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Complex Binding Interactions between Multicomponent Mixtures and Odorant Receptors in the Olfactory Organ of the Caribbean Spiny Lobster *Panulirus argus*

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Abstract

Our study was designed to examine how components of complex mixtures can inhibit the binding of other components to receptor sites in the olfactory system of the spiny lobster *Panulirus argus*. Biochemical binding assays were used to study how two- to six-component mixtures inhibit binding of the radiolabeled odorants taurine, L-glutamate and adenosine-5'-monophosphate to a tissue fraction rich in dendritic membrane of olfactory receptor neurons. Our results indicate that binding inhibition by mixtures can be large and is dependent on the nature of the odorant ligand and on the concentration and composition of the mixture. The binding inhibition by mixtures of structurally related components was generally predicted using a competitive binding model and binding inhibition data for the individual components. This was not the case for binding inhibition by most mixtures of structurally unrelated odorants. The binding inhibition for these mixtures was generally smaller than that for one or more of their components, indicating that complex binding interactions between components can reduce their ability to inhibit binding. The magnitude of binding inhibition was influenced more by the mixture's precise composition than by the number of components in it, since mixtures with few components were sometimes more inhibitory than mixtures with more components. These findings raise the possibility that complex binding interactions between components of a mixture and their receptors may shape the output of olfactory receptor neurons to complex mixtures.

Introduction

Most odorants that are important signals for animals are mixtures of many compounds. Yet how such complex signals are encoded by olfactory receptor neurons (ORNs) has received little attention. In contrast, the transduction and coding of single odorant compounds has been much better studied (Ache, 1994; Zufall *et al.*, 1994; Restrepo *et al.*, 1996), leading to the following scheme. Odorant molecules are detected by receptors located on the dendrites of ORNs. The activated receptors then stimulate G-protein-modulated inhibitory or excitatory second messenger cascades. These cascades affect the membrane potential by gating ion channels either directly or indirectly through phosphorylation of proteins. Individual chemoreceptor cells of many species can express more than one type of receptor protein (Kang and Caprio, 1991; Troemel *et al.*, 1995; Glendinning and Hills, 1996; Kashiwayanagi *et al.*, 1996; Cromarty and Derby, 1997), second messenger cascade (Dionne and Dubin, 1994; Ache and Zhainazarov; 1995) and ion channel, including those with opposing effects (Ache, 1994; Restrepo *et al.*, 1996). Therefore, the net response of an ORN to an odorant compound depends on which receptor proteins on the cell are activated by that odorant and the nature of the coupling of the activated receptors to effector pathways.

The response of an ORN to a mixture is not entirely predictable when it is based only on the responses to the components presented individually. Such unpredictable events have been called mixture interactions (Laing *et al.*, 1984), and include mixture suppression and enhancement, in which the response to a mixture is less than (suppression) or greater than (enhancement) predicted from the responses to the individual components. The predictability of the response of an ORN to a mixture should be considerably improved if it is known how each component of the mixture affects the various transduction cascades of that cell. This includes knowing not only the actions of each component alone but also how the components modulate the transduction of each other. One example is that odorants might directly bind to ion channels and suppress or enhance their activity (Kurahashi *et al.*, 1994; Kawai *et al.*, 1997).

Another is that the components in a mixture might inhibit or facilitate the binding of one another to their receptor sites (Dethier, 1982; Olson and Derby, 1995). In this second example, these binding interactions might be competitive, in which case the components compete for and either activate (agonism) or do not activate (antagonism) the same sites on the receptor. The binding interactions can also be non-competitive, in which the components bind to different sites on a receptor molecule but can nonetheless allosterically facilitate or inhibit the binding of each other to their respective sites. For example, responses to binary and even larger mixtures by gustatory and olfactory systems of catfish are generally predictable when it is known whether or not the components are competitive agonists (Kang and Caprio, 1991; Kohbara and Caprio, 1996).

