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Definition of a Pharmacophore for the Metabotropic Glutamate Receptors Negatively Linked to Adenylyl Cyclase

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Abstract—(2*S*,3*S*,4*S*)-α-Carboxycyclopropylglycine (L-CCG I) and *trans*-1-amino-(1*S*,3*R*)-cyclopentanedicarboxylic acid ((1*S*,3*R*)-ACPD), partially constrained L-glutamate analogs known to be agonists at the metabotropic glutamate receptors (mGluRs) adenylyl cyclase coupled, have been submitted to conformational analysis and the data obtained utilized to define a pharmacophore which takes into account the location of hydrogen bonding donating sites of the receptor. This pharmacophore has been utilized to define the agonist mGluRs↓cAMP bioactive conformation of L-Glu.

Introduction

Excitatory amino acid (EAA) receptors play a crucial role in the synaptic excitation of the mammalian CNS, modifying the efficacy of synapses involved in important functions such as the neuronal plastic changes of the sort that probably underlie learning and memory in the brain of vertebrates.[1] Hyperactivity at EAA receptors, on the other hand, has been associated with the etiology of a vast array of neurological disorders such as Huntington's chorea and dementia of Alzheimer's type. Hence, this receptor family has become the focus of an intense research activity aimed at understanding its overall physiological function. Synaptically released L-glutamate (L-Glu, **1**), the major excitatory neurotransmitter, acts on two receptor families. The first one, termed ionotropic since it contains a cation specific ion channel, has been further subdivided in three main receptor subtypes, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) and kainate (KA). The NMDA receptor complex is the best characterized, also due to the early availability of selective agonists and antagonists. Conformationally restricted L-Glu analogs such as *cis*-(2*S*,3*R*,4*S*)-α-carboxycyclopropylglycine (L-CGAC)[2] and 4-hydroxy L-pipecolic acid-4-sulfate (L-HPIOS),[3] in particular, have been employed in the definition of a NMDA receptor agonist pharmacophore,[4,5] in the derivation of the NMDA L-Glu bioactive conformation[6] and in the construction of a pseudoreceptor model.[5,7]

The metabotropic glutamate receptor family (mGluRs), first reported in 1986,[8-10] has been shown to be functionally and physiologically different from the ionotropic one although being also involved in the activation of the biochemical machinery leading to neuronal plasticity.[11,12] It has recently been reported, in this connection, that the stimulation of the mGluRs present in the cerebrocortical nerve terminals enhances glutamate Ca-dependent release especially when the neurotransmitter release is studied in the presence of arachidonic acid.[13] In contrast, other reports based on electrophysiological studies seemed to suggest that stimulation of the mGluRs induces inhibition of the transmitter release at glutamatergic synapses.[14] An explanation for these apparently conflicting results may come from recent molecular cloning studies which have revealed the existence of at least six receptor subtypes, termed mGluR1 to mGluR6.[15] These six mGluRs subtypes differ in their agonist selectivities and signal transduction mechanisms and can be further classified according to their sequence similarities: mGluR1 and mGluR5 (mGluRs↑PI) are coupled to the inositol triphosphate–Ca^{2+} transduction pathway, whereas mGluR2, mGluR3, mGluR4 and mGluR6 (mGluRs↓cAMP) are linked in an inhibitory manner to adenylyl cyclase.

The pharmacological characterization of this receptor family is still far from being satisfactory. Recent electrophysiological studies carried out by Nakanishi[15] have revealed various orders of selectivity toward the mGluRs subtypes by *trans*-(2*S*,3*S*,4*S*)-α-carboxy-cyclopropylglycine ((2*S*,3*S*,4*S*)-CCG I) (**2**),[16] *trans*-1-amino-(1*S*,3*R*)-cyclopentanedicarboxylic acid ((1*S*,3*R*)-ACPD) (**3**)[17] and quisqualate (**4**).

