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Characteristics of the Amino Acid Receptor Site Mediating Formation of Cyclic Adenosine 3',5'-Monophosphate in Mammalian Brains

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SUMMARY

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Aliphatic ω-acidic α-amino acids, which are known to cause neural cell excitation when applied iontophoretically to the central nervous system, were effective in increasing the concentrations of cyclic 3',5'-AMP in cerebral cortical slices of the guinea pig and rat. L-Cysteinesulfinic acid was the most powerful. Replacement of the sulfinyl group with a carboxyl group (aspartic acid) or a sulfonyl group (cysteic acid) reduced the activity to less than half that caused by the sulfinate. Elongation of the aliphatic carbon chain from aspartate (C_3) to glutamate (C_4) did not alter the activity, but further elongation to α-aminoadipate (C_5) and α-aminopimelate (C_6) decreased the activity in a manner depending on the chain length. L-Cysteinesulfinic acid was more powerful than its higher homologue. The L isomers of the enantiomorphs of active amino acids were more potent than the corresponding D isomers. However, there appeared to be no significant difference in the efficacy of the two stereoisomers at their optimal concentrations. The structure-activity relationships were similar with respect to both the amino acid-elicited accumulations of cyclic AMP in brain tissue and the iontophoretic effects on firing rates of spinal and cortical neurons.

INTRODUCTION

In a previous paper (1) we demonstrated that certain aliphatic acidic amino acids, e.g., aspartate and glutamate, were capable of stimulating the formation of adenosine cyclic 3',5'-monophosphate in cerebral cortical slices of the guinea pig. The effect of the amino acids differed from that of biogenic amines and adenosine. An effect additive with respect to the maximum produced by either a biogenic amine or adenosine was observed when either amino acid (aspartate or glutamate) was added at its optimal concentration. The effect of amino acids was also distinguishable from the cyclic AMP-stimulating action of depolarizing agents such as ouabain, veratridine, or high concentrations of K^+. Stimulation by these agents was diminished either by omission of Ca^{++} from the incubation medium or by addition of membrane stabilizers, such as cocaine. The effect of amino acids was Ca^{++}-independent and was not inhibited by cocaine. These findings suggested that these amino acids may belong to a new class of stimulants

for the cyclic AMP-generating system in the brain. Before our previous paper appeared, Ferrendelli *et al.* (2) also reported the stimulatory effect of L-glutamate on the levels of both cyclic AMP and cyclic GMP in mouse cerebellum slices.

By means of iontophoretic techniques, glutamate and aspartate have been shown to be potent excitatory agents of central neurons. Their respective α-decarboxylation products, such as γ-aminobutyric acid, depressed the same neurons (3–7). Our preliminary survey of various amino acids (1) revealed that, although glutamate and aspartate were very effective stimulants for cyclic AMP formation, γ-aminobutyric acid was completely without effect. In order to examine whether the cyclic AMP-stimulating effect of these agents has any relevance to their iontophoretic properties, we have now investigated the effect of various amino acid derivatives on the formation of cyclic AMP in cortical slices and compared them with the iontophoretic properties of these substances.

MATERIALS AND METHODS

Animals. Male Hartley guinea pigs (300–340 g) and male Sprague-Dawley rats (185–210 g) were used. They were stunned by a blow on the head and their brains were removed.