The Caribbean spiny lobster *Panulirus argus* has been used to demonstrate the importance of several transduction events in generating responses of ORNs to binary mixtures. For example, it is known from physiological studies that individual ORNs can have multiple types of receptors (Cromarty and Derby, 1997), second messengers (Boekhoff *et al.*, 1994) and ion channels (Michel and Ache 1992; Hatt and Ache, 1994), which can mediate either excitation or inhibition. Two mechanisms of mixture suppression are known. Mixture suppression can be caused by components of binary mixtures inhibiting the binding of each other through competitive or non-competitive antagonism, as demonstrated by radioligand binding (Olson and Derby, 1995; Daniel *et al.*, 1996; Burgess and Derby, 1997). In addition, mixture suppression can result from components of a mixture activating different and opposing ionic conductances in single cells (Ache, 1994).

Daniel *et al.* (1996) compared the ability of several models to predict the responses of ORNs to binary odorant mixtures and found that the responses of ORNs to these mixtures were best predicted by a non-competitive model with a correction for binding inhibition. This model incorporates excitatory or inhibitory responses of ORNs to the two components and reciprocal binding inhibition between the components. It is based on binding inhibition data for the radioligands taurine, L-glutamate, and adenosine-5′-monophosphate (Olson and Derby, 1995; Burgess and Derby, 1997).

Our current study was designed to examine the importance of binding inhibition to the coding of mixtures that contain up to seven components. A biochemical binding assay was used to study how two- to six-component mixtures inhibit binding of the radiolabeled odorants taurine, L-glutamate and adenosine-5′-monophosphate to a tissue rich in dendritic membrane from ORNs. Our aims were to determine the magnitude of binding inhibition for complex mixtures and whether the binding inhibition for complex mixtures could be predicted from the inhibition caused by individual components.

Materials and methods

Chemicals

[^{3}H]Adenosine-5′-monophosphate (sp. act. 19.4–22.0 Ci/mmol) and [^{3}H]L-glutamate (49.0 Ci/mmol) were purchased from Amersham Life Sciences (Arlington Heights, IL). [^{3}H]Taurine (sp. act. 19.7–21.0 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). All other chemicals were purchased from Sigma.

Buffers

A Tris buffer containing sucrose instead of sodium chloride, with the following composition, was used in all assays for AMP and taurine binding: 50 mM KCl, 10 mM Trizma base, 320 mM sucrose, 12.9 mM $CaCl_2 \cdot H_2O$, 23.1 mM $MgCl_2 \cdot 6H_2O$ and 25.6 mM $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.8. Levels of AMP and taurine binding were satisfactory under these buffering conditions, as in previous studies using this buffer (Olson *et al.*, 1992; Olson and Derby, 1995). However, levels of L-glutamate binding were low in this Tris-sucrose buffer (Burgess and Derby, 1997). In our current study of L-glutamate binding, we used a Tris buffer that is similar to the saline used in physiological experiments (e.g. Cromarty and Derby, 1997), except that it contained Tris rather than HEPES. This Tris buffer contained sodium rather than sucrose, since binding levels in Tris–sodium buffer were elevated compared with Tris–sucrose buffer and association was rapid and saturable, thus demonstrating binding rather than uptake (unpublished results). This Tris–sodium buffer had the following composition: 10 mM KCl, 3.5 mM Trizma base, 480 mM NaCl, 17 mM $CaCl_2 \cdot H_2O$, 16 mM $MgCl_2 \cdot 6H_2O$, 21 mM $NaSO_4 \cdot 7H_2O$ and 1.66 mM glucose at pH 7.8. All buffers were filtered using Whatman cellulose nitrate membrane filters (0.45 μm pore size).

Animals

Lateral filaments of the antennules, which represent the olfactory organ of spiny lobsters, were collected in Tris–sucrose buffer, maintained on dry ice and frozen at –80°C. For binding experiments, antennules were thawed and maintained at 4°C in either the Tris–sucrose or Tris–sodium buffer.

