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Molecular structural requirements for binding and activation of L-alanine taste receptors

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Summary. L-Alanine binds to and activates specific taste receptors of *Ictalurus punctatus*, the channel catfish. In order to determine the structural requirements for receptor binding and activation in this model system, a number of analogues of L-alanine were tested using a neurophysiological assay and a competitive ligand binding assay. These assays measured the ability of analogues to activate taste receptors and to displace L-[^{3}H]alanine from L-alanine binding sites. Of those derivatives with modifications of the sidechain, L-serine, glycine, β-chloro-L-alanine and 1-amino-cyclopropane-1-carboxylic acid were the most potent analogues with IC_{50}s similar to and neural responses slightly decremented from that of L-alanine. Derivatives containing branched sidechains or sidechains of otherwise increased volume were considerably less active. All modifications of the α-carboxylic acid and the α-amine, including amides, esters and various isosteres, led to substantial reduction in the analogues' ability to displace L-[^{3}H]alanine and, in most cases, very weak stimulatory capability. However, L-lactic acid was a reasonably strong stimulus, but a poor competitor, suggesting that it acts at a different receptor site. Overall, these results indicate the importance of the charged amine and carboxylic acid groups for binding to and activation of the receptor for L-alanine. Moreover, modifications around the chiral center of L-alanine support the hypothesis that receptor binding and activation are separate processes in this model taste system.

Keywords: Amino acids – Structure/activity – L-Alanine – Taste receptor – L-Alanine analogues – Ligand binding assay

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Introduction

Binding of stimulus molecules by a receptor binding site is the initial event in the reception of many taste stimuli (Akabas et al., 1988; Brand et al., 1989; Kinnamon, 1988). To understand how transduction of the binding event is coded into stimulus quality, it is necessary to understand the specificity of individual classes of taste receptors. Structure/activity studies contribute to this end by defining receptor specificity in terms of molecular geometrical parameters. In so doing, these studies also contribute to understanding the molecular mechanism of stimulus recognition and receptor activation in a sensory system. In this report we focus on defining the structure activity relationships for the reception of taste stimuli in a well studied model amino acid taste system, the channel catfish (*Ictalurus punctatus*) (Brand et al., 1987; Brand et al., 1989; Bryant et al., 1989a; Bryant et al., 1989b; Cagan, 1979; Cagan, 1986; Cagan and Holland, 1981; Caprio, 1975; Caprio, 1976; Caprio, 1978; Caprio, 1982; Caprio and Robinson, 1978; Caprio and Tucker, 1976; Davenport and Caprio, 1982; Kalinoski et al., 1989a; Kalinoski et al., 1989b; Kanwal and Caprio, 1983; Krueger and Cagan, 1976; Kumazawa et al., 1990; Teeter and Brand, 1987; Teeter et al., 1990).

Three independent taste receptor sites have been defined in *I. punctatus*. One very sensitive system is optimally activated by L-alanine (Caprio, 1982), and to a lesser degree by other neutral sidechain amino acids including D-alanine (Brand et al., 1987). The second, a more specific receptor, is activated by L-arginine (Bryant et al., 1989b; Caprio, 1982). The third, a low affinity receptor, is activated by L-proline (Kanwal and Caprio, 1983; Kumazawa et al., 1990). These have been characterized electrophysiologically using single unit neural recordings (Caprio, 1982; Caprio and Tucker, 1976; Davenport and Caprio, 1982), cross-adaptation experiments (Brand et al., 1987; Bryant et al., 1989b; Caprio, 1982; Caprio and Robinson, 1978; Kanwal and Caprio, 1983), and single channel patch clamp techniques (Kumazawa et al., 1990; Teeter and Brand, 1987; Teeter et al., 1990). These studies indicate that the transduction of L-arginine and L-proline is likely to be due to the direct coupling of receptors to cation channels (Kumazawa et al., 1990; Teeter et al., 1990). Transduction of L-alanine, on the other hand, appears to be mediated by stimulation of inositol-1,4,5-trisphosphate metabolism (Kalinoski et al., 1989b). Adenylate cyclase is also stimulated by L-alanine but with a time course more consonant with an adaptation mechanism.

Biochemical studies using competitive ligand binding techniques have defined the enantiomeric specificity of alanine binding and transduction (Brand et al., 1987); D- and L-alanine act through both independent and common receptor sites. Heterogeneity in the neutral amino acid (L-Alanine) receptor site(s) has also been detected (Cagan, 1986); at one of the postulated sites, L-threonine, L-serine and L-alanine are effective inhibitors of the binding of L-[^{3}H]alanine, while at the other site binding of L-[^{3}H]alanine is inhibited by glycine, L-serine, L-alanine and D-alanine. Early structure activity studies of this model taste system measured only neurophysiological responses to natural amino acids, several amino acid derivatives and peptides (Caprio, 1976; Caprio, 1978). However, the relationship between receptor site occupation and activation was not

assessed. Therefore, using an extensive series of alanine derivatives, we have incorporated a competitive ligand binding assay with the measurement of taste neural activity to further understand the parameters of molecular geometry that underlie stimulus recognition and receptor activation.

Materials and methods

Competitive ligand binding assays

Binding activity to a partial membrane preparation (Fraction P2) from taste epithelial membranes for L-[^{3}H]alanine was assayed in 0.01 M Tris-HCl, pH 7.8, 1 mM $CaCl_2$ using the standard ultra-filtration assay of Kreuger and Cagan (1976). Fraction P2 was prepared from epithelial scrapings obtained from barbels and skin anterior to the operculi, as described (Brand et al., 1987; Krueger and Cagan, 1976). These areas of sensory epithelium are innervated by a branch of the facial taste (VII) nerve. P2 was stored at -20°C in 1 mM L-alanine (Cagan, 1979). Before assays, L-alanine was removed by rinsing. For lactic acid and analogues known to decompose at pH 7.8 (esters), the pH was adjusted to 7.0 by addition of the appropriate amount of HCl or NaOH. Nonspecific binding was determined with unlabeled L-alanine present at 23 mM. Structural analogues of alanine were evaluated as competitors of L-[^{3}H]alanine binding by simultaneous incubation of each competitor and L-[^{3}H]alanine with Fraction P2. L-[^{3}H]alanine was present at 10^{-6} M, a concentration near to the K_D, and the analog concentration was varied from 10^{-8}–10^{-3} M. Binding assays were performed in triplicate at each of the 6 concentrations of analog and replicated at least twice with the exception of analogs with IC_{50}s greater than 1000 μM which were tested once. The capacity to inhibit ligand binding is reported as IC_{50}, that concentration which causes 50% inhibition of control levels of L-[^{3}H]alanine binding. The IC_{50} value was determined graphically from the plot of the log of the concentration of analog vs % inhibition of L-[^{3}H] alanine binding.

