonized by 4-carboxy-3-hydroxyphenylglycine (4C3H-PG) in the spinal cord and thalamus (Jones, Porter, Birse, Pook, Sunter, Udvarhelyi, Wharton, Roberts, and Watkins, 1992; Eaton, Salt, Udvarhelyi, Wharton, and Watkins, 1992). As shown in both Fig. 4, D and E, application of 4C3H-PG (0.5 mM) did not appear to antagonize the responses to 1SR,3RS-ACPD (20 μ M) in retinal ON bipolar cells ($n = 5$). A rapid inward transient was observed after application of 4C3H-PG in Fig. 4 D but this transient is much more rapid than the slow changes induced by bath application so it probably does not reflect antagonism by 4C3H-PG. A similar inward transient was not observed in other trials with 4C3H-PG (e.g., Fig. 4 E). In addition to its absence of antagonism to 1SR,3RS-ACPD, 4C3H-PG (0.5 mM) failed to block responses to L-AP4 (5 μ M) (data not shown, $n = 5$), indicating that it is not an L-AP4 receptor antagonist.

The results of Fig. 4 suggest that 4C3H-PG is not an antagonist to ACPD (or L-AP4) in ON bipolar cells. Instead, as shown in Fig. 5, 4C3H-PG appears to be an agonist in ON bipolar cells. Like L-AP4, 4C3H-PG (1 mM) evoked an outward current accompanied by a decrease in membrane noise (Fig. 5 A). This outward current reversed at +10 mV and was accompanied by a conductance decrease (Fig. 5 B). 4C3H-PG (0.5–1.0 mM) evoked an outward current and conductance decrease in three of four cells tested. These results suggest that, like 1S,3R and 1S,3S-ACPD, 4C3H-PG is also an agonist at L-AP4 receptors.

The results described above strongly suggest that the ionic current evoked by 1S,3R-ACPD in mudpuppy ON bipolar cells is generated by the activation of L-AP4 receptors. This supports other findings that the only glutamate receptors in ON bipolar cells of urodele amphibians are L-AP4 receptors (Nawy and Jahr, 1991; Thoreson and Miller, 1993).

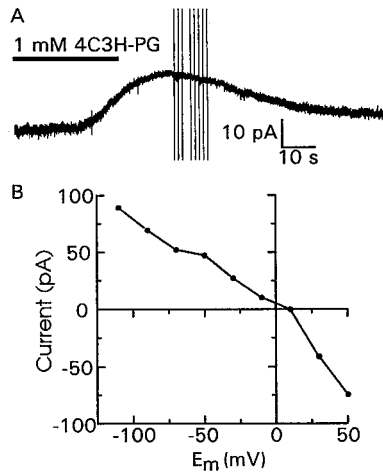
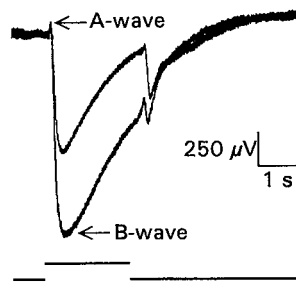


FIGURE 5. (A) Membrane current of an ON bipolar cell in response to the mGluR antagonist, 4C3H-PG (1 mM). The vertical transients are due to 150 ms voltage steps used to determine the cell's current/voltage relationship in the presence of the drug. (B) Current/voltage relationship calculated by subtracting the control current/voltage relationship from that obtained in the presence of the 4C3H-PG.

ERG Pharmacology

Taking advantage of the absence of other glutamate receptors in mudpuppy ON bipolar cells, we examined the sensitivity of L-AP4 receptors to the four enantiomers of ACPD. The washout of L-AP4 and ACPD responses prevented the acquisition of reliable concentration/response curves using the whole cell patch clamp technique. However, the b-wave of the ERG reflects the activity of ON bipolar cells in the mudpuppy and can be used as an assay for L-AP4 receptor activity (Stockton and Slaughter, 1989; Peterson et al., 1991). 1S,3R-ACPD is not taken up by plasma membrane glutamate uptake (Winter and Ueda, 1993), so the superfused concentration of the ACPD enantiomers is presumably the concentration which reached the cells.

Fig. 6 shows examples of ERGs recorded in a mudpuppy retina in control Ringer's solution and in the presence of 1S,3R-ACPD (80 μ M). The control and test responses are superimposed. The b-wave was measured from the peak of the a-wave to the peak of the b-wave. The fractional response of the ERG b-wave was calculated from the



measured from the peak of the a-wave to the peak of the b-wave. In this example, the amplitude of the control b-wave was 1,558 μ V while the b-wave in the presence of ACPD was 942 μ V, yielding a fractional response in the presence of ACPD of 0.60. Note that 1S,3R-ACPD selectively attenuated the b-wave without affecting the a-wave.

