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1-AMINO-APDC, A PARTIAL AGONIST OF GROUP II METABOTROPIC GLUTAMATE RECEPTORS WITH NEUROPROTECTIVE PROPERTIES

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Abstract: The synthesis of the 1-amino derivative of (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid (1-amino-APDC), a selective metabotropic glutamate ligand, is disclosed. This compound acts as a partial agonist of the group II mGluRs and shows pronounced neuroprotective properties in the NMDA model of cell toxicity.

The amino acid glutamate plays a pivotal role in biological processes ranging from memory and learning to neuronal degeneration. This major excitatory amino acid (EAA) acts through disparate glutamate receptors, which can be categorized into two distinct types, the so-called ionotropic receptors and the metabotropic receptors.[1] The ionotropic glutamate receptors, or iGluRs, are associated with integral cation-specific ion channels and include the *N*-methyl-D-aspartate (NMDA), 2-amino-3-(5-methyl-3-hydroxyisoxazol-4-yl)propanoic acid (AMPA), and kainate subtypes. On the other hand, the metabotropic receptors are coupled to cellular effectors through GTP-binding proteins. The metabotropic glutamate receptors, or mGluRs, have been distinguished pharmacologically from the iGluRs by the use of the mGluR-selective agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) generally through measurements involving phosphoinositide hydrolysis or Ca^{2+} mobilization. To date the use of expression cloning techniques has led to the identification of eight mGluR subtypes, which have been placed into three major categories based on their molecular structure, signal transduction mechanisms, and pharmacological properties. Group I mGluRs (mGluR1 and 5) are coupled to phosphoinositide (PI) hydrolysis, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) are negatively linked to adenylyl cyclase activity. The group I receptors are more sensitive to quisqualic acid than they are to ACPD, the group II receptors are more sensitive to ACPD than to quisqualic acid, and the group III receptors are most sensitive to 2-amino-4-phosphonobutyric acid (L-AP4).[2]

In order to better characterize the roles of GluRs in physiological processes,[3-5] there is an important need to identify novel, high affinity ligands that are family and subtype specific.[6] While a number of mGluR selective compounds have been described to date, such as LY354740,[7] ABHxD-I,[8] and 1-benzyl-APDC,[9] the goal of having agonists, partial agonists, and antagonists of exquisite selectivity for each of the known subtypes has not been achieved. Herein we report on a derivative of (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid (APDC), namely its 1-amino derivative, which acts as a partial agonist of group II receptors. As APDC itself is a full agonist of the group II receptors,[7] the present finding opens up a new avenue for mGluR compound design.

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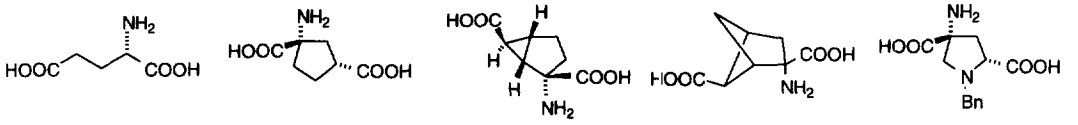
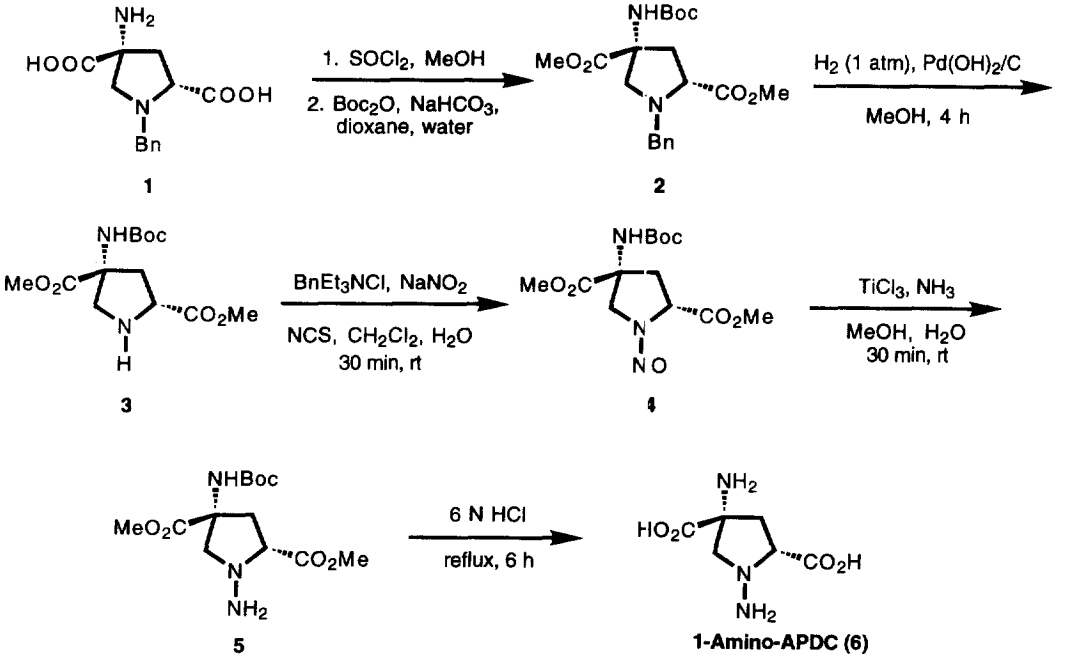


