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L-Cysteine Sulfinic Acid as an Endogenous Agonist of a Novel Metabotropic Receptor Coupled to Stimulation of Phospholipase D Activity

VALERIE BOSS, KAREN M. NUTT, and P. JEFFREY CONN

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

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SUMMARY

A substantial body of research implicates L-cysteine sulfinic acid (L-CSA) as a neurotransmitter. However, all physiological actions of L-CSA that have been pharmacologically characterized are mediated by cross-activation of glutamate receptors, and no receptor has been identified that is primarily activated by L-CSA. We report that a receptor exists in adult rat hippocampus that is activated by L-CSA but is insensitive to several other endogenous excitatory amino acids (EAAs), including L-glutamate, L-aspartate, and L-homocysteic acid. This receptor is coupled to an increase in the activity of phospholipase D (PLD). The L-CSA-induced PLD response is not blocked by ionotropic glutamate receptor antagonists but is mimicked by the metabotropic glutamate receptor (mGluR) agonist (1*S*,3*R*)-amino-1,3-cyclopentanedicarboxylic acid. The agonist pharmacology of the PLD-coupled response is generally similar to that of mGluRs but clearly differs from that of any particular mGluR that has been characterized to date. Furthermore, this receptor is not significantly blocked by (*RS*)-α-methyl-4-carboxyphenylglycine, which blocks a variety of mGluR-mediated responses. L-CSA has little effect on mGluRs coupled to phosphoinositide hydrolysis or the potentiation of cAMP responses in adult hippocampus, indicating that L-CSA is not a broad mGluR agonist. It is commonly thought that EAAs act on the same receptor families, all of which use glutamate as their primary agonist. However, the finding that L-CSA acts on a glutamate-insensitive receptor suggests that different receptor families might exist for different EAAs.

L-CSA is an EAA that fulfills most of the criteria for serving as a neurotransmitter in mammalian brain (for reviews, see Refs. 1–3). The necessary enzymes for L-CSA synthesis and degradation are present in nerve endings and L-CSA is released in a calcium-dependent manner upon depolarization. Furthermore, L-CSA is a substrate for a high affinity Na^+-dependent uptake system. L-CSA is structurally similar to L-glutamate and L-aspartate and has long been known to have excitotoxic effects similar to those of these EAA neurotransmitters. In addition, L-CSA induces a variety of physiological and biochemical responses (for reviews, see Refs. 1–3). However, L-CSA cross-reacts with iGluRs (4, 5) and has recently been shown to activate some mGluRs in immature brain (6). It has been proposed that L-CSA may also activate non-glutamate receptors (for review, see Ref. 3). However, the effects of L-CSA that have been fully characterized are mediated by cross-activation of L-glutamate receptors, and no receptors that are selectively activated by L-CSA have been characterized. Consequently, the question of whether L-CSA is an endogenous neurotransmitter with actions independent of those mediated by L-glutamate receptors has yet to be resolved.

We have identified an EAA receptor that is coupled to activation of PLD and is insensitive to 1 mM L-glutamate (7). PLD catalyzes the breakdown of membrane phospholipids, particularly PC. PC hydrolysis yields PA and free choline (for reviews, see Refs. 8–10). PA, a potential second messenger compound, is converted by phosphatidate phosphohydrolase to DAG. DAG activates protein kinase C and plays a key role in signal transduction. The activation of various neurotransmitter receptors has been shown to increase DAG, but such receptors identified to date are coupled to phospholipase C, which hydrolyzes phosphoinositides to produce DAG. The activation of novel receptors coupled to the PLD-catalyzed metabolism of PC could potentially induce much higher levels of DAG than those associated with phospholipase C-coupled phosphoinositide hydrolysis, because PC is the major phospholipid component of the cell membrane.

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ABBREVIATIONS: L-CSA, L-cysteine sulfinic acid; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; PC, phosphatidylcholine; PA, phosphatidic acid; DAG, diacylglycerol; ACPD, amino-1,3-cyclopentanedicarboxylic acid; PET, phosphatidylethanol; KRB, Krebs' Ringer bicarbonate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-APV, D-2-amino-5-phosphonopentanoic acid; L-HCA, L-homocysteic acid; L-CCG-I, (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine; L-AP3, L-2-amino-3-phosphonopropionic acid; CHPG, 4-carboxy-3-hydroxyphenylglycine; MCPG, α-methyl-4-carboxyphenylglycine; 4-CPG, 4-carboxyphenylglycine; VIP, vasoactive intestinal peptide; 2-CA, 2-chloroadenosine; ISO, isoproterenol; PLD, phospholipase D; EAA, excitatory amino acid; NE, norepinephrine.