Neurophysiology

Responses to stimuli were measured using standard (Bryant et al., 1989b; Caprio, 1975) multiunit recording techniques from the maxillary branch of the facial nerve (VII), the nerve that innervates the facial barbels. Briefly, fish were initially immobilized with Flaxedil (gallamine triethiodide, 0.3 mg/kg, IM). During the course of experiments, the fish were anesthetized with MS-222 (tricaine methane sulfonate, 1:16,000, w/v) applied in the respiratory water that flowed over the gills. The nerve was exposed surgically, freed of surrounding connective tissue and bundles of axons were teased free from the whole nerve. An isolated bundle containing 10s to 100s of axons was placed on a Pt/Ir electrode and submerged under mineral oil to prevent dessication. The recordings were obtained with differential amplification (Grass P-511 AC-coupled amplifier) with the indifferent electrode contacting the connective tissue in the surgical site. The amplified action potentials were summed using a leaky integrator RC circuit with a time constant of 1 sec (Fig. 1). Response amplitudes were measured from chart recorder output and expressed as a percentage of the standard stimulus, 10^{-4} M L-alanine. To deliver stimuli, the maxillary barbel was positioned in a glass tube through which there was a constant flow (approx. 13 ml/min) of a background medium designated as "artificial pond water" (APW) (0.3 mM NaCl, 0.2 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM $NaHCO_3$, pH 7.1–7.3). Stimuli were introduced into the APW flow in 1 ml volumes using an HPLC loop injector valve. This caused only a transient cessation of flow that resulted in little or no mechanical neural response. Because of potential hydrolysis under normal solution conditions (APW), esters were prepared fresh immediately before use in 5 mM sodium phosphate, pH 7.0 and stored on ice. The barbel was rinsed with APW for at least 4 mins between test stimuli to prevent interactions between sequential stimuli. Only bundles that responded well to 10^{-6} M L-alanine and 10^{-6} M L-arginine were used for

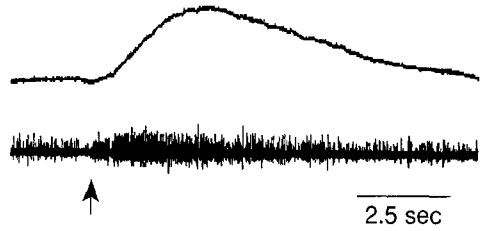


Fig. 1. Neural response to 10^{-4} M L-alanine. Lower trace: multiunit recording of action potentials obtained from a nerve bundle. An arrow indicates the onset of stimulation. Upper trace: the summated response of the action potentials in the lower trace. The summated response was quantified as the peak amplitude measured from the baseline. Time bar is 2.5 seconds

experiments. This served as a general indicator of the viability of the preparation. Spectrophotometric measurements of dye solution applied as a stimulus indicated that the peak concentration of stimulus at the barbel was approximately 55% of the applied concentration. Stimulus concentrations reported herein are uncorrected.

Structure/activity relationships were determined using test stimuli at 10^{-4} M. Tests of analogs were bracketed with tests of 10^{-4} M L-alanine in order to control for temporal variation in responsiveness. Responses to analogs were included for analysis only if bracketing tests were within 7.5% of one another. Responses are reported as a percentage of the response to 10^{-4} M L-alanine (defined as 100%). Responses to control stimuli (APW blanks) ranged from 0–9% of responses to 10^{-4} M L-alanine. An analog yielding a neural response of 50% corresponded in efficacy to a concentration of 10^{-6} M L-Ala (Fig. 2). Any analog containing more than 0.1% of L-Ala contamination would have generated a significant neural response, as indicated in Figure 2, the stimulus concentration/neural response function. Therefore, before biological evaluation, analogs were tested for purity using either thin layer chromatography with ninhydrin or HPLC following derivatization with phenylisothiocyanate (Bidlingmayer et al., 1984). Analogs were repurified if more than 0.05% L-alanine was detected.

Statistical analyses consisted of pairwise comparisons using the Tukey test (Tables 1–5), paired t-tests with a significance level of 0.05 and Pearson correlation coefficients.

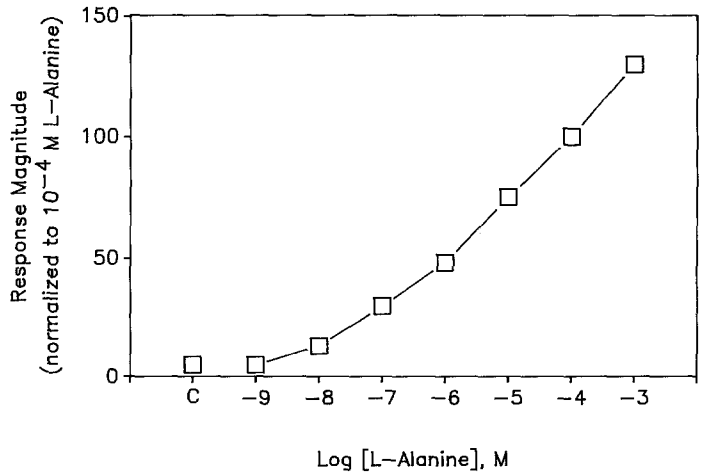


Fig. 2. Concentration-response function for taste neural response to L-alanine. Neural response was measured as described in the Materials and Methods section. *C* indicates the blank control stimulation which consisted of the background solution