Some typical ligands exhibiting mGluR activity.

Chemistry

We have previously reported a synthesis of the 1-benzyl derivative of (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid (**1**)[9] using *cis*-4-hydroxy-D-proline as the starting material (Scheme 1). Esterification of this compound with thionyl chloride and methanol followed by reaction with di-*tert*-butyl dicarbonate gave the desired methyl ester intermediate **2**. This fully protected pyrrolidine **2** was debenzylated by hydrogenolysis using $Pd(OH)_2/C$ as catalyst. The free amine **3** was readily converted to the corresponding *N*-nitrosamine in high yield under phase-transfer conditions by means of sodium nitrite and *N*-chlorosuccinimide.[10] Following a published reduction procedure, **4** was converted to the corresponding hydrazine **5** using aqueous titanium trichloride.[11] Lastly, the final deprotection step was accomplished using 6N HCl to deliver 1-amino-APDC (**6**).

Scheme 1. Synthesis of 1-Amino-APDC from 1-Benzyl-APDC



Pharmacological Evaluation and Results

The activity of amino-APDC was tested in cell lines expressing the individual subtypes of metabotropic glutamate receptors (mGluRs). Chinese hamster ovary (CHO) cells were used to produce cell lines expressing mGluR1a and mGluR5a (group I), mGluR2 (group II), and mGluR6 (group III) receptors as well as the chimeric receptor mGluR3/1a which combines the pharmacological properties of mGluR3 (group II) with the activation of phospholipase C.[12] The mGluR4 receptor (group III) was expressed in baby hamster kidney (BHK) cells.

The activity at phospholipase C-coupled receptors (mGluR1a, mGluR5a, and mGluR3/1a) was determined by measurements of their ability to increase the hydrolysis of membrane phosphoinositides.[13] CHO cells expressing mGluR1, mGluR5 or mGluR3/1a cultured in 96-well plates, were incubated overnight in glutamine-free culture medium supplemented with 0.75 μCi [^{3}H]*myo*-inositol to label the cell membrane phosphoinositides. Before the experiments, cells were washed 2 times with 0.5 mL of Locke's solution (156 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.6 mM glucose, and 20 mM Hepes, pH 7.4) followed by addition of 20 mM LiCl to block the degradation of inositol phosphates. The receptor ligands were added, and the cells were incubated for 40 min at 37°C. The reaction was terminated by aspiration of the medium, and the inositol phosphates were extracted for 10 min with 0.5 mL of 0.1 M HCl. The separation of [^{3}H]inositol phosphates was performed by anion exchange chromatography. To the collected fractions was added 10 mL of scintillation fluid, and the radioactivity was measured.

The activity at receptors coupled negatively to adenylyl cyclase (mGluR2, mGluR4, mGluR6) was determined by measurement of their ability to decrease the forskolin-induced elevation of cAMP formation.[12] Cells expressing these receptors were cultured on 96-well culture plates. Before experiments, cells were washed three times and preincubated 10 min at 37°C in Locke's medium containing 300 μM isobutylmethylxanthine to inhibit the activity of phosphodiesterases which degrade cAMP. Then 5 μM forskolin was added without or with the mGluR agonists, and the incubation was continued for 10 min. After the incubation, the medium was rapidly aspirated, and the cAMP was extracted for 10 min with 0.1 M HCl and measured by radioimmunoassay using a magnetic Amerlex RIA kit.