The PLD-coupled receptor is activated by (1*S*,3*R*)-ACPD in hippocampus. Because (1*S*,3*R*)-ACPD is a selective mGluR agonist, this receptor was initially assumed to be a subtype of mGluR (7, 11). However, the insensitivity of this receptor to 1 mM L-glutamate (in the presence of iGluR antagonists) in adult rat hippocampus suggested that it could be a receptor for some other endogenous EAA. We now present evidence that another putative neurotransmitter, L-CSA, may serve as an endogenous agonist of the PLD-coupled metabotropic EAA receptor.

Materials and Methods

Measurement of agonist-induced activation of PLD. Agonist-induced increases in PLD activity were measured using the protocol of Llahi and Fain (12), modified for use with hippocampal slices (7). PLD catalyzes the metabolism of phospholipids, resulting in the formation of PA. However, exogenously added ethanol preferentially substitutes for water in the PLD-catalyzed transphosphatidylation reaction, transferring ethanol to the phosphatidyl moiety, and PET forms in place of PA. It is generally accepted that agonist-induced PET formation is indicative of agonist-induced PLD activity (for review, see Ref. 13).

Cross-chopped hippocampal slices (350 × 350 μm) from three to five adult male Sprague-Dawley rats were incubated for 15 min in KRB and then rinsed well with warmed KRB. Unless otherwise noted, all incubations were carried out in an atmosphere of 95% O_2/5% CO_2 in a shaking water bath at 37°. Slices were incubated for 2.5–3 hr in the presence of [^{32}P]orthophosphate, at a final concentration of 60–100 μCi/ml, in 10–15 ml of KRB. They were then rinsed thoroughly with KRB. Gravity-packed slices (25 μl) were added to tubes containing the iGluR antagonists CNQX (50 μM) and D-APV (100 μM), in KRB, and were incubated for 15 min. In some assays, other receptor antagonists were added as well. Receptor agonists and ethanol (final concentration, 170 mM) were added, and the slices were incubated for an additional 30 min. All experiments were performed in triplicate. Control sets of triplicate samples were included in which antagonists or agonists were replaced with KRB. In addition, one set of triplicate samples was always included in which ethanol was omitted and replaced by KRB. The reaction was stopped with 1.2 ml of chloroform/methanol (1:2, v/v).

Equal volumes (500 μl) of chloroform and methanol were added, and the tubes were mixed thoroughly. Low-speed centrifugation was used to separate the phases. Five hundred microliters of the organic phase were dried under N_2 and then 10 μl of chloroform/methanol (9:1, v/v) were added to resuspend each sample. Samples were spotted onto silica gel HL plates (Analtech, Newark, DE), and PET was separated from other phospholipids using a solvent system of chloroform/methanol/acetic acid (6:15:2, by volume). PET standards were spotted onto each plate in every assay. In addition, some assays included standards for PA and PC, which were used to verify that the chromatography protocol separated these compounds from PET. Spots containing ^{32}P radioactivity were identified by autoradiography. The plates were exposed to iodine to reveal the positions of phospholipid samples and standards. For each sample (including the control samples in which ethanol was excluded) the spot in the position corresponding to [^{32}P]PET was scraped into a scintillation vial. All other radioactive spots were scraped into a second vial. Scintillation fluid was added to the vials and the radioactivity was counted. The result for each individual sample was analyzed as the percentage of total ^{32}P radioactivity incorporated into phospholipids that was converted to [^{32}P]PET. PLD activity was expressed as [([^{32}P]PET/total ^{32}P-phospholipids) × 100].

The radioactivity comigrating with [^{32}P]PET, expressed as [([^{32}P]PET/total ^{32}P-phospholipids) × 100], in the ethanol-free controls ranged from 0.03 to 0.28, with a mean ± standard error of 0.15 ± 0.01 for 65 assays. This was considered to be background radioactivity that failed to rinse out of the tissue slices, because it is well established that PET forms only in the presence of ethanol (for review, see Ref. 13). This background radioactivity was subtracted from the mean determined for each ethanol-containing triplicate set.