Tissue preparation

Our method of collecting a pellet rich in membranes from ORNs of aesthetasc sensilla is similar to that described previously (Olson *et al.*, 1992; Olson and Derby, 1995; Burgess and Derby, 1997). Aesthetasc sensilla contain (i) dendrites of ORNs, in which are located their odorant-binding receptor sites (Blaustein *et al.*, 1993), and (ii) distal processes of supporting cells (Grünert and Ache, 1988). Thus, by selectively collecting aesthetasc sensilla, we can harvest a tissue fraction highly enriched in olfactory

dendritic membrane and their olfactory receptor proteins. Aesthetasc sensilla were manually removed from antennules by scraping with a scalpel and placing them in Tris buffer at 4°C. All experimental procedures were performed at this temperature. The sensilla were homogenized with a glass–glass homogenizer. The homogenized tissue was then sonicated and centrifuged at 6000 *g* for 10 min. The supernatant (S1) was removed and stored at 4°C, and the precipitate (P1) was resuspended in incubation buffer and centrifuged again under the same conditions. The supernatant was again removed, combined with the S1 and centrifuged at 150 000 *g* for 30 min. The S2 was discarded while the pellet was resuspended in 1 ml of buffer and centrifuged again under the same conditions. The resulting P2 was resuspended in Tris buffer. Transmission electron microscopy of the P2 tissue fraction verified that it is highly enriched in plasma membrane (unpublished data).

Binding assay

Binding activity of the olfactory tissue was assayed by incubating aliquots of the P2 tissue fraction and radiolabeled odorants in buffer at 4°C. The protein concentration of the tissue placed in each tube was ~15–25 μg. Protein concentrations were measured according to Bradford (1976) using bovine serum albumin as a standard. The P2 tissue fraction was incubated in 1 μM radiolabeled odorant in a total volume of 50 μl for 60 min. Following incubation, bound radioligand was separated from free radioligand by rapid filtration under vacuum through 0.45 μm pore-size cellulose acetate filters (HAWP, Millipore, Bedford, MA), presoaked in 0.3% polyethylenimine in buffer, using a Hoefer vacuum filtration manifold. Filters were washed with two 5 ml volumes of cold buffer until dry. They were then dissolved by placing them in scintillation vials containing 1.3 ml of ethylene glycol monomethylether for 30 min. Ecolite(+) scintillation fluid (ICN Biomedical, Costa Mesa, CA) was added to the vials and radioactivity was measured using a Beckman LS 6500 liquid scintillation counter.

Inhibition assays

Previous studies have shown that the association of AMP, L-glutamate and taurine to *P. argus* olfactory membrane is rapid, reversible and specific, which is indicative of binding to olfactory receptors (Olson *et al.*, 1992; Olson and Derby, 1995; Burgess and Derby, 1997). These studies show that inhibition assays can be used to examine how an unlabeled odorant compound can affect binding of another odorant to the P2 tissue fraction. In the current study, inhibition binding assays were used to examine the effect of complex odorant mixtures on the binding of the radiolabeled odorants AMP, L-glutamate and taurine. Total binding was measured as the amount of binding of 1 μM tritiated odorant in the absence of any unlabeled compound. Non-specific binding was determined by incubating 1 μM tritiated odorant and 1 mM of the unlabeled version of the same odorant. Specific binding was defined as the difference between the total binding and the non-specific binding. The inhibition of binding of the radioligands to the P2 tissue fraction was determined in the presence of numerous compounds and mixtures at a range of concentrations (see next section). These experiments were performed 2–4 times in triplicate at each concentration. Inhibition curves were used to compare the amount of binding inhibition in the presence of different mixtures.

Single compounds and mixtures as inhibitors

The odorant compounds used in this study are listed in Table 1. These compounds were selected because each is a component of natural food odors for spiny lobsters (Carr *et al.*, 1996). In addition, these single compounds have been studied in several electrophysiological, behavioral and biochemical studies. In general, these compounds, when tested individually, have been shown to be partial and, in most cases, non-competitive inhibitors of AMP, L-glutamate and taurine binding (Olson and Derby, 1995; Burgess and Derby, 1997).