FIGURE 6. DC intraretinal electroretinogram (ERG) from the mudpuppy retina. Recording pipette was placed just proximal to the outer limiting membrane. The ERGs in control Ringer's solution and in the presence of 1S,3R-ACPD (80 μ M) are superimposed. The peaks of the a- and b-waves are noted in the control ERG. The amplitude of the b-wave was

ratio of the amplitude of the b-wave in the presence of the drug to the amplitude in control Ringer's. Note that 1S,3R-ACPD suppresses the b-wave without suppressing the a-wave.

Fig. 7 shows the mean fractional response of the ERG b-wave as a function of log concentration of 1S,3R-ACPD (*open circles*) and 1S,3S-ACPD (*filled circles*). The results show that 1S,3R-ACPD is 4.7 times more potent than 1S,3S-ACPD. The 1R isomers had little effect on L-AP4 receptors (data not shown). At a concentration of 5 mM, 1R,3S-ACPD suppressed the b-wave by 20% ($n = 3$), but this degree of suppression can be accounted for by the contamination of 1R,3S-ACPD by $\sim 1\%$ 1S,3R-ACPD (Sunter, Edgar, Pook, Howard, Udvarhelyi, and Watkins, 1991). 1R,3R-ACPD (3.2 mM, $n = 1$; 5 mM, $n = 2$) did not suppress the b-wave.

As described in the Methods, IC_{50} 's were determined by fitting a theoretical binding curve to the data (Fig. 7, *solid lines*). The rank order potency and IC_{50} 's for L-AP4 (Peterson et al., 1991) and the ACPD stereoisomers are: L-AP4 (1.1 μM) \gg 1S,3R-ACPD (89 μM) $>$ 1S,3S-ACPD (417 μM) \gg 1R,3S-ACPD; 1R,3R-ACPD (> 5 mM).

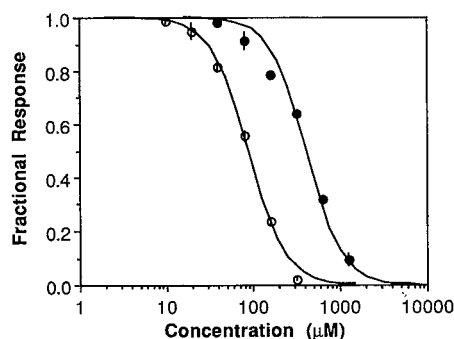


FIGURE 7. Concentration/response relationships for 1S,3R-ACPD (*open circles*) and 1S,3S-ACPD (*filled circles*) at L-AP4 receptors. The mean fractional response of the ERG b-wave (b/b_{max}) is plotted as a function of log agonist concentration. Each curve was repeated four times. Error bars = \pm SEM. The data were fit by a theoretical binding equation where the IC_{50} and Hill coefficient (h) were allowed to vary (see Methods). For 1S,3R-ACPD, $IC_{50} = 89 \mu\text{M}$, $h = 2.06$; for 1S,3S-ACPD, $IC_{50} = 417 \mu\text{M}$, $h = 2.15$.

DISCUSSION

The results of this study reveal a consistent similarity between the effects of L-AP4 and the mGluR agonist, 1S,3R-ACPD, in mudpuppy ON bipolar cells. (a) Both drugs evoked identical currents which indicates that they close the same ion channels (Fig. 1). This is consistent with a recent abstract which reported that 1S,3R-ACPD and the endogenous photoreceptor transmitter produce similar currents in ON bipolar cells (Slaughter and Tian, 1993). (b) The responses to both drugs washed out in parallel (Fig. 2). The parallel washout suggests that both drugs act through the same intracellular second messenger pathway. (c) The rate of washout of responses to L-AP4 and 1S,3R-ACPD were both increased by the cGMP-PDE inhibitors, zaprinast or dipyridamole, suggesting that both L-AP4 and 1S,3R-ACPD activate cGMP-PDE (Fig. 3). (d) Prior activation of L-AP4 receptors occluded the response to

1SR,3RS-ACPD, whereas the non-AP4, mGluR antagonists, L-AP3 and 4C3H-PG, had no effect on responses to ACPD (Fig. 4). These results indicate that 1S,3R-ACPD is an agonist at L-AP4 receptors in retinal ON bipolar cells of the mudpuppy.

One could argue that a more direct way to test whether both ACPD and L-AP4 act at the same receptors would be to determine if they occupy the same binding sites using radioligand binding assays. However, the binding of ^3H -L-AP4 or L-AP4 displaceable ^3H -glutamate is pharmacologically distinguishable from electrophysiologically defined L-AP4 receptors suggesting that the binding site is not the L-AP4 receptor recognition site (Fagg and Lanthorn, 1985; Crooks, Robinson, Koerner, and Johnson, 1986). Therefore, binding studies would not provide an unequivocal answer to the question of whether these two compounds act at the same or different receptors.