Chemistry

Ammonia-carboxy borane (Spielvogel et al., 1980) was purchased from Boron Biologicals, Inc., Raleigh, NC. Diisopropyl [1-[bis(trimethylsilyl)amino]ethyl] boronate (Duncan et al., 1989) was a gift from Dr. D. Matteson, Washington State University, Pullman, WA. All other starting materials and a number of analogues used in this study were obtained from Sigma, Aldrich, Advanced Chemtech or Fluka. Melting points were determined using a Mel-Temp apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a Brüker AM250 MHz FT-NMR spectrometer and are reported in parts per million (ppm, δ) downfield from tetramethylsilane as an internal standard. IR spectra were recorded using a Perkin Elmer 237 grating infrared spectrometer. Thin layer chromatography was carried out using aluminum-backed EM silica $60F_{254}$ plates (Merck). Products were visualized with UV, ninhydrin or fluorescamine. Optical rotations were measured on a Perkin Elmer Model 241 polarimeter using a 10 cm pathlength cell at 25°C. Microchemical analysis and molecular weight determination by DCI/MS were carried out by Oneida Research Services, Whitesboro, NY. Amino acid analysis was performed at the Protein Analytical Laboratory, School of Dental Medicine, University of Pennsylvania. Molecular volumes were calculated using Dr. C. Still's MacroModel, Version 2.5 (Still et al.).

Synthesis

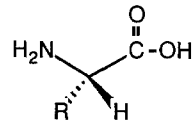
Compound **11** (Table 1) was prepared using methods from the literature (Hanessian and Sahoo, 1984) as was the N-benzylated derivative of 1 (30) (Velluz et al., 1954).

D,L-2-Acetylamino-4-pentynoic Acid **(10a)** – To a solution of D,L-2-amino-4-pentynoic acid **(10)** (1.0 g, 9.0 mmol) in HOAc (30 mL) was added triethylamine (2.0 g, 20 mmol). The solution was heated to 50°C and acetic anhydride (1.6 g, 16 mmol) was added slowly with stirring. After 1 hr, almost complete product formation was observed by tlc (butanol, acetic acid, water; 3:1:1). The material was concentrated to an oil and the remaining HOAc was removed by azeotroping the mixture with toluene (4 × 10 mL). To the residue was added ethanolic HCl (15%, HCl in EtOH, 10 mL) along with EtOAc (50 mL). The material was concentrated and mixed with acetone (100 mL). After filtration, the residue was washed with acetone (30 mL). The supernatant was concentrated to an oil which crystallized under high vacuum to give D,L-2-acetylaminopentynoic acid **(10a)**. Recrystallization (EtOAc/hexanes) yielded 1.2 g (88%), mp 137°C, ^{1}H NMR (DMSO-d_6) δ 1.87 (s, 3 H), 2.57 (m, 2 H), 2.89 (t, 1 H), 4.33 (q 1 H), 8.26 (d, 1 H).

L-2-Amino-4-pentynoic Acid **(10-L)** – A portion of the above acetylated derivative **(10a-D,L;** 100 mg, 0.6 mmol) was added to water (23 mL) and the pH was adjusted to 7.0–7.2. To this solution was added Acylase I (Sigma, 1700 units/mg, 0.25 mg, 1 unit hydrolyzes N-Ac-L-methionine at a rate of 1.0 micromol/min, 425 units). The suspension was gently shaken at 37°C for 48 hrs. The suspended protein was filtered off and the resulting liquid was passed through a cation exchange column (0.7 × 10 cm) containing 4 mL of AG 50W-X8 resin in the hydrogen form. The column was washed with 100 mL of water followed by 120 mL of 1 M NH_4OH. The basic eluent was collected and concentrated to dryness. Recrystallization (MeOH/Et_2O) afforded 46 mg (92%) of **10-L**, mp 205–206°C, $[\alpha]_D = -38.0°$ (C = 1.0, water), DCI/MS (m/z); 114.03. Anal. $C_5H_7NO_2$; C,H,N. To prove the optical purity of **10-L**, a portion (15 mg) was treated with 10% Pd/C, H_2 at atmospheric pressure for 2 hrs. The catalyst was filtered off and the material was concentrated to dryness, mp 303°C, $[\alpha]_D = +22.8°$ (c = 10, 20% HCl). Literature values (Windholz et al., 1983); mp 305°C, $[\alpha]_D = +23°$ (c = 10, 20% HCl).

L-Alanine-anilide · HCl **(21)** – To a solution of Boc-L-alanine (2 g, 10.6 mmol) and THF (100 mL) was added N-methylmorpholine (1.08 g, 10.7 mmol). The solution was cooled to 4°C and isobutylchloroformate (1.43 g, 10.5 mmol) was added with stirring. After stirring proceeded for 20 min, aniline (0.98 g, 10.5 mmol) in THF (20 mL) was added dropwise. The reaction stirred for 3 hrs followed by filtration. The supernatant was concentrated under a vacuum to a yellow thick oil. The oil was dissolved in EtOAc (100 mL) and washed with 1 N HCl (3 × 50 mL) followed by saturated $NaHCO_3$ solution (3 × 50 mL) and water

Table 1. Sidechain modifications of L-alanine: binding inhibition and neural activity



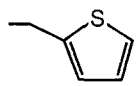
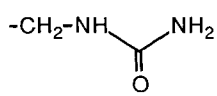
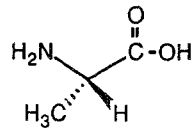
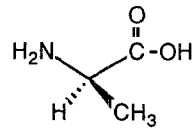
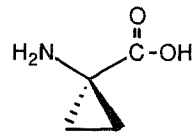
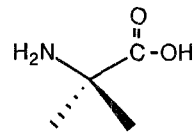
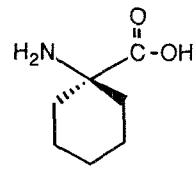
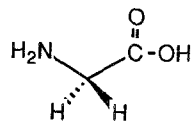
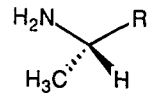
#	R	$IC_{50}(\mu M)$	Neural response	
1	$-CH_3$	3.5	100%[a]	(24)
2	$-CH_2Cl$	2.5	88.6 +/− 13.7%[a,b]	(8)
3	$-CH_2NH_2$	14	69.1 +/− 16.9%[b–e]	(12)
4	$-CH_2OH$	2	58.6 +/− 15.6%[d–f]	(10)
5	$-CHOH$ $\quad\vert$ $\quad CH_3$	190	33.5 +/− 14.0%[f–h]	(18)
6	$-CH_2CN$	38	56.4 +/− 14.4%[c–f]	(16)
7	$-CH_2SH$	22	52.5 +/− 14.7%[e,f]	(17)
8	$-CH{=}CH_2$	45	77.5 +/− 11.3%[a–c]	(5)
9	$-CH_2CH_3$	20	59.1 +/− 15.1%[c–e]	(16)
10	$-CH_2C{\equiv}CH$	30	76.0 +/− 19.3%[b–d]	(5)
11	$-CH_2CH{=}CH_2$	30	48.6 +/− 12.4%[e–g]	(8)
12	$-CH_2CH_2CH_3$	170	37.4 +/− 16.4%[f–h]	(11)
13	$-CH_2CHCH_3$ $\quad\quad\vert$ $\quad\quad CH_3$	>1000	23.6 +/− 3.2%[h]	(8)
14		>1000	21.1 +/− 5.5%[h]	(3)
15		>1000	27.8 +/− 7.1%[g–h]	(5)