Measurement of agonist-induced phosphoinositide hydrolysis. Phosphoinositide hydrolysis was assayed in cross-chopped (350- × 350-μm) hippocampal slices from adult male Sprague-Dawley rats by measuring the formation of [^{3}H]inositol monophosphates from [^{3}H]phosphatidylinositol (prelabeled by incorporation of [^{3}H]inositol), as described previously (14, 15). The iGluR antagonists CNQX (50 μM) and D-APV (100 μM) were applied for 15 min in all experiments, and then receptor agonists (or KRB in control samples) were applied for an additional 45 min. All incubations were performed at 37°. Data are presented as the ratio of radioactivity (mean ± standard error) in [^{3}H]inositol monophosphates from triplicate samples of slices treated with L-CSA or (1*S*,3*R*)-ACPD (in KRB) to radioactivity in [^{3}H]inositol monophosphates from triplicate samples of control slices containing KRB without receptor agonists.

Measurement of agonist-induced cAMP accumulation. cAMP accumulation was determined using the method of Shimizu *et al.* (16), with some modifications (17–19). The conversion of [^{3}H]adenine to [^{3}H]cAMP in the presence of various receptor agonists was measured in cross-chopped (350- × 350-μm) hippocampal slices from adult male Sprague-Dawley rats. Slices were initially incubated for 15 min in KRB at 37°, rinsed with warmed KRB, and incubated for an additional 40 min in 15 ml of KRB containing 30 μCi of [^{3}H]adenine and 10 μl of unlabeled adenine. After several rinses with warmed KRB, 25-μl aliquots of gravity-packed slices were added to incubation tubes containing fresh KRB. CNQX (50 μM) and D-APV (100 μM) were added to all samples (to block iGluRs) before the addition of receptor agonists, and the tissue was incubated for 15 min at room temperature. Triplicate samples containing receptor agonists or appropriate controls were incubated for 15 min at 37°. The final volume in all tubes was 500 μl. All incubations were conducted in an atmosphere of 95% O_2/5% CO_2. The reaction was stopped by addition of 50 μl of 77% trichloroacetic acid and 25 μl of 10 mM cAMP (added as a carrier). The tissue was homogenized and subjected to centrifugation (15 min, 37,000 × *g*). A 25-μl aliquot of the supernatant was added to 4 ml of scintillant and used for the determination of incorporation of radioactivity into the tissue. [^{3}H]cAMP in the remaining supernatant was determined from sequential elution through Dowex and then alumina columns. Data were expressed as percentage conversion (mean ± standard error) of the [^{3}H]adenine incorporated into the slices to [^{3}H]cAMP.

Materials. The EAA receptor ligands ibotenate, quisqualate, L-CCG-I, (1*S*,3*R*)-ACPD, L-HCA, L-AP3, (*S*)-CHPG, (*R*)-CHPG, (*RS*)-MCPG, (*S*)-4-CPG, β-*N*-methylamino-L-alanine, CNQX, and D-APV were obtained from Tocris Neuramin (Bristol, UK). L-Glutamate, L-aspartate, L-CSA, L-proline, L-serine-*o*-phosphate, adenine, VIP, 2-CA, ISO, NE, and tetrodotoxin were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipid standards were purchased from Avanti Polar Lipids (Pelham, AL). [^{32}P]Orthophosphate (carrier free, 8800 Ci/mmol) was obtained from DuPont/NEN (Boston, MA). [^{3}H]Inositol and [^{3}H]adenine were purchased from American Radiolabeled Chemicals (St. Louis, MO).

Results

L-CSA selectively activates the PLD-coupled receptor. In a previous report, we showed that 1 mM L-glutamate does not increase PLD activity in adult hippocampal slices (7). However, previous studies indicated that 1 mM L-glutamate has relatively little effect on mGluR-mediated increases in phosphoinositide hydrolysis in adult hippocampal slices (20, 21). This is likely due to the presence of uptake systems that lower the effective concentration of L-glutamate. At higher concentrations (10 mM), L-glutamate saturates these uptake mechanisms and elicits a robust phosphoinositide hydrolysis response (for review, see Ref. 22). Thus, we compared the phosphoinositide hydrolysis and PLD responses to 1 mM and 10 mM L-glutamate, to determine whether L-glutamate induces a PLD response at concentrations that are clearly effective at activat-

ing phosphoinositide hydrolysis-coupled mGluRs. Consistent with previous findings, L-glutamate elicited a small but significant phosphoinositide hydrolysis response at 1 mM and a robust response at 10 mM (Fig. 1). In contrast, neither 1 mM nor 10 mM L-glutamate increased PLD activity in the adult rat hippocampus (Fig. 1). Thus, it is unlikely that L-glutamate uptake is solely responsible for the lack of sensitivity of the PLD-coupled receptor to L-glutamate. Consistent with this, we found that 1 mM L-glutamate did not increase PLD activity in the presence of the L-glutamate uptake blocker *trans*-pyrrolidine-2,4-dicarboxylic acid (100 μM) (data not shown.)