Amino acids and their derivatives. DL-α-Aminopimelic acid, L-cysteine-sulfinic acid, α-methyl-DL-glutamic acid, *N*-methyl-L-glutamic acid, formimino-L-glutamic acid (hemibarium salt), *N*-acetyl-L-aspartic acid, and DL-β-amino-*n* butyric acid were obtained from Sigma Chemical Company. L-Homocystine, *O*-phosphoryl-DL-serine, L-glutamic acid γ-methyl ester, L-pyroglutamic acid, and DL-homocysteine were purchased from Fluka, AG, Switzerland; L-cysteic acid, glutaric acid, and oxalacetic acid, from Nakarai Chemicals, Japan; *N*-methyl-DL-aspartic acid, from K & K Laboratories; and γ-amino-β-hydroxybutyric acid, from Calbiochem. L-Homocysteinesulfinic acid and L-homocysteic acid were prepared from L-homocysteine according to Watkins (8). 2-Amino-3-phosphonopropionic acid was a gift from Dr. A. F. Isbell, Texas A & M University. β-Aminoglutamic acid and β-methylglutamic acid were synthesized by the methods of Romeo and Magno (9) and Kim and Cocolas (10), respectively. The identities of the synthesized compounds were confirmed from their nuclear magnetic reasonance spectra, and purity was ascertained by chromatography. Sources of other compounds were described in the previous paper (1).

Measurement of cyclic AMP formation. Preparation and incubation of brain slices and measurement of formation of cyclic AMP in the slices were carried out as reported previously (1). Briefly, the principle of the method depends on (a) prior labeling of the intracellular ATP pool by incubation of the slices with [^{14}C]adenine (3.7 μM, 42 μCi/μmole), (b) subsequent incubation of the labeled slices with test agent(s) adjusted to pH 7.3 ± 0.15 in an incubation medium containing 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 10 mM D-glucose, and 25 mM $NaHCO_3$, and (c) radioassay of the formed cyclic [^{14}C]AMP after purification by thin-layer chromatography. Formation of radioactive cyclic AMP was expressed as percentage conversion, which refers to the percentage of radioactivity of cyclic [^{14}C]AMP as compared to the total radioactivity taken up in the slice sample.

In certain experiments endogenous cyclic AMP was assayed in slices after incubation with or without a test agent. This assay was carried out by the protein binding method of Brown *et al.* (11) after purification from trichloracetic acid extracts of the slices as described previously (1). Results were expressed per milligram of protein. The trichloroacetic acid precipitates of the slices were digested in 1 N NaOH, and aliquots of the digest were used for determination of protein according to Lowry *et al.* (12).

RESULTS

Structure-activity relationships. The effects of various amino acids and their derivatives on formation of cyclic AMP in incubated slices of guinea pig cerebral cortex are shown in Table 1. Among various analogues and homologues of aspartic

TABLE 1

Structure-activity relationships of various amino acids and related compounds

Cyclic AMP formation was measured in cortical slices of guinea pig brain, with the prior labeling radioassay method described in the text, after incubation for 8 min in the presence or absence of the test compound (1 mM and/or 10 mM). Results of separate experiments carried out in duplicate or triplicate were normalized to the average effect of 10 mM L-aspartate or L-glutamate, one of which was included in each experiment as a reference.

Compound tested	Cyclic AMP formation[a]		Electrophysiological activity[a]
	At 1 mM	At 10 mM	
		% conversion	
None		0.27 ± 0.08	0
L-Aspartic acid	3.1	8.3 ± 1.7	+++
D-Aspartic acid	2.4	6.4	++ (+)
L-Glutamic acid	3.0	8.1 ± 0.9	+++
D-Glutamic acid	2.8	5.7	++ (+)
DL-α-Aminoadipic acid		4.5	+
DL-α-Aminopimelic acid	0.2	1.2	+
L-Cysteic acid	1.4	7.2	+++
L-Cysteinesulfinic acid	5.7	18.5	+++
L-Homocysteic acid	1.4	5.9	NR
L-Homocysteinesulfinic acid	4.9	9.3	NR
O-Phosphoryl-L-serine	0.3	0.3	0
DL-2-Amino-3-phosphonopropionic acid	0.3	0.2	NR
Glycine	0.3	0.4	– –
L-Alanine	0.5	1.4	– –
DL-α-Amino-*n*-butyric acid		0.3	0
L-Serine		0.8	0
L-Homoserine	0.2	1.0	NR
L-Methionine		0.3	NR
L-Leucine	0.2	0.3	0
DL-Isoleucine	0.2	0.3	0
L-Tyrosine	0.3		0
L-Cysteine	0.3		0
L-Homocysteine	0.2	0.2	NR
β-Alanine	0.4	1.7	– – –
Taurine		0.2	– –
DL-β-Amino-*n*-butyric acid	0.3	0.2	–
γ-Amino-*n*-butyric acid	0.2	0.3	– – –
β-Aminoglutaric acid	0.7	0.8	++ (+)
DL-α,ε-Diaminopimelic acid	0.2	0.4	0
α-Methyl-DL-glutamic acid	0.4	0.6	0
β-Methyl-*meso*-glutamic acid	1.7	4.7	NR
N-Methyl-DL-aspartic acid		2.3	++
N-Methyl-L-glutamic acid	0.3	0.5	0[b]
N-Acetyl-L-aspartic acid	0.4	0.4	0
N-Formimino-L-glutamic acid	0.3	0.3	NR
L-Asparagine	0.2	0.4	0
DL-Glutamine	0.3	0.3	NR
L-Glutamine	0.2	0.6	0