In addition to showing that 1S,3R-ACPD is an agonist at L-AP4 receptors, the results of this study indicate that L-AP4 receptors are the only metabotropic glutamate receptors in mudpuppy ON bipolar cells. This finding is consistent with other electrophysiological studies which suggest that L-AP4 receptors are the only glutamate receptors in ON bipolar cells of urodele amphibians (Nawy and Jahr, 1991; Thoreson and Miller, 1993) and with in situ hybridization experiments indicating that mGluR1-4 expression is limited to amacrine and ganglion cells (Tanabe-Ohuchi, Yoshimura, Shigemoto, and Honda, 1992).

The absence of other glutamate receptor types in ON bipolar cells made it possible to determine concentration/response relationships for the four enantiomers of ACPD acting at L-AP4 receptors relatively uncontaminated by their effects at other glutamate receptors. Knowledge of the rank-order potency of mGluR agonists at retinal L-AP4 receptors in vivo is important for confirming that a cloned receptor, e.g., mGluR6, is the retinal L-AP4 receptor.

The responses of ON bipolar cells are reflected in the b-wave of the ERG (Stockton and Slaughter, 1989) and the b-wave was used as an assay of ON bipolar cell responses. To assess the correspondence of rank-order potencies obtained using the ERG with the potencies observed with whole cell recordings, we compared response ratios obtained using whole cell recording techniques with the response ratios determined from concentration/response curves for ACPD enantiomers (Fig. 7) and L-AP4 (Peterson et al., 1991). From concentration/response curves, the responses to 80 and 160 μM 1S,3R-ACPD and 640 μM 1S,3S-ACPD should be 57, 78, and 72%, respectively, of the response to 5 μM L-AP4. The whole cell currents evoked by these compounds were 56% ($n = 2$), 110% ($n = 3$), and 82% ($n = 4$), respectively, of the currents evoked by L-AP4 in the same cells. Given the difficulty in obtaining reliable concentration/response data from whole cell recordings due to the washout of whole cell currents (Figs. 2 and 3), these ratios agree reasonably well with the ratios predicted from the concentration/response curves obtained using the ERG.

The rank-order potency of ACPD enantiomers at retinal L-AP4 receptors appears similar to their rank-order potency at non-AP4, mGluRs in brain. Both retinal L-AP4 receptors and brain mGluRs exhibit a strong stereospecificity, preferring 1S isomers over 1R isomers (Schoepp et al., 1991*b*). Given this stereospecificity, the effects of racemic 1SR,3RS- and 1SR,3SR-ACPD at mGluRs in brain are probably mediated largely by the 1S,3R- and 1S,3S-enantiomers, respectively. Therefore, the finding

that in hippocampus and cerebral cortex 1SR,3RS-ACPD is 3.2 and 4.1 times more potent, respectively, than 1SR,3SR-ACPD in stimulating IP₃ production (Schoepp et al., 1991a) correlates well with the present finding that 1S,3R-ACPD is 4.7 times more potent than 1S,3S-ACPD. The common preference of 1S over 1R isomers and of 1S,3R over 1S,3S suggests that there is some similarity between the binding sites of retinal L-AP4 receptors and brain, non-AP4 mGluRs. Two other characteristic mGluR agonists, ibotenate and quisqualate, also appear to be agonists at retinal L-AP4 receptors (Slaughter and Miller, 1985). These similarities between the pharmacology of L-AP4 receptors and non-AP4, mGluRs support the hypothesis that 1S,3R-ACPD is an agonist at all metabotropic glutamate receptors whereas L-AP4 activates a limited subset of this receptor class (Thomsen et al., 1992; Nakanishi, 1992; Tanabe et al., 1993). None of the mGluR agonists thus far tested are selective for non-AP4 over AP4 mGluRs.

There are currently no known L-AP4 receptor antagonists (Koerner and Johnson, 1992). The similarity between the binding sites of L-AP4 receptors and non-AP4 mGluRs raises the possibility that non-AP4, mGluR antagonists may also act as antagonists at L-AP4 receptors. Three putative mGluR antagonists were examined in the present study, but none of them are L-AP4 antagonists. In some preparations, L-AP4 can act as a weak mGluR antagonist (Nicoletti, Meek, Iadarola, Chuang, Roth, and Costa, 1988; Schoepp and Johnson, 1988). The present results suggest that another mGluR antagonist, 4C3H-PG, is also an L-AP4 receptor agonist (Fig. 5). A third mGluR antagonist, L-AP3, does not appear to be either an agonist or antagonist at L-AP4 receptors (Fig. 4; Slaughter and Miller, 1985). It will be of interest to see if any future mGluR antagonists prove to be L-AP4 antagonists.

1S,3R-ACPD is selective for mGluRs over ionotropic glutamate receptors and is therefore useful for demonstrating the presence of mGluRs. However, the present results indicate that it is not useful for discriminating between AP4 and non-AP4 mGluRs. Discriminating between these mGluR subtypes requires the use of other compounds, such as L-AP4.

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