The finding that L-glutamate does not act at the PLD-coupled receptor in adult rat hippocampal slices brings into question the hypothesis that L-glutamate is the endogenous agonist of this receptor in adults. Thus, we investigated the effects of other putative EAA neurotransmitters on the PLD-coupled receptor. A number of EAAs elicit physiological or biochemical responses in the central nervous system, and it is possible that one or several of them could activate PLD-coupled EAA receptors. Of these EAAs, L-glutamate, L-aspartate, L-CSA, and L-HCA have been shown to fit most of the criteria for serving as neurotransmitters in the brain (for reviews, see Refs. 3 and 23–25). Furthermore, all of these EAAs are released upon stimulation of Schaffer collaterals (26, 27), which form synapses with hippocampal CA1 pyramidal cells. We found that 1 mM L-CSA induced a significant increase in PLD activity in hippocampal slices, whereas 1 mM concentrations of L-glutamate, L-aspartate, and L-HCA were without effect (Fig. 2, *inset*). L-CSA elicited a dose-dependent increase in PLD activity in rat hippocampal slices in the presence of iGluR antagonists, with an approximate EC_{50} of 500 μM (Fig. 2). The PLD response induced by 1 mM L-CSA was not significantly decreased in the presence of 1 μM tetrodotoxin, suggesting that this response is not dependent upon L-CSA-induced increases in cell firing (data not shown).

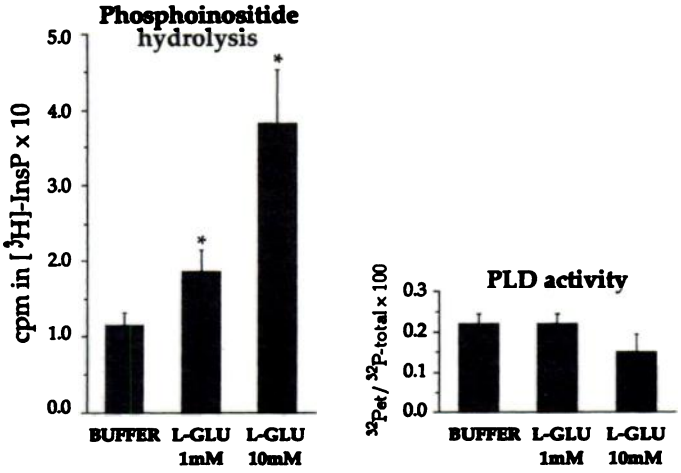

Fig. 1. L-Glutamate stimulates phosphoinositide hydrolysis but does not increase PLD activity in slices from adult rat hippocampus. *Left*, the turnover of membrane phosphoinositides was assayed in the absence and presence of L-glutamate (*L-GLU*) (1 mM and 10 mM). Data are represented as means ± standard errors for four separate experiments, each performed in triplicate. *, $p < 0.025$ (paired *t* test). *Right*, PLD activity was measured in the presence and absence of L-glutamate (1 mM and 10 mM). PLD activity was expressed as [^{32}P]PET/^{32}P-total × 100, where ^{32}P-total includes all ^{32}P-labeled phospholipids. Data are represented as means ± standard errors for three separate experiments, each performed in triplicate. The iGluR antagonists CNQX (50 μM) and D-APV (100 μM) were included in these and all subsequent experiments.

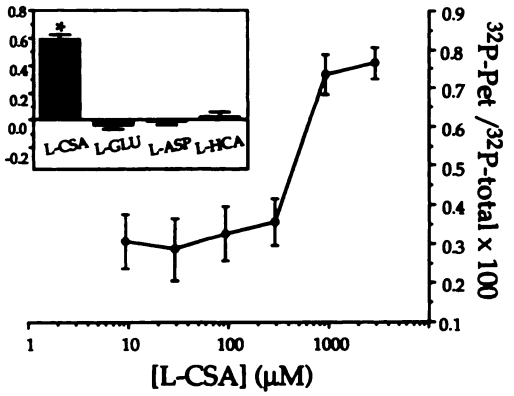

Fig. 2. L-CSA increases PLD activity in a concentration-dependent manner. PLD activity was expressed as in Fig. 1. Basal PLD activity was 0.29 ± 0.06 (five experiments). *Inset*, effect of 1 mM concentrations of L-CSA, L-aspartate (*L-ASP*), and L-HCA on PLD activity, compared with basal levels. For experiments using L-CSA and L-HCA, basal PLD activity was 0.36 ± 0.02 (four experiments); for those using L-glutamate (*L-GLU*) and L-aspartate, basal PLD activity was 0.38 ± 0.03 (three experiments). Data are represented as means ± standard errors for three or four separate experiments, each performed in triplicate. For *inset* only, data were normalized by subtraction of basal PLD activity. *, $p < 0.001$ (paired *t* test).