(TABLE 1—*Continued*)

Compound tested	Cyclic AMP formation[a]		Electrophysiological activity[a]
	At 1 mM	At 10 mM	
	% conversion		
L-Glutamic acid γ-methyl ester	0.3	0.3	0
L-Glutamic acid α,γ-diethyl ester	0.2	0.3	0
L-Pyroglutamic acid	0.2	0.3	0
Glutathione (reduced)	0.3	0.3	NR
Arginine	0.2	0.3	NR
Pyruvic acid	0.2	0.2	NR
Succinic acid	0.2	0.4	0
Glutaric acid	0.4	0.2	0
α-Ketoglutaric acid	0.3	0.3	NR
Oxalacetic acid	0.2	0.3	NR

[a] Data taken from ref. 2. + and − indicate excitant and depressant activities, respectively; NR indicates not reported.

[b] With the DL isomer.

acid which we investigated, L-cysteinesulfinic acid was outstandingly active. The structure-activity relationships reported in Table 1 may be summarized as follows. The presence of an α-amino, an α-carboxyl, and an ω-acidic group is a prerequisite for activity. Modifications of either one or two of the three essential groups always diminish or abolish activity. Of the acidic or electronegative groups located at the ω-position, the sulfinyl group is most active, followed by carboxyl and sulfonyl; phosphoryl and phosphonyl groups are ineffective. Variation of the number of carbon atoms in the chain connecting the α-amino and ω-acidic groups shows that peak activity is associated with separation of these groups by 2 (or 3) carbon atoms. Attachment of a bulky group to the carbon chain diminishes the activity. Finally, the L isomers of the aspartic and glutamic acid enantiomorphs are slightly more powerful than the D isomers at 10 mM.

With the iontophoretic technique relative effects among aspartic, glutamic, cysteic, cysteinesulfinic, and homocysteic acids have been reported to vary slightly, depending on the region of the central nervous system examined (3, 13, 14). In our hands, however, cysteinesulfinic acid was by far the most efficacious stimulant of cyclic AMP accumulation in cerebral cortical slices of rat and guinea pig (Fig. 1 and Table 2). If the sulfinic acid moiety were inhibitory toward cyclic 3′,5′-nucleotide phosphodiesterase, this could be responsible for the selective superiority of the sulfinic acid among the excitant amino acids, and represent its sole mode of action. Neither aspartic acid nor glutamic acid has been shown to possess inhibitory action toward the phosphodiesterase (1). However, L-cysteinesulfinic acid at 1.0 mM concentration, where the half-maximal effect for cyclic AMP formation was obtained, also failed to inhibit the phosphodiesterase activity when tested at both low and high substrate concentrations (data not shown) (see ref. 1 for the assay method).