As continuation of our work in the area, we have studied the biological activity of (2*S*,3*S*,4*S*)-CCG I (**2**), (1*S*,3*R*)-ACPD (**3**) and QUIS (**4**) *in vitro* in rat striatal slices by monitoring: (1) the inhibition of forskolin (30 μM) stimulated cAMP accumulation; (2) the formation of [^{3}H]-inositol phosphates. It should be pointed out, in this connection, that the striatum receives a major input from

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layers III–V of the cortex, where mGluR2 receptors are heavily expressed[14] and that mGluR3 receptors are also heavily expressed in cortical and in striatal neurons and in glial cells. mGluR4 receptors, on the other hand, are not significantly expressed either in the cortex or in the striatum.[18] The data so obtained have then been utilized to define a pharmacophore model for the mGluR↓cAMP subtypes and for defining the agonist bioactive conformation of L-Glu (**1**). The results are reported herein.

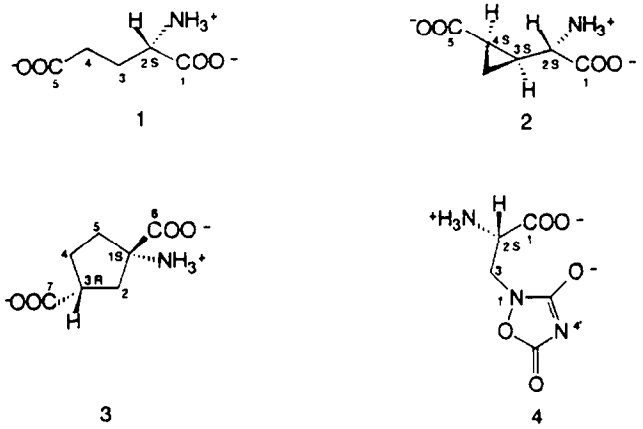

Figure 1. L-Glu (**1**); (2*S*,3*S*,4*S*)-CCG I (**2**); (1*S*,3*R*)-ACPD (**3**); QUIS (**4**)

Results and Discussion

In order to study the effect of different agonists on the mGluRs linked to the inhibition of adenylyl cyclase, striatal slices were prepared and incubated at 37 °C *in vitro* in oxygenated Krebs solution containing 1mM IBMX. The addition of forskolin (30 μM) to these slices increased their cyclic cAMP content from 4.7 ± 0.2 to 103 ± 10 pM/mg protein. (2*S*,3*S*,4*S*)-CCG I (**2**), (1*S*,3*R*)-ACPD (**3**) and QUIS (**4**) inhibited the accumulation of the cAMP in forskolin-treated slices in a concentration-dependent manner. The maximal inhibition was approximately 50%. The half-maximally effective concentration was 0.9 μM for (2*S*,3*S*,4*S*)-CCG I (**2**), 20 μM for (1*S*,3*R*)-ACPD (**3**) and 300 μM for QUIS (**4**) (see Table 1).

Table 1. Affinity profile for mGluRs agonists[a]

compound	EC_{50} - PIP2 (μM)	EC_{50} - ↓cAMP (μM)
2	30	0.9
3	25	20
4	15	300

[a]Data derived from at least five experiments conducted in duplicate. S.E. was within 20% of the reported values.

Thus the order of potency of the three tested compounds was (2*S*,3*S*,4*S*)-CCG I > (1*S*,3*R*)-ACPD >> QUIS, in qualitative agreement with that found by Nakanishi in mGluR2 transfected cells.[15] In separate experiments slices were incubated for 2 h with [^{3}H]-inositol (20 μCi/ml) in order to label phosphoinositides. The effects of mGluR agonists on the stimulation of phosphoinositide metabolism were evaluated by exposing the slices for 15 min to molecules under study and by monitoring the formation of inositol monophosphate (IP), inositol-1,4-diphosphate (IP_2) and inositol-1,3,4-triphosphate (IP_3). QUIS (**4**) increased inositol phosphate formation 2.2-fold over the control value with an EC_{50} of 15 μM, while (2*S*,3*S*,4*S*)-CCG I (**2**) and (1*S*,3*R*)-ACPD (**3**) increased inositol phosphate formation 1.8-fold with EC_{50} values of 30 and 25 μM, respectively (Table 1). These data suggest that different mGluRs agonists may differentiate the receptors linked to inositol phosphates formation from receptors linked to inhibition of cAMP accumulation. In particular (2*S*,3*S*,4*S*)-CCG I (**2**) interacted in a relatively selective manner with the receptors associated with the cyclase system, while QUIS (**4**) preferentially interacted with the receptors linked to the phosphoinositide cycle.