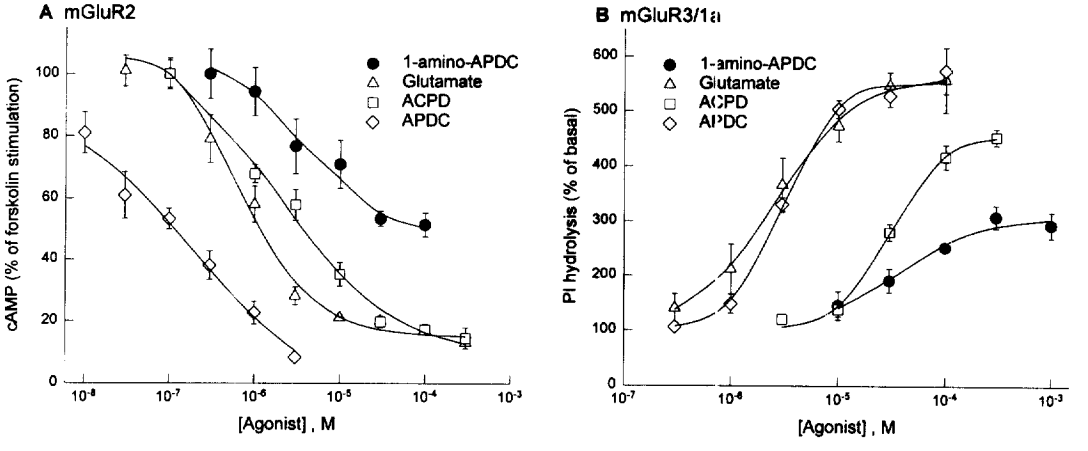


Figure 1. Dose response curves for 1-amino-APDC and reference compounds in CHO cells expressing mGluR2 (**A**) and mGluR3/1a (**B**) receptors. Values are means ± SEM from 3-4 experiments.

Table 1. EC_{50} values (μM) for 1-aminoAPDC and other agonists in CHO cells expressing mGluR2 and chimeric mGluR3/1a receptors

	mGluR2	mGluR3/1a
1-amino-APDC	4.8 ± 2.8	52 ± 17
Glutamate	0.61 ± 0.23	2.5 ± 0.41
ACPD	2.1 ± 0.61	30 ± 3.9
APDC	0.20 ± 0.19	3.0 ± 0.38

EC_{50} values were calculated by fitting the normalized data to the logistic equation by non-linear regression. Values are means ± SEM from 3-4 experiments.

Table 2. Effects of 1 mM 1-amino-APDC on group I and group III mGluRs

Receptor		% of maximal activity
Group I	mGluR1a	5 ± 2
	mGluR5a	3 ± 2
Group III	mGluR4a	8 ± 4
	mGluR6	23 ± 15

Values are means ± SEM from 3 experiments and represent % of maximal activity obtained with 1 mM glutamate for mGluR1 and 5, and with 1 mM AP4 for mGluR4 and 6.

The measurements of the activity of 1-amino-APDC at the individual mGluRs showed that this compound activates preferentially group II receptors. Dose-response curves shown in Figure 1 indicate that 1-amino-APDC acts as a partial agonist of both mGluR2 and mGluR3/1a receptors. At both receptors, the maximal stimulation reaches about 50% of the effect obtained with full agonists. This is compared with the agonist action of glutamate, APDC, and (±)-ACPD. The calculated EC_{50} values for 1-amino-APDC and the reference compounds at group II mGluRs are shown in Table 1. Among other mGluRs, 1-amino-APDC showed no activity as agonists or antagonists at group I receptors (mGluR1a and mGluR5a) nor at the group III receptor mGluR4a. However, at 1 mM concentration, a weak partial agonist activity is observed at the mGluR6 receptor (Table 2).