Possible antagonists. According to reports by Curtis and his associates (3, 15), the excitation of central neurons by iontophoretically applied aspartate or glutamate could be blocked by the simultaneous application of equivalent or lesser amounts of one of the electrophysiologically depressant amino acids: γ-aminobutyric acid, taurine, or glycine. In contrast, we observed no such antagonism in our experiment on the receptor mediating cyclic AMP formation (Table 3).

The electrophysiological effect of the excitatory amino acids has been reported to be abolished by tetrodotoxin applied iontophoretically. This was interpreted to

TABLE 2

Relative effects of excitant amino acids on accumulation of cyclic AMP in cerebral cortical slices of guinea pigs and rats

Slices were exposed to a 10 mM concentration of each stimulant for 10 min, and formation of cyclic AMP in the slices during the exposure was determined by the methods described in the text. Total cyclic AMP, expressed per milligram of protein, and radioactive cyclic AMP, expressed as percentage conversion, were determined in separate experiments. Data are the average of three experiments ± standard error for guinea pig slices, and represent individual values of two experiments for rats.

Stimulant	Cyclic AMP formation		
	Guinea pig		Rat
	pmoles/mg protein	*% conversion*	*% conversion*
None	12 ± 1.9	0.3 ± 0.1	0.5, 0.4
L-Cysteic acid		6.9 ± 0.9	3.0, 3.1
L-Cysteininesulfinic acid	974 ± 81	18.5 ± 3.4	6.2, 7.0
L-Aspartic acid	502 ± 23	8.3 ± 1.7	3.0, 3.3
L-Glutamic acid	487 ± 24	8.1 ± 1.4	2.8, 3.4

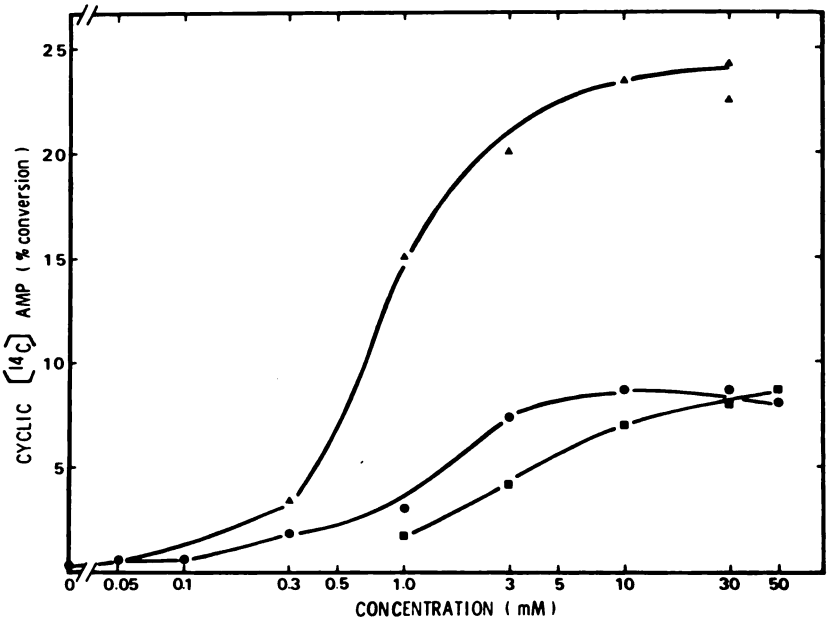

FIG. 1. *Dose-response curves for stimulated formation of radioactive cyclic AMP during incubation with various acidic amino acids in guinea pig cerebral cortical slices labeled by prior incubation with [^{14}C]adenine*

Slices were labeled with [^{14}C]adenine and then incubated with L-cysteinesulfinic acid (▲——▲), L-aspartic acid (●——●), or D-aspartic acid (■——■) for 15 min. After homogenization of the slices with trichloracetic acid, radioactive cyclic AMP in the homogenate was measured and expressed as the rate of conversion from total incorporated radioactivity as described under MATERIALS AND METHODS.

indicate the involvement of Na^+ influx in their mechanism of action (16). Formation of cyclic AMP stimulated by certain depolarizing agents, such as veratridine and batrachotoxin, was also blocked by simultaneous addition of tetrodotoxin (17). However, the stimulatory effect of glutamate and aspartate on brain cyclic AMP formation was not influenced at all by tetrodotoxin (Table 3).