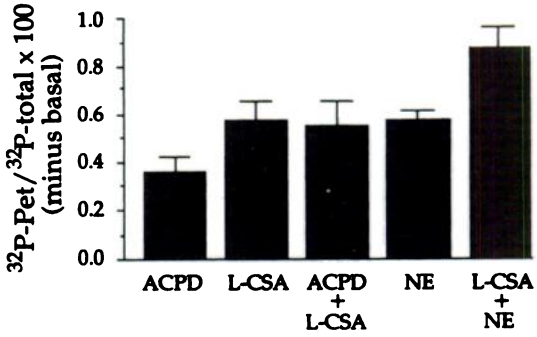

Fig. 3. PLD reponses elicited by (1*S*,3*R*)-ACPD (100 μM) and L-CSA (1 mM), and NE (100 μM) are not additive. The PLD responses induced by coapplication of (1*S*,3*R*)-ACPD and L-CSA did not differ significantly from the greater response (L-CSA alone). In contrast, the PLD response to L-CSA and NE (100 μM) together was significantly greater than the response elicited by either L-CSA ($p < 0.01$) or NE ($p < 0.02$) (paired *t* tests). Data are represented as means ± standard errors of four experiments, each performed in triplicate. Basal PLD activity (0.28 ± 0.02) was subtracted from all values presented.

The PLD-coupled receptor is pharmacologically distinct from known iGluRs and mGluRs. The L-CSA-sensitive receptor coupled to PLD activation is not blocked by selective iGluR antagonists or activated by selective iGluR agonists (7), suggesting that it is pharmacologically distinct from members of the iGluR family. However, the mGluR agonist (1*S*,3*R*)-ACPD increases PLD activity in a manner similar to that of L-CSA (7). The PLD responses elicited by 1 mM L-CSA and 100 μM (1*S*,3*R*)-ACPD were not additive (Fig. 3). In contrast, the PLD responses to 1 mM L-CSA and 100 μM NE were partially additive (Fig. 3). Although these data do not completely rule out the possibility that (1*S*,3*R*)-ACPD and L-CSA activate distinct receptor subtypes, these data are consistent with the hypothesis that these compounds act at the same receptor.

Because the PLD response was activated by (1*S*,3*R*)-ACPD, the effects of other compounds known to interact with mGluRs were examined. PLD was activated by a variety of mGluR agonists, as well as the putative mGluR antagonist L-AP3 (Fig.

4). The rank order of potency of agonists at stimulating this response was quisqualate > L-CCG-I > (1*S*,3*R*)-ACPD > L-AP3 = ibotenate > L-CSA. These data suggest that the overall agonist profile of the L-CSA-sensitive receptor is similar to that of the mGluRs. However, this profile is clearly different from that of any specific mGluR studied to date (for review, see Ref. 28).

Recent studies have demonstrated that various phenylglycine derivatives are antagonists or weak agonists of mGluRs (29–32). For instance, MCPG competitively blocks (1*S*,3*R*)-ACPD-stimulated phosphoinositide hydrolysis and a variety of mGluR-mediated electrophysiological responses (31, 32). In contrast, MCPG (1 mM) was virtually inactive as an antagonist of the PLD-coupled receptor. Other phenylglycine derivatives examined [(*R*)-CHPG and (*S*)-CHPG, each at 1 mM] slightly decreased the PLD response to L-CSA but also slightly increased basal PLD activity (data not shown), suggesting that they may have weak partial agonist or antagonist activity. However, these effects were not statistically significant ($p < 0.05$). (*S*)-4-CPG (1 mM), which competitively antagonizes mGluR-mediated increases in phosphoinositide hydrolysis and thalamic cell firing (31, 32), slightly but significantly ($p < 0.05$, paired *t* test) reduced the PLD response elicited by L-CSA (data not shown). However, (*S*)-4-CPG also increased basal PLD activity, suggesting that it may have partial agonist effects. Thus, the overall effects of the phenylglycine derivatives were minimal, even at a fairly high concentration (1 mM), suggesting that these compounds are unlikely to be of practical value as antagonists at the PLD-coupled receptor.