The availability of potent and relatively selective agonists such as (2*S*,3*S*,4*S*)-CCG I (**2**) and (1*S*,3*R*)-ACPD (**3**), which are conformationally restricted analogs of L-Glu (**1**) makes possible the definition of a pharmacophore model for the mGluR↓cAMP receptor subtypes. In addition, quisqualate (**4**), was chosen as probe for testing the model validity.

All the molecules under study were considered as fully ionized species, i.e. as existing with a protonated amine and two ionized carboxylates at physiological pH. While a systematic search around rotatable bonds (see Experimental Section) was sufficent to explore the (2*S*,3*S*,4*S*)-CCG I (**2**) conformational surface, the twenty stable conformations given by free interconversion around pseudotorsional angles had to be considered for the cyclopentane moiety of (1*S*,3*R*)-ACPD (**3**).[19] The aminoacidic moiety and the distal carboxylate were thus added, with the correct stereochemistry, in position 1 and 3 respectively. The rotations about the symmetric carboxylate were subsequently studied in the 0–179° range. In this way 156 and 166 rotamers free of bad steric interactions were generated for (1*S*,3*R*)-ACPD (**3**), (2*S*,3*S*,4*S*)-CCG I (**2**), respectively. An analogous procedure was followed to generate rotamers of **4** to be used later as probes for the model validity (see Table 2). The former two conformational ensembles were thus taken as basis for the pharmacophore search and first submitted to geometry optimization.

Table 2. Conformational search settings and results

Compound	Rotatable Bonds	Increment	Range	Rotamers
2	C1 - C2	30°	0 - 179°	166[a]
	C2 - C3	30°	0 - 359°	130[b]
	C4 - C5	30°	0 - 179°	
3	C1 - C6	30°	0 - 179°	156[a]
	C3 - C7	30°	0 - 179°	171[b]
4	C1 - C2	30°	0 - 179°	374[a]
	C2 - C3	30°	0 - 359°	309[b]
	C3 - N4	30°	0 - 359°	

[a]Rotamers obtained by the systematic search.
[b]Rotamers obtained after the two phases energy minimization.

A major problem in treating highly charged small molecules is to decide whether to include or not the electrostatic term in the force field. Since amino acids are likely to act as fully ionized species, it would be appropriate to consider them in this form in modeling studies. Geometry optimization of highly charged molecules in absence of explicit solvent, however, can produce internally hydrogen-bonded conformers with a geometry distorted by coulombic attraction.[5] This problem can be partly overcome either by assigning an appropriate value to the dielectric constant (e.g. 80), thus simulating a water environment, or by defining a distance dependent dielectric function. On the other hand, even if such attenuation functions were capable of simulating correctly the medium, they cannot mimic the explicit receptor–ligand interactions (i.e. hydrogen bonds, ionic interactions, etc.) which can stabilize particular unexpected conformations. Since what one needs in this phase of the receptor mapping approach is only a realistic estimation of the energy contents associated with minimized internal molecular strain, we decided to carry out all the computations without considering charges, thus avoiding the problem of taking into account ambigous and not directly observable quantities such as point charges, dielectric constant and attenuation functions.[4,20–22]

The two conformational collections previously generated were submitted to energy minimization. For each ligand, the generated conformations were minimized first by keeping fixed the torsional angles and then, in a separate operation, fully optimized in order to localize the relative energy minima. This two-phase optimization strategy thereby provides an energetically relative estimate of the entire conformational surface in the first phase, while providing true minima in the second phase. The two sets of conformations were merged and limited to unique structures within 4 Kcal/mol of the global energy minimum resulting in 130 and 171 rotamers for (2*S*,3*S*,4*S*)-CCG I (**2**) and (1*S*,3*R*)-ACPD (**3**) respectively.