Prior binding, electrophysiological and behavioral experiments show that specific combinations of components in binary mixtures produce mixture interactions (Daniel and Derby, 1991; Derby *et al.*, 1991; Lynn *et al.*, 1994; Olson and

Table 1 Compounds tested as inhibitors

Structurally unrelated analogues for all three radioligands	Structurally related analogues for [^{3}H]AMP binding	Structurally related analogues for [^{3}H]glutamate binding	Structurally related analogues for [^{3}H]taurine binding
Adenosine-5′-monophosphate (AMP, A)	xanthosine-5′-monophosphate (XMP, X)	*N*-methyl-D-aspartic acid (NMDA, M)	hypotaurine (H)
Betaine (B)	6-chloropurine (Ch)		β-alanine (β)
L-Cysteine (C)			
L-Glutamate (G)			
Ammonium chloride (N)			
DL-Succinate (S)			
Taurine (T)			

Derby, 1995; Daniel *et al.*, 1996; Burgess and Derby, 1997). Our current study examines the interactions between components in mixtures composed of a larger number of compounds. However, the number of all possible mixtures containing between two and six components based on seven compounds is too large to study. Therefore, the 36 mixtures listed in Tables 2–4 were tested as inhibitors in our study. Our selection of mixtures was biased towards those containing AMP, glutamate, taurine and cysteine, since these compounds are highly excitatory and/or inhibitory for ORNs (e.g. Steullet and Derby, 1997), and in prior biochemical studies the basic binding properties of receptors for AMP, glutamate and taurine have been characterized (Olson and Derby, 1995; Burgess and Derby, 1997). These 36 mixtures are also currently being tested in electrophysiological experiments of ORNs to evaluate the functional importance of the binding interactions described here.

In addition to these odorants, competitors for each receptor system were tested so that their inhibitory effects could be compared with the effects of mixtures of non-competitive odorants (e.g. Cromarty and Derby, 1997). Competitors were identified based on three criteria: (i) they are structurally similar to each other; (ii) based on binding studies, they compete for the same receptors; and (iii) based on electrophysiological studies, they act as agonists for the same receptors (for AMP receptors: Derby *et al.*, 1984; Olson and Derby, 1995; for taurine receptors: Olson and Derby, 1995; Cromarty and Derby, 1997; for L-glutamate receptors: Burgess and Derby, 1997). For the AMP and taurine receptors, two structurally related competitors were tested individually and combined as a mixture; for the glutamate receptors, only one competitor was tested (Table 1).

The effects of a mixture can be predicted from knowing (i) if the components are competitors and (ii) the inhibitory effect of each individual component. A mixture of purely competitive compounds, each of which is presented at a concentration that produces the same amount of binding inhibition, is expected to inhibit the binding of the radiolabeled odorant only as much as either of that mixture's individual components. Inhibition by a mixture of non-competitive compounds, each acting through a different binding site from the others, should be greater than that of any component and should approach the sum of the inhibition by the components if all of their effects are indeed independent of each other. Mixtures composed of both competitive inhibitors and non-competitive inhibitors are expected to produce inhibition between the two extremes of purely competitive and purely non-competitive inhibition. If the results are different from these, binding interactions are indicated. Examples of binding interactions in which a mixture produces less binding inhibition than an equimolar concentration of any of its components have been reported for some binary mixtures (Olson and Derby, 1995).

In each experiment, components in the mixtures were equimolar to each other and tested over various concentrations from 10^{-7} to 10^{-3} M. Therefore, the total concentration of the inhibitor varied depending on the number of components in a given mixture. The percentage of specific binding of the radioligand was then quantified at these various concentrations, as described below.

Data analysis

Specific binding is the difference between the total binding and the non-specific binding. The ability of odorants to inhibit specific binding was expressed as a percentage of specific binding in the absence of inhibitors, and was calculated as follows:

$$\% \text{ inhibition} = (1 - SB_I/SB_0) \times 100 \quad (1)$$

where SB_I is the specific binding in the presence of an unlabeled inhibitor I and SB_0 is specific binding in the absence of inhibitors. This value was used to compare the inhibition by different mixtures. From the inhibition/competition assays, inhibition curves were generated using INPLOT (Graph Pad, Inc).

It was also necessary to calculate the inhibition caused by single components at concentrations not actually measured so that inhibition by single components and mixtures could be compared at concentrations of complex mixtures. For example, two-component mixtures were tested at 0.2, 2 and 20 μM, but their components were tested at 0.1, 1, 10, 100 and 1000 μM. Thus, the inhibition data for each component were fitted with either a linear or non-linear regression equation which was used to determine the binding inhibition at any concentration, including 2 μM for comparison with two-component mixtures, 3 μM for comparison with three-component mixtures and so forth. Regression analysis was performed using INPLOT.