In addition to the phenylglycine derivatives, a wide variety of EAAs and EAA analogs were tested for their ability to antagonize the PLD response elicited by (1*S*,3*R*)-ACPD. Most of these compounds had little or no effect on PLD activity, but β-*N*-methylamino-L-alanine, L-serine-*o*-phosphate, and L-proline (all at 1 mM) inhibited the (1*S*,3*R*)-ACPD-induced PLD response. However, each of these compounds also inhibited the PLD response elicited by 100 μM NE and/or decreased basal PLD activity, which suggests that their inhibition of PLD activity was probably not mediated by specific actions on EAA receptors (data not shown).

L-CSA-induced activation of PLD is developmentally regulated. Although L-glutamate does not activate the PLD-coupled receptor in adult animals, previous reports suggested that L-glutamate does elicit this response in slices from immature animals (11). Thus, we compared the effect of L-CSA with that of L-glutamate in hippocampal slices from 9–10-day-old rats, to determine whether L-CSA is more efficacious than glutamate in immature animals. Consistent with previous experiments (11), L-glutamate (1 mM) elicited a significant ($p < 0.05$) increase in PLD activity in hippocampal slices from immature rats (610 ± 160% of basal). However, L-CSA and (1*S*,3*R*)-ACPD also induced significantly larger increases in PLD activity in slices from 9–10-day-old animals (1100 ± 300 and 660 ± 180% of basal, respectively) than in slices from adults (350 ± 30 and 240 ± 10%, respectively). These data suggest that the PLD-coupled metabotropic EAA receptor undergoes a decline during postnatal development, resembling the developmental regulation of various mGluRs (20, 33–35). Thus, although L-glutamate may activate the PLD-coupled receptor in immature animals, L-CSA is the most efficacious endogenous agonist at both developmental stages. This is consistent with the hypothesis that L-CSA is the primary endogenous agonist of this receptor.

L-CSA does not significantly activate mGluRs coupled to phosphoinositide hydrolysis or potentiation of cAMP responses. The similarity of the agonist profile of the L-CSA-sensitive PLD-coupled receptor to those of the mGluRs suggests that this receptor may belong to the same superfamily as the mGluRs. Although L-glutamate is clearly an agonist of the mGluRs that have been characterized thus far, the possibility exists that L-CSA may also be an endogenous mGluR agonist. Therefore, we determined the effects of L-CSA on phosphoinositide hydrolysis and the potentiation of cAMP responses to agonists of receptors that are positively coupled to adenylate cyclase via G_s. Both of these are well characterized mGluR-mediated responses that are activated by (1*S*,3*R*)-ACPD in hippocampal slices. Consistent with previous reports (36, 37), (1*S*,3*R*)-ACPD induced a concentration-dependent increase in phosphoinositide hydrolysis in hippocampal slices from adult animals. In contrast, L-CSA only slightly increased phosphoinositide hydrolysis at 1 mM (but not at 3.2 mM), and this effect was not statistically significant (Student's *t* test) (Fig. 5).

Also consistent with previous studies (19, 35), (1*S*,3*R*)-ACPD (100 μM) markedly potentiated VIP (100 nM)-, 2-CA (10 μM)-,

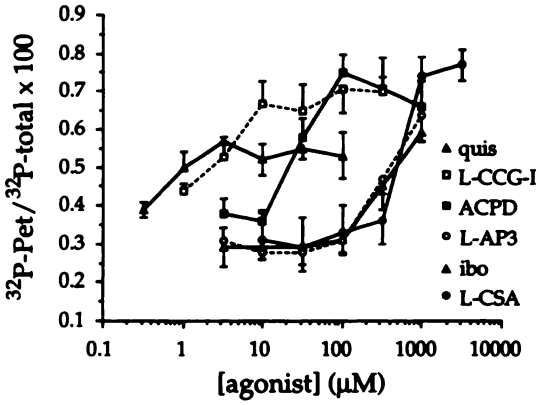

Fig. 4. A variety of mGluR agonists activate the PLD-coupled L-CSA receptor. Dose-responses curves for PLD activation are illustrated for quisqualate (*quis*), L-CCG-I, (1*S*,3*R*)-ACPD, L-AP3, ibotenate (*ibo*), and L-CSA. The respective basal PLD levels (mean ± standard error) were 0.36 ± 0.03 (three experiments), 0.43 ± 0.01 (three experiments), 0.34 ± 0.04 (four experiments), 0.31 ± 0.03 (five experiments), 0.29 ± 0.06 (three experiments), and 0.30 ± 0.04 (three experiments).