In order to select from these large lists of energetically allowed conformers pairs which meet the geometrical requirements necessary to interact comparably well with a common receptor site, we have decided to follow the computational protocol APOLLO developed by Snyder and coworkers.[5] The first step of the procedure is to select the common points which have to be superimposed. In the present case, in analogy with what has already been reported for the NMDA receptor complex,[5] we decided to choose five points; the nitrogen and the two carboxylate oxygens in the zwitterionic aminoacidic group and the two oxygens in the distal carboxylate. It was then assumed that the aminoacidic moiety binds a ionic receptor site and that the distal carboxylate behaves as a hydrogen bonding acceptor. Moreover, in order to take into consideration the possibility of the distal carboxylates to bind to the same receptor point from different spatial orientation we have utilized the possibility offered by the APOLLO procedure to add dummy atoms to hydrogen bond acceptor atoms and extending them in order to reach the putative receptor points.[5,23] These extended lone pairs (ELP's) are then used in the matching procedure in place of the corresponding ligand atoms. Accordingly, we have added two ELP's, having *syn* and *anti* orientation respectively, to each of the distal carboxylate oxygens in **2** and **3**. In the same way, ELP's were added to the distal carboxylate of **1** and, finally, one ELP was added to the 4' nitrogen of the heterocycle and to the hydroxy oxygen in **4**. The average hydrogen bond angles and distances were taken from the literature.[24]

In order to evaluate the validity of the fit for any pair of conformations, five points were thus matched: N^{+}, $O1^{\delta-}$, $O^{\delta-}$, ELP1, ELP2 (see Figure 2). Furthermore, in order to take into account the equivalence of carboxylate extended lone pairs (*syn* and *anti* in a pairwise manner) these structural features were superimposed in all possible ways. The whole matching procedure (implemented as SPL macro within SYBYL[25]) required the evaluation of 211,152 RMS (root mean squares) values.

The pairwise superimpositions so obtained were ranked according to the RMS values. By this way, it was possible to dispose for each ligand of a number of rotamers useful for the definition of the pharmacophore model. A list of the best conformational sets, with their energies, is reported in Table 3.

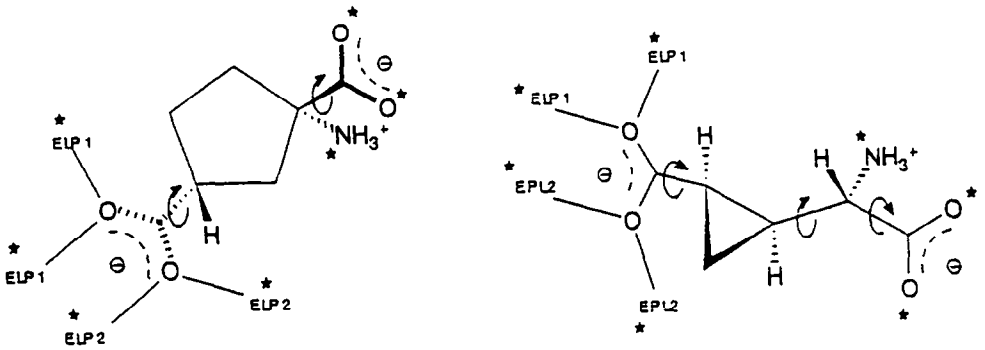

Figure 2. Illustration of the five points used in the conformational matching

Table 3. Ranking of the six best superimpositions

Conf. of 2[a]	Conf. of 3[a]	ΔE2(Kcal/mol)[b]	ΔE3(Kcal/mol)[b]	RMS[c]
1	1	0.9425	1.2288	0.1212
2	2	0.2783	0.9229	0.1246
3	3	0.3947	0.9361	0.1284
4	4	0.7249	1.0633	0.1307
5	5	0.6697	0.7152	0.1341
6	6	0.1520	0.7981	0.1357

[a]Rotamers of **2** and **3** involved in best matches.
[b]Energy deviation from the global minimum.
[c]Root mean squares of each pairwise superimposition.

It is worth noting that only *syn* lone pairs of carboxylates, known to form more favourable hydrogen bonds with respect to *anti* ones,[26] contribute to the definition of our pharmacophore (Figures 3 and 6).