In order to further establish its selectivity, 1-amino-APDC was tested for possible activity at ionotropic glutamate receptors by measuring $^{45}Ca^{2+}$ influx in primary cultures of cerebellar neurons.[14] Cells, cultured in 96-well plates, were incubated for 15 min at room temperature in Locke's buffer containing 1 μCi/ml of $^{45}CaCl_2$ and the indicated additions. Agonist activity at all ionotropic glutamate receptors was measured in Mg^{2+}-free medium including 1 μM glycine to allow for activity at NMDA receptors, and 10 μM cyclothiazide to inhibit desensitization of AMPA receptors. Antagonist activity at NMDA receptors was measured in a Mg^{2+}-free medium in presence of 100 μM NMDA, 1 μM glycine, and 10 μM NBQX to inhibit non-NMDA receptors. Antagonism of kainate action was tested in the presence of 1 mM Mg^{2+}, 50 μM kainate and 1 μM MK-801 to inhibit NMDA receptors, while the effects on AMPA action were measured in the presence of 1 mM Mg^{2+}, 30 μM AMPA, 10 μM cyclothiazide, and 1 μM MK-801. As shown in Table 3, 1-amino-APDC, used at 1 mM concentration, was not an agonist or an antagonist of NMDA, kainate, or AMPA receptors.

Table 3. Effect of 1 mM 1-amino-APDC on the activity of ionotropic glutamate receptors in primary cultures of cerebellar neurons

Addition	Control	1-amino-APDC
	$^{45}Ca^{2+}$ Influx (nmol/mg protein)	
None	2.53 ± 0.25	2.59 ± 0.51
NMDA 100 μM	5.40 ± 0.57	4.56 ± 0.14
Kainate 50 μM	1.82 ± 0.18	1.47 ± 0.11
AMPA 30 μM	2.56 ± 0.21	2.57 ± 0.10

Values are means ± SEM from six measurements performed on two separate preparations of neuronal cultures. Effects of NMDA, kainate, and AMPA are expressed as net stimulation after subtracting the basal values.

Neuroprotective Effects of 1-Amino-APDC

The neuroprotective effects of 1-amino-APDC were tested in primary cultures of mouse cortical neurons plated on a layer of mouse glial cells.[15] Neuronal cell death was induced by the application of 250 μM NMDA for a period of 10 min in the absence or in the presence of the tested drugs. This was followed by a 24 hour incubation in normal culture medium to allow for development of neuronal cell death. Cell death was quantified by measurement of the amounts of lactate dehydrogenase (LDH) released from dying neurons during this 24 hour period. Figure 2 shows the protective effect of 1-amino-APDC against NMDA toxicity. L-CCG-I, a relatively selective agonist of group II mGluRs, was used as a reference compound. Control toxicity refers to the amount of LDH released after the action of NMDA without protective agents. The basal levels of LDH release measured in the absence of the toxic stimulus were subtracted from all the values. As shown in Figure 2, 100 μM 1-amino-APDC provides a neuroprotection against NMDA toxicity similar to that elicited by 30 μM L-CCG-I.

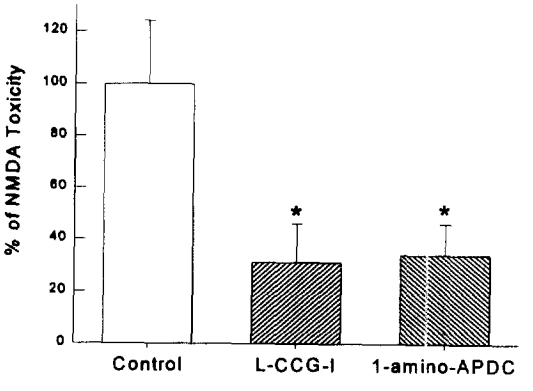


Figure 2. Protective effect of 1-amino-APDC (100 μM) and L-CCG-I (30 μM) against toxicity induced by NMDA (250 μM) in cultures of mouse cortical neurons. Values represent means ± SEM from 8 measurements; *$p<0.05$ by Dunnett's test.

Summary

In summary, 1-amino-APDC is a partial agonist of group II metabotropic glutamate receptors. At both receptors, the maximal stimulation reaches about 50% of the effect obtained with full agonists. The EC_{50} values are 4.8 ± 2.8 μM for mGluR2 and 52 ± 17 μM for mGluR3/1a. The pharmacology of this compound is thus distinct from that of 1-benzyl-APDC, which we had shown previously to be a selective agonist of mGluR6 (EC_{50} = 20 μM) with some weak antagonist activity at mGluR2 and mGluR5.[9] In a model of NMDA-induced neuronal cell death, 1-amino-APDC, acting as an agonist of group II mGluRs, provides a level of neuroprotection which is comparable to that achieved with L-CCG-I. As 1-amino-APDC represents one of the first mGluR ligands to be reported that possesses partial agonist properties, it represents a valuable, new lead structure. As partial agonists are never fully capable of activating their receptor targets, such ligands may offer a safer approach to therapeutic development.[16]

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