DISCUSSION

In 1960 Curtis and his associates (3) reported an extensive survey of the "excita-

TABLE 3

Inhibition of glutamate-stimulated formation of cyclic AMP by possible antagonists

Slices were first labeled with [^{14}C]adenine and then incubated for 13 min with one of the various antagonist candidates at the concentrations indicated in the presence of either 2 mM L-glutamic acid or 0.05 mM veratridine. Formation of cyclic AMP in the slices during the incubation was measured as described in the text, and results are presented as the average ± standard error for the numbers of experiments indicated in parentheses, or as individual values from two experiments.

Stimulant	Possible antagonist	Cyclic AMP
		% conversion
None	None	0.3 ± 0.1 (9)
L-Glutamic acid	None	5.5 ± 0.7 (7)
L-Glutamic acid	γ-Aminobutyric acid, 2 mM	5.3, 5.4
L-Glutamic acid	γ-Aminobutyric acid, 4 mM	6.5, 6.0
L-Glutamic acid	Taurine, 4 mM	5.9, 6.2
L-Glutamic acid	Glycine, 4 mM	6.1, 6.6
L-Glutamic acid	Tetrodotoxin, 0.05 mM	5.5, 5.9
L-Glutamic acid	Theophylline, 0.05 mM	0.8 ± 0.1 (4)
Veratridine	Tetrodotoxin, 0.05 mM	0.5 ± 0.1 (4)
Veratridine	None	17.6 ± 2.1 (3)

tory activity" of iontophoretically applied amino acids and related compounds in the spinal cord and cerebral cortex of the cat. Interestingly, the structure-activity relationships they obtained were very similar to those involved in mediating the "formation of cyclic AMP". Thus aspartic, glutamic, and cysteic acids were the strongest excitatory compounds tested by them. Modification of the molecular structures of aspartic and glutamic acids led to impairment or complete abolition of the activity in both iontophoretic and biochemical studies. Another interesting analogy of the activity in both iontophoretic and biochemical studies was the action of *N*-methyl derivatives of aspartic and glutamic acid: *N*-methylaspartic acid was found to have modest activity while *N*-methylglutamic acid possessed almost none. A minor difference was observed with β-aminoglutaric acid, which was very active as an iontophoretic excitant but not as a cyclic AMP stimulant. All other findings shown in the structure-activity study (Table 1) and summarized under RESULTS are in good agreement with the results obtained with the electrophysiological technique (2, 14).

As far as the antagonism experiment is concerned, results obtained in the present study with γ-aminobutyric acid and tetrodotoxin are quite inconsistent with the findings reported for the iontophoretic properties. Ferrendelli *et al.* (2) were also unable to show any attenuation by γ-aminobutyric acid of the glutamate-elicited elevation of cyclic AMP in mouse cerebellum slices. It appears very likely that the antagonism observed electrophysiologically between the excitatory and depressant amino acids is not a phenomenon occurring at a single receptor but is a final manifestation of two actions mediated through two separate receptors, one for the excitatory amino acids and the other for the depressants. In spite of the fact that L-alanine and β-alanine are electrically depressant amino acids (3), they possessed weak activity in stimulating the formation of cyclic AMP (Table 1). This inconsistency might be resolved if the depressant amino acids have a dual action on both excitant and depressant receptors. The cyclic AMP-generating system might be linked only to the excitant receptor. Tetrodotoxin could block propagation of the excitatory response without affecting the excitatory amino acid receptor directly.