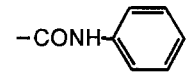
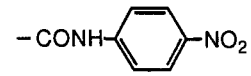
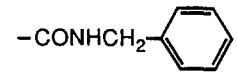
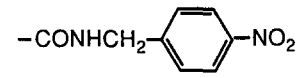
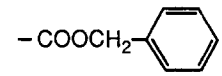
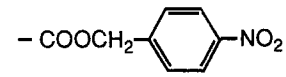
Table 2. Achiral sidechain modifications of L-alanine: binding inhibition and neural activity

#		$IC_{50}(\mu M)$	Neural response	
1		3.5	100%[a]	(24)
16		8	32.0 +/− 5.2%[d]	(9)
17		3.7	78.3 +/− 9.3%[b]	(12)
18		28	35.1 +/− 18.7%[d]	(17)
19		>1000	12.5 +/− 8.0%[e]	(6)
20		3	62.3 +/− 9.4%[c]	(15)

Tables 1–5. The IC_{50} and neural activity of compounds were determined as described in the Materials and methods section. IC_{50}s represent means of multiple determinations with the exception of compounds that had an IC_{50} greater than 1000 μM which were tested once. Numbers of animals tested are indicated in parentheses. A repeated measures ANOVA was used to test if neural activity measures for individual analogues were different. Mean neural responses that are marked by different superscript letters are significantly different at the level of $p < 0.05$

Table 3. Carboxylic acid modifications of L-alanine: binding inhibition and neural activity

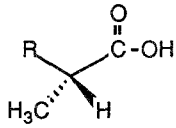


#	R	$IC_{50}(\mu M)$	Neural response	
1	-COOH	3.5	100%[a]	(24)
21		>1000	50.2 +/− 15.2%[b]	(11)
22		~1000	70.5 +/− 20.4%[b]	(10)
23		>1000	54.0 +/− 15.5%[b]	(10)
24		>1000	51.1 +/− 9.8%[b]	(10)
25		~1000	45.7 +/− 18.6%[b]	(5)
26		550	62.8 +/− 9.2%[b]	(5)
27	$-COOCH_3$	>1000	52.3 +/− 18.8%[b]	(5)
28	$-CH_2OH$	>1000	22.1 +/− 8.7%[c]	(5)

(2 × 20 mL). The organic layer was dried ($MgSO_4$) and concentrated to offwhite crystals. Crystallization (EtOAc/Hex) afforded 2.5g (89%) of **21a**. A portion of this material (1 g, 2 mmol) was dissolved in THF (50 mL) and HCl was bubbled through for 5 min. The solution was allowed to stand for 1/2 hr followed by concentration to an oil. The product crystallized on treatment with Et_2O. Yield; 0.70g (93%), mp 248°C w/dec., ^1H NMR (DMSO-d_6) δ 1.49 (d, 2 H), 4.12 (q, 1 H), 7.09 (t, 1 H), 7.33 (t, 2 H), 7.68 (d, 2 H), 8.43 (s, 3 H), 8.68 (s, 1 H), $[\alpha]_D$ = +28.1° (c = 7.1, water), DCI/ME; (m/z); 165. Anal. $C_9H_{13}N_2OCl$; C,H,N.

L-Alanine-paranitroanilide · HCl (**22**) – To a solution of Boc-L-alanine (2 g, 10.6 mmol) and THF (100 mL) was added N-methylmorpholine (1.08 g, 10.7 mmol). The solution was cooled to 4°C and isobutylchloroformate (1.43 g, 10.5 mmol) was added with stirring. After stirring proceeded for 20 min, paranitroaniline (1.46 g, 10.6 mmol) in THF (20 mL) and N-methylmorpholine (0.2 g, 2.0 mmol) was added dropwise. When the amine had been added, the reaction was heated to 50°C and allowed to stir for 2 hrs. After cooling, the solution was filtered and the supernatant was concentrated under a vacuum to a yellow thick oil. The oil was dissolved in CH_2Cl_2 (100 mL) and washed with 1 N HCl (3 × 50 mL)

Table 4. α-Amine modifications of L-alanine: binding inhibition and neural activity



#	R	$IC_{50}(\mu M)$	Neural response	
1	$-NH_2$	3.5	100%[a]	(24)
29	$-OH$	>1000	79.4 +/− 18.2%[b]	(13)
30	$-NHCH_2-$ 	>1000	43.2 +/− 13.9%[c]	(8)
31	$-NHCH_3$	>1000	31.9 +/− 14.0%[d]	(9)