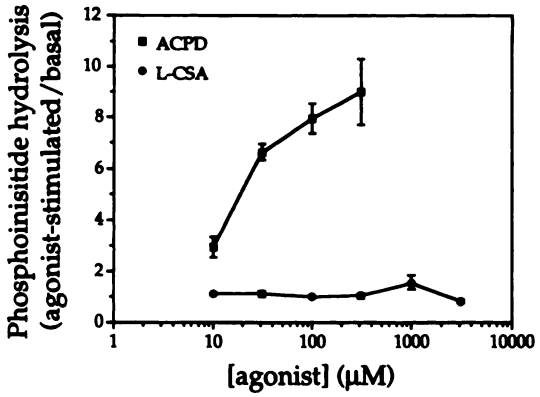

Fig. 5. L-CSA is not an agonist of phosphoinositide hydrolysis-coupled mGluRs in adult rat hippocampus. Data are presented as radioactivity in [^{3}H]inositol monophosphate extracted from slices incubated in the presence of added EAAs [L-CSA or (1*S*,3*R*)-ACPD] divided by radioactivity in [^{3}H]inositol monophosphate extracted from slices incubated in the absence of added EAAs (i.e., drug/no drug). Data are means ± standard errors for three experiments, each performed in triplicate.

and ISO (10 μM)-induced increases in cAMP accumulation. However, L-CSA (1 mM) did not mimic the actions of (1*S*,3*R*)-ACPD on cAMP responses to any of these receptor agonists (Fig. 6).

Because ethanol was routinely included in the PLD assay but not the assays for phosphoinositide hydrolysis and cAMP formation, control experiments were performed in which agonist-induced phosphoinositide hydrolysis and cAMP accumulation were measured in the presence of 170 mM ethanol (the concentration used in PLD assays). L-CSA had a similar lack of effect on phosphoinositide hydrolysis and the potentiation of cAMP responses in the presence or absence of ethanol (data not shown).

Discussion

Taken together with evidence that L-CSA fits most of the criteria for a neurotransmitter in brain, the data reported here suggest that L-CSA is an endogenous agonist of the hippocampal PLD-coupled receptor. L-CSA has a fairly low potency (EC_{50} = 500 μM) for activating PLD in adult hippocampus, which is reminiscent of the low potency of L-glutamate at mGluRs in brain slices from adult rats, relative to slices from 1–2-week-old pups (for review, see Ref. 22). Because L-glutamate and L-CSA are thought to share a common transporter (38), the low potency of L-CSA in adult brain, like that of L-glutamate, is likely to be partially due to high affinity L-CSA uptake.

Unlike L-CSA, L-glutamate and several other endogenous EAAs that are released from synaptic terminals in the hippocampus do not elicit a PLD response in adult rats. We found that the PLD-coupled receptor was not activated by the putative endogenous EAA receptor agonist L-glutamate even at 10 mM, a concentration that overcomes L-glutamate uptake and evokes a marked phosphoinositide hydrolysis response in brain slices from adult rats. In addition, we found that 1 mM L-glutamate did not increase PLD activity in the presence of the L-glutamate uptake blocker *trans*-pyrrolidine-2,4-dicarboxylic acid. These results suggest that the PLD-coupled receptor is insensitive to L-glutamate in adult hippocampus. It should be noted that Holler *et al.* (11) reported that 1 mM L-glutamate plus a glutamate uptake blocker, α-hydroxy-β-hydroxamate, induced a slight increase in PLD activity in adult rat hippocampus, relative to basal levels. However, α-hydroxy-β-hydroxamate elicited a slight PLD response when added alone, and those authors did not demonstrate that the response to L-glutamate plus α-hydroxy-β-hydroxamate was greater than the response elicited by α-hydroxy-β-hydroxamate alone. Thus, the interpretation of these data is difficult.