The model we propose is certainly subjected to the moderate angle and distance variations which hydrogen bonding and ionic interactions can tolerate with a negligible impact on their energy. However, Figure 3 shows how the six best pairwise superimpositions reported in Table 3, adopt the same extended disposition of the pharmacophoric points. The variations of the RMS values observed in Table 3 are mainly due to the relative rotations around carboxylate rotatable bonds. Figure 4 shows the proposed bioactive conformations of both (2*S*,3*S*,4*S*)-CCG I (**2**) and (1*S*,3*R*)-ACPD (**3**), based on the best fit of Table 3.

Thus, our pharmacophore model may retain some uncertainty with regard to the relative orientation of the carboxylate oxygens but it is actually stable for what concerns the extended disposition of functional groups.

The validity of our model has been further validated by superimposing all the previously generated (see Table 2) QUIS (**4**) rotamers on the pharmacophore model. The best fit (RMS = 0.52) involves a rotamer within 1.5 Kcal/mol above the global minimum. While this geometrical match seems to indicate a possible complementarity between QUIS (**4**) and the putative mGluR↓cAMP agonist pharmacohore, other features can account for the weak affinity of **4**. Thus, when the above QUIS rotamer was superimposed to the putative pharmacophore model (see Figure 5) it was shown to possess an additional volume which can be responsible for the lower affinity.

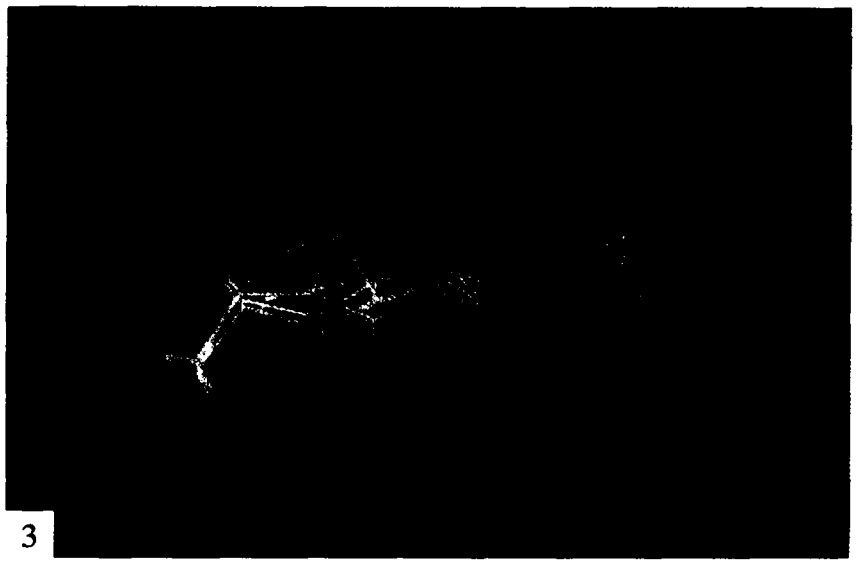

Figure 3. Superimposition of the six best pairwise matches, defining the pharmacophore model. In yellow the region of favourable hydrogen bonding interaction is reported (computed by means of the GRID/GRIN program).[33] The extended lone pairs are represented in magenta

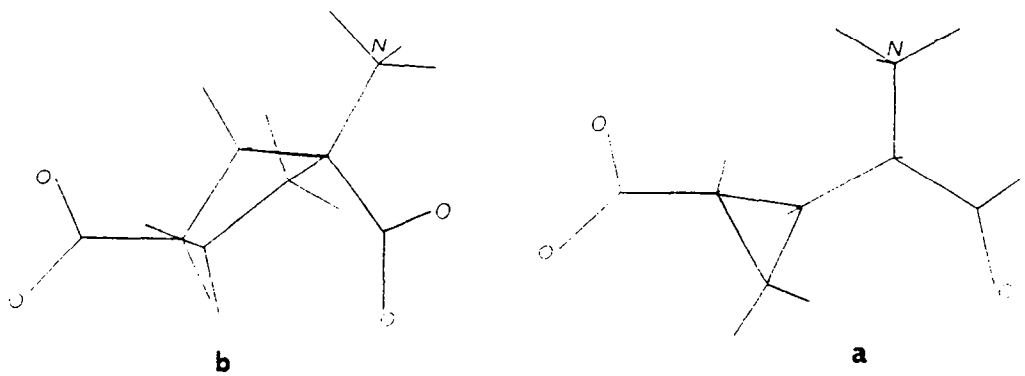

Figure 4. Proposed bioactive conformation of: (a) (2*S*,3*S*,4*S*)-CCG I (**2**); (b) (1*S*,3*R*)-ACPD (**3**)