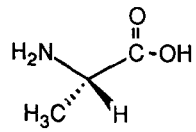
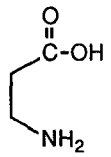
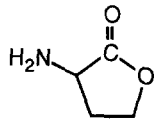
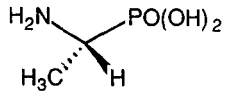
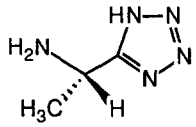
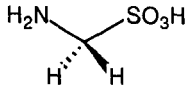
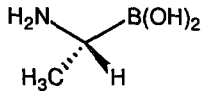
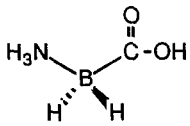
followed by saturated $NaHCO_3$ solution (3 × 50 mL) and water (2 × 20 mL). The organic layer was dried ($MgSO_4$) and concentrated to a solid. Crystallization (EtOAc/Hex) afforded 2.7 g (83%) of **22a**. A portion of this material (1 g, 3.2 mmol) was dissolved in THF (50 mL) and HCl was bubbled through for 5 mins. The solution was allowed to stand for 1/2 hr followed by concentration to an oil. Crystallization from EtOAc/Hex yielded 0.5 g (75%) of **22.** mp 210–212°C, ^1H NMR (DMSO-d_6) δ 1.51 (d, 2 H), 4.22 (q, 1 H), 7.97 (d, 2 H), 8.40 (d, 2 H), 8.40 (s, 3 H), $[\alpha]_D = +34.0°$ (c = 2.4, water), DCI/MS; (m/z); 210. Anal. $C_9H_{12}N_3O_3Cl \cdot 1/4\ H_2O$; C,H,N.

L-Alanine-benzylamide · HCl **(23)** – Compound **23** was prepared in a similar manner to Compound **21** using benzylamine (1.14 g, 10.6 mmol). Crystallization from toluene/hexanes afforded 2.6 g (88%) of **23a** as white crystals. A portion of this material (1 g, 3.6 mmol) was dissolved in CH_2Cl_2 and dry HCl gas was bubbled through the solution for 5 mins. The solution was allowed to stand for 45 mins. Concentration and crystallization from EtOH/Et_2O yielded 0.6 g (94%) of **23**, mp 143–144°C, ^1H NMR (DMSO-d_6) δ 1.41 (d, 3 H), 3.91 (m, 1 H), 4.31 (d, 2 H), 7.31 (m, 5 H), 8.37 (broad s, 3 H), 9.18 (t, 1 H), $[\alpha]_D = +9.5°$ (c = 2.8, MeOH), DCI/MS (m/z); 179. Anal. $C_{10}H_{15}N_2OCl \cdot 1/8\ H_2O$; C,H,N.

L-Alanine-p-nitrobenzylamide · HCl **(24)** – Compound **24** was prepared in a similar manner to Compound **21** using p-nitrobenzylamine · HCl (2 g, 10.6 mmol). Crystallization (toluene) afforded 3.2 g (84%) of **24a**. The product is obtained on treatment of **24a** (1 g, 2.8 mmol) with dry HCl in THF (similar to **21**). Yield, 0.7 g (97%), mp 228–229°C, ^1H NMR (DMSO-d_6) δ 1.41 (d, 3 H), 3.95 (m, 1 H), 4.48 (d, 2 H), 7.58 (d, 2 H), 8.20 (d, 2 H), 8.32 (broad s, 3 H), 9.40 (t, 1 H), $[\alpha]_D = +14.0°$ (c = 1.1, MeOH), DCI/MS (m/z); 224. Anal. $C_{10}H_{14}N_3O_3Cl$; C,H,N.

L-2-(N-Benzyloxycarbonyl)aminopropionitrile **(35a)** – A solution of phosphoryl chloride (1.2 g, 7.8 mmol) in CH_2Cl_2 (2 mL) was added over 15 mins to an ice cooled solution of Z-L-Ala-NH_2 (2.2 g, 10 mmol) in pyridine (15 mL) and stirred at 0°C for 1.5 hrs. To this solution was added CH_2Cl_2 (50 mL) and the layers were separated. The aqueous layer was washed with CH_2Cl_2 (30 mL). All organic layers were pooled and washed with 1 N HCl until aqueous washes were acidic. The organic layer was dried ($MgSO_4$) and concentrated to yield 1.5 g (74%) of **35a.** mp 78°C,, IR (film); 2300 cm^{-1} (CN), ^1H NMR ($CDCl_3$) δ 1.57 (d, 3

Table 5. Bioisosteres and derivatives of L-alanine: binding inhibition and neural activity

#		$IC_{50}(\mu M)$	Neural response	
1		3.5	100%[a]	(24)
32		61	43.4 +/− 13.0%[b]	(18)
33		>1000	30.7 +/− 17.5%[b,c]	(13)
34		>1000	28.2 +/− 10.8%[d]	(9)
35		>1000	22.3 +/− 7.7%[c]	(6)
36		>1000	17.5 +/− 7.8%[c]	(6)
37		>1000	3.2 +/− 4.5%[c]	(5)
38		>1000	15.7 +/− 10.6%[c]	(6)

H), 4.69 (m, 1 H), 5.20 (s, 2 H), 7.37 (s, 5 H), $[\alpha]_D = -62.3°$ (c = 5.9, $CHCl_3$), DCI/MS (m/z); 205. Anal. $C_{11}H_{12}N_2O_2$; C,H,N.

L-1-(5-Tetrazolyl)-1-(N-benzyloxycarbonyl)aminoethane **(35b)** – To DMF (4 mL) was added **35a** (0.8 g, 3.9 mmol), NaN_3 (0.26 g, 4.1 mmol), and NH_4Cl (0.23 g, 4.3 mmol). The suspension was heated with stirring to 95–100°C for 24 hrs. The solution was cooled and 1 N HCl (20 mL) was added. The solution stirred and within a minute, a heavy white precipitate formed. This was filtered and washed with cold water (100 mL). The product was dissolved in MeOH and concentrated to yield 600 mgs (62%) of **35b** as a white crystal-

line solid. mp 137–138°C, ^{1}H NMR ($CDCl_3$) δ 1.62 (d, 3 H), 3.0–2.5 (broad s, 1 H), 5.11 (s, 2 H), 5.25 (m, 1 H), 6.65 (broad s, 1 H), 7.32 (s, 5 H), $[\alpha]_D = -34.6°$ (c = 2.2, MeOH), DCI/MS (m/z); 248. Anal. $C_{11}H_{13}N_5O_2 \cdot 1/8\ H_2O$; C,H,N.