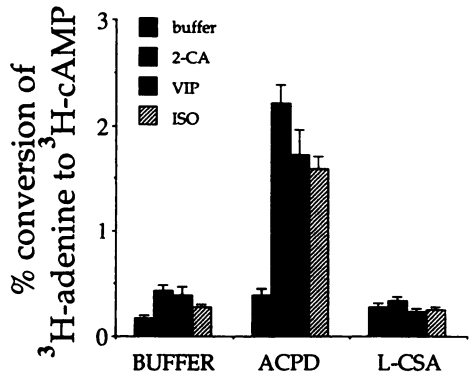

Fig. 6. L-CSA does not potentiate the cAMP responses induced by agonists of other receptors. Accumulation of cAMP induced by 2-CA (10 μM), VIP (100 nM), or ISO (10 μM) was measured in the absence of EAA agonists (*BUFFER*) or in the presence of (1*S*,3*R*)-ACPD (100 μM) or L-CSA (1 mM). Data are expressed as the percentage of radioactivity incorporated into slices that was converted to [^{3}H]cAMP (percentage conversion) and are shown as means ± standard errors of four separate experiments, each performed in triplicate.

The PLD-coupled L-CSA receptor is activated by a variety of mGluR agonists (for reviews, see Refs. 28 and 39); however, there are clear differences between the agonist profile of the PLD-coupled receptor and those of the mGluRs characterized to date. L-AP3 is a highly efficacious agonist of the receptor mediating the PLD response. Similarly, L-AP3 is an agonist of the mGluR that mediates an inhibition of forskolin-stimulated cAMP accumulation in brain slices. However, that mGluR (40) is clearly activated by 1 mM L-glutamate in adult hippocampal slices and is much less sensitive to quisqualate than is the PLD-coupled receptor. L-AP3 has little effect on the (1*S*,3*R*)-ACPD stimulation of phosphoinositide hydrolysis mediated by cloned mGluRs or on most electrophysiological responses elicited by the activation of mGluRs by (1*S*,3*R*)-ACPD. Furthermore, L-AP3 is a partial agonist or an antagonist of (1*S*,3*R*)-ACPD-induced increases in phosphoinositide hydrolysis and of some previously characterized mGluRs (for review, see Ref. 28). In addition, the PLD-coupled receptor is relatively insensitive to a series of phenylglycine derivatives that are antagonists or weak partial agonists of a variety of mGluRs.

L-CSA does not appear to be a general mGluR agonist, because it has little effect on mGluRs coupled to phosphoinositide hydrolysis or potentiation of cAMP responses in hippocampal slices from adult rats. A recent study provides evidence that L-CSA activates mGluRs coupled to phosphoinositide hydrolysis in cortical slices from 6-day-old rat pups, with an EC_{50} of 471 μM (6). This is likely due to the profound developmental regulation of mGluRs coupled to phosphoinositide hydrolysis and the robust response to even low-efficacy agonists that can be measured in slices from immature animals.

L-CSA appears to be an efficacious agonist of the PLD-coupled receptor in immature rat hippocampus. In agreement with previous findings (11), other EAAs, including L-glutamate, also induce substantial PLD responses in hippocampal slices from 9–10-day-old pups. However, these EAAs are not as efficacious as L-CSA in stimulating the PLD response in slices from immature animals. The simplest explanation for these results is that there is a single subtype of PLD-coupled EAA receptor that undergoes a developmental decline. Alternatively, the relative uptake or metabolism of L-glutamate and L-CSA, rather than the PLD-coupled receptor, might be developmentally regulated. It is also possible that multiple subtypes of PLD-coupled receptors exist, including a juvenile form that is activated by L-glutamate and is not present in adults.

Our results are consistent with the hypothesis that L-CSA is a neurotransmitter in the brain and serves as an endogenous agonist of a novel receptor that is coupled to activation of PLD. The finding that L-glutamate does not significantly activate the PLD-coupled receptor in brain slices from adults does not necessarily imply that glutamate is not an endogenous agonist of this receptor. This is especially true in immature animals, where L-glutamate elicits a significant PLD response. However, the data reported here, taken together with the large body of literature implicating L-CSA as a neurotransmitter in the hippocampus, suggest that L-CSA is more likely than L-glutamate

to be the primary endogenous agonist of a PLD-coupled metabotropic EAA receptor in adult rats. A common conception regarding EAA neurotransmitter function is that all EAAs act on members of an EAA receptor family that is composed of various iGluRs and mGluRs, all of which use L-glutamate as the primary endogenous agonist. The present finding that L-CSA may act on a glutamate-insensitive receptor suggests that specific receptor families might exist for different endogenous EAAs. Thus, the actions of various EAAs could be more diverse than was previously appreciated.

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Send reprint requests to: P. Jeffrey Conn, Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322.