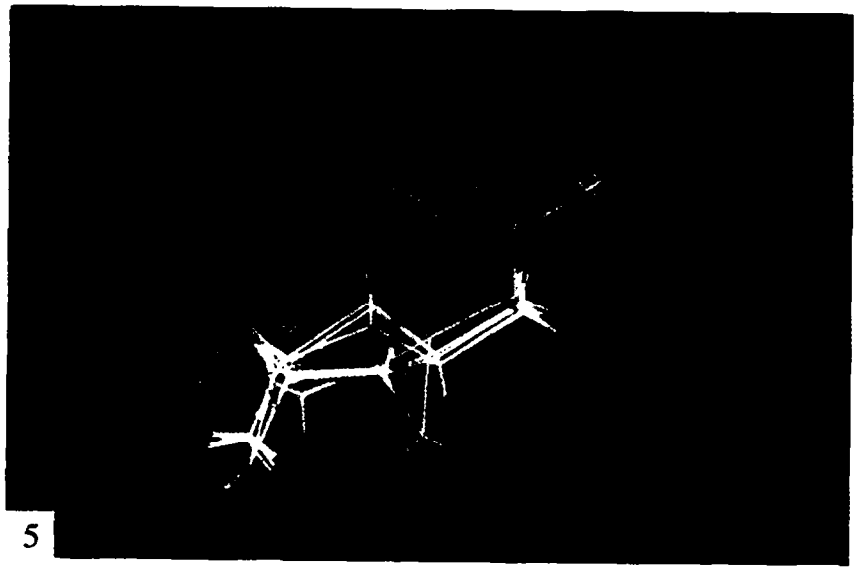

Figure 5. Superimposition of **4** (green) on the six best pairwise matches. In yellow the region of additional volume is reported

Finally, when L-Glu (**1**) itself was superimposed to the pharmacophore model, it was shown to adopt an extended conformation characterized by a *trans* orientation of both C2–C3 and C3–C4 rotatable bonds (τ_{c2-c3} = 176.9°, τ_{c3-c4} = -174.0°, see Figure 6), thus confirming recent hypothesis.[27] Previously it was reported that L-Glu interacts with the NMDA receptor site of the NMDA receptor complex in a more folded conformation in which the C2–C3 rotatable bond resides in a near *gauche* orientation while the C3–C4 bond adopts a near eclipsed (120–140°) one.[6] While this latter conformation was reported to reside within 3 Kcal/mol (AMBER calculation) from the solution favoured β-form,[28] the bioactive mGluR2 conformation of L-Glu requires a smaller amount (1.0 Kcal/mol) of energy to fit the pharmacophore, differing from the β-form only by a 120° rotation around the C3–C4 rotatable bond.