L-1-(5-Tetrazolyl)-1-aminoethane · HCl **(35)** – To **35b** (600 mg, 2.4 mmol) was added 6 N HBr in HOAc (2 mL). This stirred for 45 mins. The material was concentrated to an oil and water (15 mL) was added. This solution was passed through a cation exchange column (0.7 × 20 cm) containing 8 mL AG 50W-X8 resin in the hydrogen form. The column was washed with 1 M NH_4OH. The basic eluent was concentrated to dryness and 1N HCl (15 mL) was added. This material was concentrated to yield 300 mg (83%) of **35.** mp 205–206°C, ^{1}H NMR (DMSO-d_6) δ 1.41 (d, 3 H), 3.95 (m, 1 H), 8.38 (broad s, 3 H), $[\alpha]_D = -10.6°$ (c = 1.5, water), DCI/MS (m/z); 144. Anal. $C_3H_8N_5Cl$; C,H,N.

1-Aminoethyl boronic acid **(37)** – Compound **37** was produced by the addition of diisopropyl [1-[bis(trimethylsilyl)amino]ethyl] boronate to H_2O. The racemic mixture was assayed without removal of the isopropanol and trisilanol byproducts.

Results and discussion

Both a receptor binding assay and an *in vivo* taste neural preparation were used to assess the relationship between the molecular structure of a taste stimulus and receptor binding and subsequent activation. Receptor binding studies were performed by testing structural analogues of L-alanine for their ability to inhibit the binding of L-[^{3}H]alanine to a previously characterized (Brand et al., 1987; Bryant et al., 1989a; Bryant et al., 1989b; Cagan, 1979; Cagan, 1986; Cagan and Holland, 1981;) L-alanine binding site in a partially purified membrane preparation obtained from catfish taste epithelium. In parallel studies, the ability of these analogues to activate taste receptors was determined by recording from the barbel facial nerve of an anesthetized channel catfish (Bryant et al., 1989b).

As shown in Table 1, and Fig. 3 and 4, chirality, size and linearity of the sidechain appear to be major factors in determining the extent to which deriva-

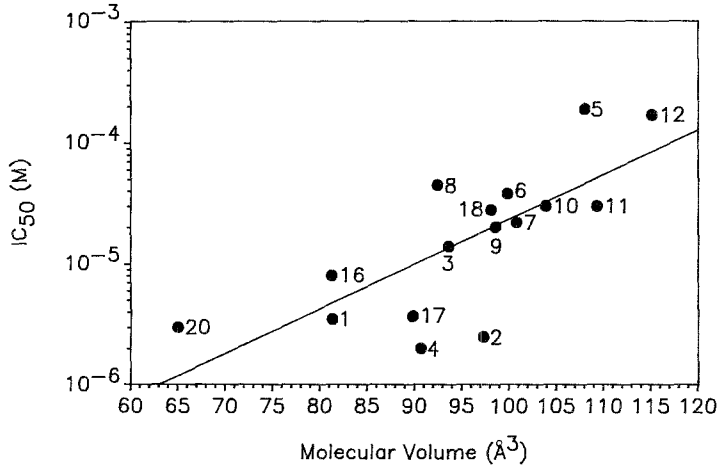


Fig. 3. Relationship of IC_{50} to molecular volume. A series of analogues comprising sidechain modifications of L-alanine is plotted, using the corresponding identification numbers from Tables 1 and 2 and the text. IC_{50} and molecular volumes were determined as described in the Materials and Methods section. The least squares linear regression is also plotted. The Pearson correlation coefficient of the two variables is 0.612

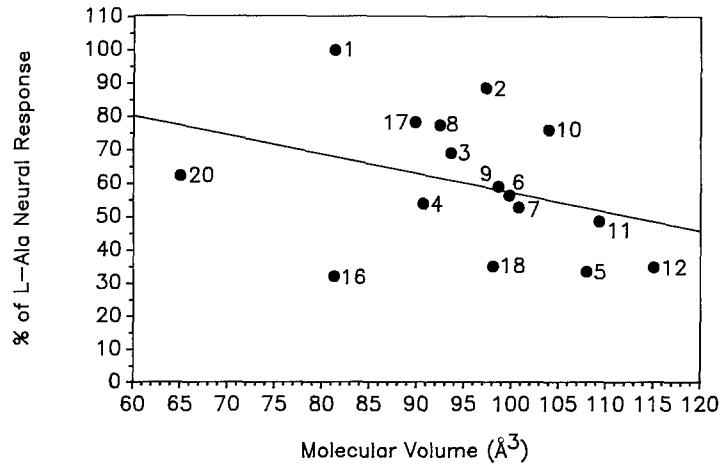


Fig. 4. Relationship of neural response to molecular volume. The same compounds as in Fig. 3 are plotted here. Neural responses were determined as described in the Experimental section. The least squares linear regression is also plotted. The Pearson correlation coefficient of these two variables is −0.343

tives of L-alanine first, bind to the receptor and second, generate neural responses. Figure 3, illustrating the relationship between molecular volume and IC_{50} (see Methods-Competitive Ligand Binding Assay), indicates that these variables are correlated (Pearson correlation, $r = 0.612$). Although there is same scatter around the regression line, it is clear that molecular volume is an important though not singular determinant of binding of the analogues to the alanine binding site. Compounds that are found above the regression line are, for their volume, inefficient inhibitors. This may be due to excess or insufficient disposition of molecular bulk near the alpha-carbon, i.e. compounds **5**, **8** and **20**. Those compounds found below the regression line, **2**, **4** and **17**, bind more efficiently than would be predicted by the regression relationship. Factors such as the presence of electronegative atoms in the sidechain may contribute to this enhanced binding for some of these compounds (see discussion of electronegativity below). With respect to the relationship between molecular volume and receptor activation, there appears to be a trend relating these two variables, although due to the low correlation (Fig. 3, Pearson correlation, $r = -0.343$), molecular volume alone is not a good predictor of neural response. For the linear sidechains, there is a significant decrease ($p < 0.05$) in stimulatory efficacy and an increase in IC_{50} with the addition of 2 and 3 methylenes (**9** and **12**, respectively) to the L-alanine sidechain. It is clear that if the sidechain of a derivative is long (**9** and **12**), branched (**5** and **13**) or bulky (**14**, **15** and **19**), binding inhibition is weak implying that bulky analogues do not bind well to the L-alanine site and consequently, neural response is very low.