As already postulated in several reports (18–20), the stimulatory effect of depolarizing agents on cyclic AMP accumulation is likely to be a secondary one mediated by adenosine (or related compounds) and other endogenous substances which can be

released from intracellular compartments into the extracellular space by depolarizing stimuli. Preferential release of glutamate and aspartate from neural preparations by depolarizing stimuli (21, 22), taken together with our finding that combination of either one of the amino acids with adenosine resulted in additive or supra-additive synergism, suggests that these two amino acids are probably the endogenous substances that are released into the extracellular space by stimulation and are subsequently capable of enhancing cyclic AMP accumulation in combination with adenosine. Although Ferrendelli *et al.* (2) attributed the effect of glutamate on the levels of cyclic AMP and cyclic GMP to the ability of this amino acid to produce depolarization, the ineffectiveness of both γ-aminobutyric acid and tetrodotoxin on the glutamate-elicited accumulation of cyclic AMP makes it difficult to support their interpretation. Tetrodotoxin does inhibit the formation of cyclic AMP stimulated by veratridine or ouabain (17). Furthermore, one should be aware that a high concentration of EGTA[1] (2.5 mM), used in their experiments to demonstrate Ca^{++} dependence of the glutamate effect, is likely to inhibit the elevation of cyclic AMP elicited with every type of agent, including histamine (17). With a low concentration of EGTA (0.2 mM) sufficient to block the depolarization-elicited formation of cyclic AMP (1, 17), we could not detect Ca^{++} dependency in the glutamate-elicited accumulation of cyclic AMP in guinea pig cerebral slices (1).

Another possible mechanism for the amino acid-elicited accumulation of cyclic AMP may be reduction of intracellular ATP levels by aspartic and glutamic acids, a phenomenon probably associated with the uptake of these amino acids (23). The lowering of ATP levels might lead to efflux of adenosine (24), and the released adenosine could cause cyclic AMP accumulation. In support of this hypothesis, we observed that diethyl glutamate, which antagonized the iontophoretic activity of L-glutamate, but not at the site for its uptake (25), was ineffective in inhibiting the glutamate-elicited formation of cyclic AMP in our preliminary study; furthermore, theophylline inhibits the cyclic AMP formation elicited not only by adenosine but also by glutamic and cysteinesulfinic acids (1) (Table 3). However, this possibility is contradicted by the observation that cysteinesulfinic acid possesses much higher efficacy toward the cyclic AMP formation than adenosine when tested at their respective optimal concentrations.

The cyclic AMP-stimulating functions of catecholamines (26, 27), histamine (26, 28), and perhaps adenosine (18, 20, 29) have been assumed to reside in their respective receptors, which are identical, or directly associated, with the regulatory unit of the adenylate cyclase system. It is now possible to consider a hypothetical amino acid receptor, through which the accumulation of cyclic AMP is mediated. Forces involved in the interaction between the receptor and the agonistic amino acids appear to be mainly electrostatic, because every amino acid agonist possesses at least three ionizable groups: two acidic groups, at both ends of the aliphatic chain, and one amino group. One can depict the spatial relationship between the agonistic amino acid and the three-charge receptor as shown in Fig. 2. In this figure the three charged sites of the receptor are placed on a plane, i.e., the plane of the paper, so that their positions correspond to the triangle formed by the three ionized centers of the amino acid molecule. The distances between the three points of the triangle were determined by means of Dreiding molecular models of D- and L-aspartic and glutamic acids, based on the principle that the three ionized centers of the four agonistic molecules should be situated so as to correspond to the three charge sites of the common receptor. Within this cavity of the receptor membrane, the higher homologues of the dicarboxylic amino acids, e.g., α-aminoadipic and α-aminopimelic acids, would of necessity cause distortion of the aliphatic chain skeleton during interaction with the charged groups on the receptor, and therefore would have only limited