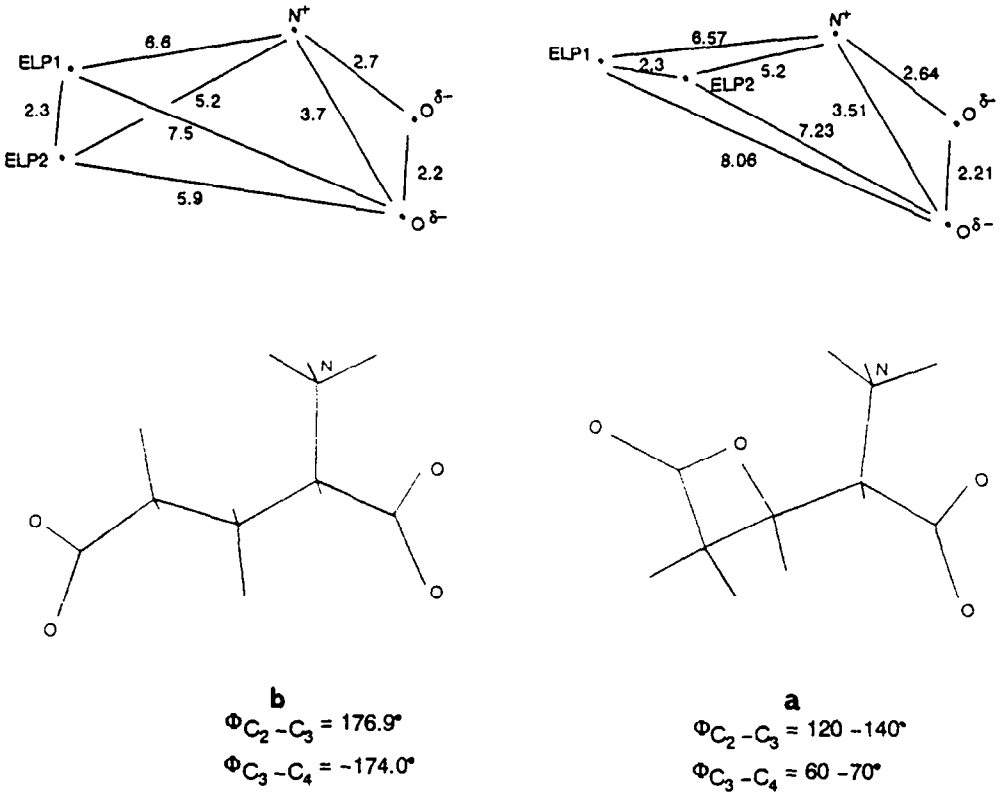

Figure 6. (a, right) NMDA site of the NMDA receptor complex agonist pharmacophore model (from Ref. 5) and the folded bioactive L-Glu (**1**) conformation. (b, left) mGluR↓cAMP pharmacophore model and extended bioactive L-Glu (**1**) conformation. The 3D constructs are expressed as distances between points based upon the first best superimposition

Experimental Section

Pharmacology

The cAMP content of striatal slices was evaluated using an Amersham radio-immuno assay kit. IBMX (3-isobutyl-1-methylxanthine 1 mM) was constantly present in the incubating medium in order to prevent phosphodiesterases and the action of adenosine on the cyclase system.[29] [^{3}H]-Inositol phosphates accumulation was monitored according to Berridge *et al.*[30] in the presence of lithium (10 mM) as previously described.[31]

All commercially available agonists were taken from Tocris Neuramin (U.K.), while the synthesis of **2** was carried out as described below.

Chemistry

(2*S*,3*S*,4*S*)-α-Carboxycyclopropylglycine (**2**) was prepared by a route involving as key step the not stereospecific addition of the carboethoxycarbenoid generated by the dirhodium–tetraacetate decomposition of ethyl diazoacetate to protected L-vinylglycine.[6] The four diastereoisomeric α-carboxycyclopropylglycines thus obtained were then separated by a variety of chromatographic and chemical methods, in analogy with what was described for the four D-α-carboxycyclopropylglycines.[32]

Molecular modeling

All the computations were carried out by means of SYBYL (ver. 6.0)[25] molecular modeling software running on a ESV workstation. The structures of (2*S*,3*S*,4*S*)-CCG I (**2**), (1*S*,3*R*)-ACPD (**3**), L-Glu (**1**), QUIS (**4**) and (*S*,*S*)-*t*-ADA (**5**) were built from the standard SYBYL fragment library and subsequently geometrically optimized by means of the TRIPOS force field. BFGS energy minimization was continued until the RMS energy gradient was less than 0.05 Kcal/mol Å. The conformational analyses were performed by means of the SEARCH module of SYBYL, by defining the carboxylate bonds as rotatables in the range 0–179° range with 30° increment while the others have been defined in the whole 0–359° range.

Conclusions

The capability of conformationally restrained L-Glu (**1**) analogs such as (2*S*,3*S*,4*S*)-CCG I (**2**) and (1*S*,3*R*)-ACPD (**3**) to inhibit the cAMP intracellular accumulation in rat striatal slices has been instrumental in defining a pharmacophore model for the mGluR↓cAMP receptor subtypes. This heuristic model accounts for an extended disposition of ligands as well as for a L-Glu (**1**) mGluR↓cAMP bioactive conformation, and provides a volume exclusion region. Some putative ligands have been designed on the basis of such a model and the results will be communicated in due course.

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