The effect of introducing unsaturated carbon-carbon bonds into linear carbon sidechains has also been examined (Table 1, **8**, **10** and **11**). For the pentane series, as unsaturation increases from alkane to alkyne (**12** to **10**), there is a significant ($p < 0.05$) increase in neural response and parallel decrease (almost 6-fold) in IC_{50}. As the level of unsaturation increases, the volume of the sidechain

decreases. Thus, the pattern observed may be a further indication that binding of L-alanine to receptor sites and subsequent receptor activation are sensitive to sidechain volume.

Of the derivatives containing electronegative groups off the beta carbon of **1** (L-alanine) (**2**–**7**), **2** and **4** are the most similar to **1** in their ability to inhibit the binding of L-[^{3}H]alanine. Indeed, these compounds are more efficient inhibitors of L-[^{3}H]alanine binding than would be expected on the basis of their sidechain size (see Fig. 3). Although the electronegative character of the sidechain may help overcome the effect of increased molecular size in some cases (**2** and **4**), electronegativity appears not to be a general determinant of binding inhibition because there is no overall relationship (Pearson correlation, $r = 0.165$) between IC_{50} and sidechain electronegativity (values of electronegativity obtained from Inamoto and Mazuda, 1982).

A number of compounds in Table 3 (**21**–**27** and **29**), have not been included in the above discussion due to their extremely weak interaction ($IC_{50} > 1$ mM) with the L-alanine binding site. These compounds retain substantial neural activity (> 50% compared to L-alanine(100%)) while having little or no ability to displace L-[^{3}H]-alanine from its binding site. These results strongly suggest that these compounds are not acting at L-alanine binding sites, but at previously uncharacterized receptor sites. (Unpublished neurophysiological studies indicate that compound **29**, L-lactic acid acts at a receptor site independent from the receptor for L-alanine.)

The interaction of D-alanine (**16**) with L-[^{3}H]alanine binding sites has been examined previously (Brand et al., 1987). D-alanine inhibits the binding of L-[^{3}H]alanine to the taste tissue preparation with an apparent K_I of 10 μM. It also binds to a lower affinity population of D-alanine binding sites at which L-alanine is not an efficient inhibitor. This is in good agreement with results presented here, which confirm that **16** is a weak partial agonist at the L-alanine site. Thus, the neural response to **16** is below that predicted on the basis of an IC_{50} of 8 μM (Table 2).

The importance of how the disposition of sidechain volume in close proximity to the alpha carbon affects binding and receptor activation can be seen in the results obtained from compounds **20**, **1**, **16**, **17**, **18**, the molecular volumes of which are 65.0, 81.3, 81.3, 89.3 and 98.1 Å^3, respectively. Above a molecular volume of 90 Å^3 (**18**), the ability to inhibit L-[^{3}H]alanine binding (see also Fig. 3) is decreased. Given that the other compounds bind reasonably well to a receptor ($IC_{50} < 10^{-5}$ M), what determines their ability to activate the receptor? With the minimal sidechain volume, **20** is a good competitor of L-[^{3}H]alanine binding. Addition of an α-methyl in the L configuration produces **1**, the most potent agonist. The addition to **20** of other variously positioned methyl groups yields compounds of similarly low IC_{50}'s but variable agonist activity. Whether these compounds activate alanine receptors efficiently or not appears to be determined by the presence and orientation of a β-methyl or -methylene group. For example, **16**, D-alanine, as mentioned previously, inhibits L-[^{3}H]alanine binding well but is only a weak agonist. The addition of a second β-methyl (**18**) strongly reduced inhibitory activity, most likely because of bulkiness. However, if the methyl groups of **18** are forced closer together in a cyclopropyl ring

(**17**), both inhibitory activity and neural efficacy approach that of L-alanine. This interesting result implies that the presence of a β-methyl carbon fully occupying the position corresponding to the D configuration (**16**) is an important factor in preventing activation of the receptor by bound ligand.

All modifications of the carboxylic acid to either amides, esters (Table 3) or substitutions of the acid (Table 4) cause substantial reduction in both binding inhibition and neural efficacy. This implies that the carboxylic acid of the stimulus molecule must be charged and unhindered. Similar to the observation in a series of sweet compounds (Owens, 1990), the tetrazole bioisostere (**35**) is not an adequate substitution for the carboxylic acid in the present study. Modifications of the α-amine (**30** and **31**) also result in a significant loss of inhibitory ability and neural efficacy as indicated by data in Table 4. This suggests that a charged, unhindered amine is necessary for binding to and activation of the alanine receptor. This conclusion is further supported by the fact that the amine in **38** exists as an uncharged ammonia adduct to the alpha boron (Spielvogel et al., 1980). The substitution of a hydroxyl for the L-α-amine yields a good agonist (**29**) but poor inhibitor, which, as discussed earlier, indicates that its neural activity is due to action at another type of receptor.

Substitution of the α carbon by boron (Compound **38**) results in a compound that is inactive in both ligand binding and neural assays. This substitution results in 6–7% increases in bond lengths between the α atom and the acid and amine (Spielvogel et al., 1980), as well as slight changes in several bond angles. These changes of molecular geometry may be factors contributing to the inactivity of the compound.

Using the combined approach of biochemical and neurophysiological studies, the molecular determinants of alanine receptor binding and activation have been examined. In general agreement with an earlier neurophysiological study (Caprio, 1978), the importance of charged and unhindered amine and carboxylic acid groups for receptor activation has been demonstrated. The high neural activity of esters of L-alanine reported by Caprio (1978) was likely due to free L-alanine released by alkaline hydrolysis of the ester bond. The present biochemical study demonstrates that modification of the amine and carboxylic acid eliminates all ability to inhibit L-[^{3}H]alanine binding since no antagonists or partial agonists of the L-alanine receptor are observed in this group of analogues. These results demonstrate the rigid steric and electrostatic requirements for binding of the amine and the carboxylic acid at receptor subsites.

Effective interaction of the sidechain with the alanine receptor appears to be based in part on different factors. Steric factors appear to play a large role in determining binding inhibition and stimulatory efficacy as does the configuration about the α-carbon. Although the presence of electrophilic atoms in the sidechain might confer possible energetic advantages to the ligand-receptor interaction for same stimulus compounds, no such general relationship was observed.