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

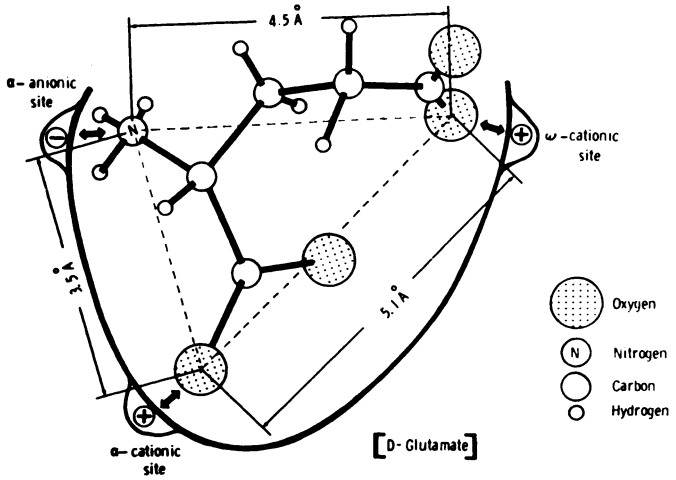

FIG. 2. *Preferred conformation of* D*-glutamate situated at three charge sites of postulated amino acid receptor*

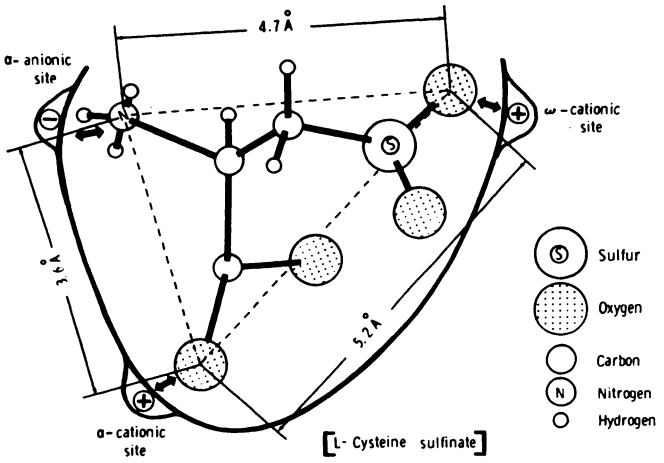

FIG. 3. *Preferred conformation of* L*-cysteinesulfinate situated at three charge sites of postulated amino acid receptor*

access to the three sites of the receptor. Modification of the aliphatic carbon chain would likewise cause steric hindrances and hence a decrease in the agonistic activity, as was the case for both α- and β-methylglutamic acid (Table 1).

L-Cysteinesulfinic acid, the strongest agonist found in the present study, also can be accommodated to this receptor model, as shown in Fig. 3; the internuclear distances of the S—C and S—O bonds are only 0.2–0.3 A longer than those of the C—C and C—O bonds (30). This receptor model, however, does not explain the superiority of the sulfinate among the agonists of cyclic AMP formation. Our study with the brain phosphodiesterase preparation did not indicate that the cyclic AMP-degrading system is responsible for the selective superiority of the sulfinate. The acidity of the ω-acidic group must be involved in some way in its agonistic activity. However, cysteinesulfonic acid, which is no less acidic than cysteinesulfinic acid,[2] was much weaker than the sulfinic acid as an agonist for cyclic AMP formation. It is

[2] On high-voltage paper electrophoresis in pyridine-acetate buffer, pH 3.4, at 100 V/cm, the rate of migration of cysteic acid to the anode was faster by 4% than that of cysteinesulfinic acid.

also interesting in this context that both phosphoryl and phosphonyl groups were almost completely devoid of agonistic action.

Another question to be solved is whether the configuration of the receptor cavity is equally suitable for both L-aspartic and L-glutamic acids, or whether L-aspartic acid is a better agonist than L-glutamic acid, just as L-cysteinesulfinic acid was stronger than L-homocysteinesulfinic acid. Investigation with conformationally restricted analogues of the amino acids should help in obtaining further information on the receptor configuration. Such a study is now in progress.

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