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References

Akabas MH, Dodd J, Al-Awqati Q (1988) A bitter substance induces a rise in intracellular calcium in a subpopulation of rat taste cells. Science 242: 985–1100

Bidlingmayer BA, Cohen SA, Tarvin TL (1984) Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 336: 93–104

Brand JG, Bryant BP, Cagan RH, Kalinoski DL (1987) Biochemical studies in taste sensation. XIII. Enantiomeric specificity of the alanine taste receptors in the catfish, *Ictalurus punctatus*. Brain Res 416: 119–128

Brand JG, Teeter JH, Cagan RH, Kare MR (eds) (1989) Chemical senses, vol 1: Receptor events and transduction in taste and olfaction. Marcel Dekker, New York

Bryant BP, Brand JG, Kalinoski DL (1989a) Receptor site specificity in taste: Characterization of amino acid taste receptors from the cutaneous taste system of *Ictalurus punctatus*. In: Chemical senses, vol 1: Receptor events and transduction in taste and olfaction. Marcel Dekker, New York, pp 35–53

Bryant BP, Harpaz S, Brand JG (1989b) Structure/activity relationships in the arginine taste pathway of the channel catfish. Chem Senses 14: 805–815

Cagan RH (1979) Biochemical studies in taste sensation. VII. Enhancement of taste stimulus binding to a catfish receptor preparation by prior exposure to the stimulus. J Neurobiol 10: 207–220

Cagan RH (1986) Biochemical studies of taste sensation. XII. Specificity of binding of taste ligands to a sedimentable fraction from catfish taste tissue. Comp Biochem Physiol 85A: 355–358

Cagan RH, Holland KN (1981) Relationship of biochemical recognition of taste compounds to behavioral and physiological responses in catfish, *Ictalurus punctatus*. Determination of Behavior by Chemical Stimuli. IRL Press, London, pp 7–15

Caprio J (1975) High sensitivity of catfish taste receptors to amino acids. Comp Biochem Physiol 52A: 247–251

Caprio J (1976) Olfactory and gustatory responses of catfish to amino acids and their derivatives. PhD Thesis. Florida State University, Tallahassee

Caprio J (1978) Olfaction and taste in the channel catfish: An electrophysiological study of responses to amino acids and their derivatives. J Comp Physiol 132: 357–371

Caprio J (1982) High sensitivity and specificity of olfactory and gustatory receptors of catfish to amino acids. In: Chemoreception in fishes. Elsevier, Amsterdam, pp 109–134

Caprio J, Robinson JJ (1978) Adaptation and cross-adaptation of the peripheral olfactory and gustatory systems of the catfish to amino acids. Soc Neurosci Abstr 4: 86

Caprio J, Tucker D (1976) Two classes of amino acid taste fibers and receptors in the catfish. Soc Neurosci Abstr 2: 152

Davenport CJ, Caprio J (1982) Taste and tactile recordings from the *ramus recurrens facialis* innervating flank taste buds in the catfish. J Comp Physiol 147: 217–229

Duncan K, Faraci WS, Matteson DS, Walsh CT (1989) (1-aminoethyl)-boronic acid: A novel inhibitor for *Bacillus stearotherophilus* alanine racemase and *Salmonella typhimurium* D-alanine:D-alanine ligase (ADP forming). Biochemistry 28: 3541–3549

Hanessian S, Sahoo SP (1984) A novel and efficient synthesis of L-vinylglycine. Tetra Lett 25: 1425–1428

Inamoto N, Mazuda S (1982) Revised methods for calculation of group electronegativities. Chem Lett: 1003–1006

Kalinoski DL, Bryant BP, Shaulsky G, Brand JG, Harpaz S (1989a) Specific L-arginine taste receptor sites in the catfish, *Ictalurus punctatus*: biochemical and neurophysiological characterization. Brain Res 488: 163–173

Kalinoski DL, Huque T, LaMorte VJ, Brand JG (1989b) Second-messenger events in taste. In: Chemical Senses, vol 1: Receptor events and transduction in taste and olfaction. Marcel Dekker, New York, pp 85–101

Kanwal JS, Caprio J (1983) An electrophysiological investigation of the oro-pharyngeal (IX-X) taste system of the channel catfish, *Ictalurus punctatus*. J Comp Physiol 150: 345–357

Kinnamon SC (1988) Taste transduction: A diversity of mechanisms. Trends Neural Sci 11: 491–496

Krueger JM, Cagan RH (1976) Biochemical studies of taste sensation. IV. Binding of L-[^{3}H]-alanine to a sedimentable fraction from catfish barbel epithelium. J Biol Chem 25: 88–97

Kumazawa T, Teeter JH, Brand JG (1990) L-proline activates cation channels in isloated catfish taste epithelial membranes. Chem Senses 15: 603

Owens WH (1990) Tetrazoles as carboxylic acid surrogates in the suosan sweetener series. J Pharm Sci 79: 826–828

Spielvogel BF, Das MK, McPhail AT, Onan KD, Hall IH (1980) Boron analogues of the alpha-amino acids. Synthesis, x-ray crystal structure, and biological activity of ammonia-carboxyborane, the boron analogue of glycine. J Am Chem Soc 102: 6343–6344

Still WC, Mohamadi F, Richards NJG, Guida WC, Lipton M, Liskamp R, Chang G, Hendrickson T, DeGunst F, Hasel W. MacroModel Version 2.5, Department of Chemistry, Columbia University, New York

Teeter JH, Brand JG (1987) L-arginine-activated cation channels from the catfish taste epithelium. Soc Neurosci Abstr 13: 361

Teeter JH, Brand JG, Kumazawa T (1990) A stimulus activated conductance in isolated taste epithelial membranes. Biophys J 58: 253–259

Velluz L, Amiard G, Heymes R (1954) No. 213-Utilisation d'intermediaires N-benzyles en synthese peptidique. I.-Chlorures de N-benzyl (alpha)-amino-acides. Bull Soc Chim Fr: 1012

Windholz M, Budavari S, Blumetti RF, Otterbein ES (eds) (1983) The Merck Index. Merck & Co, Rahway, p 964

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