Statistical analysis

Mean ± SEM values for % specific binding were determined for all mixtures at each concentration tested. For inhibition curves, the *F*-statistic was used to compare whether a one-site or two-site affinity curve best fit the data (Motulsky and Ransas, 1987). For comparison of calculated and measured values for individual components and mixtures, calculated values lying outside the 95% confidence limits of the measured values were considered significantly different.

Results

The results are organized into three sections according to binding interactions with each of the three principal natural odorants: taurine, AMP and L-glutamate. Within each section we describe the following: (i) inhibition by the unlabeled version of the radiolabeled odorant; (ii) inhibition by single compounds and mixtures that are

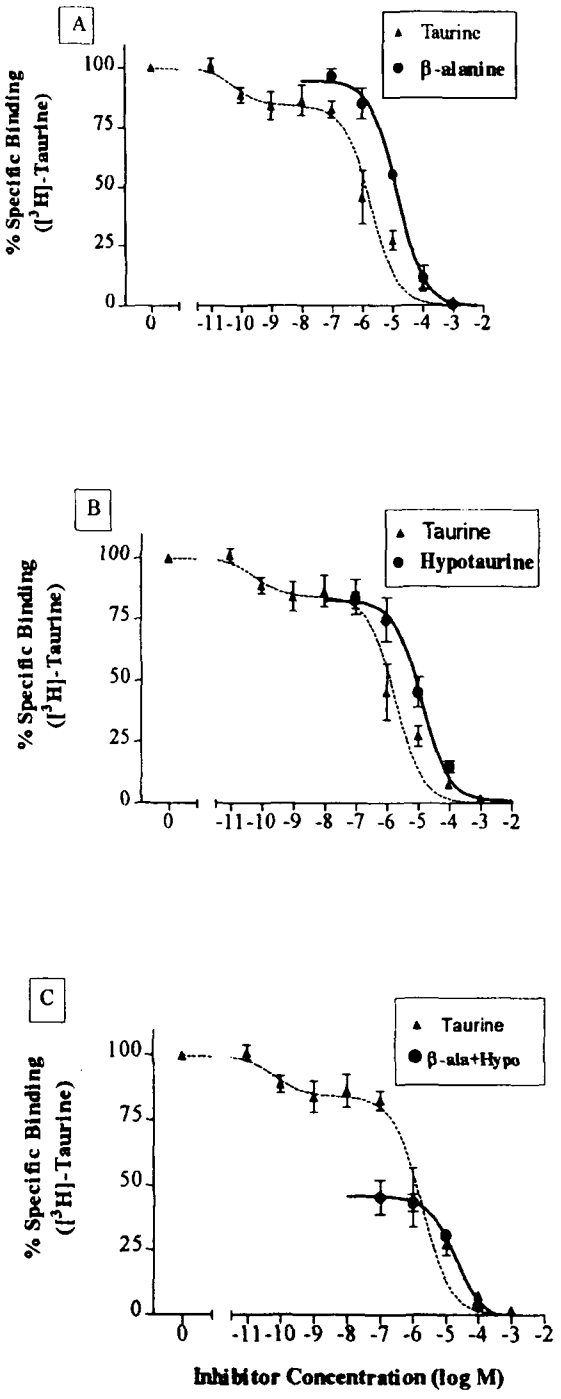

Figure 1 Inhibition of binding of 1 μM [^{3}H]taurine by unlabeled taurine (dashed line) and structural analogues of taurine (solid lines). P2 was incubated with 1 μM [^{3}H]taurine plus varying concentrations of unlabeled β-alanine (A), hypotaurine (B) or a mixture of β-alanine + hypotaurine (C). Values are means ± SEM for two experiments each run in triplicate.

structural analogues of the radiolabeled odorant; (iii) inhibition by single compounds that are structurally unrelated to the radiolabeled odorant; and (iv) inhibition by mixtures of compounds that are structurally unrelated to the radiolabeled odorant.

Taurine receptors

Inhibition by unlabeled taurine

The inhibition experiments using unlabeled taurine from 10^{-11} to 10^{-3} M showed that a two-site competition curve best fit the data ($r^2 = 0.96$, $P < 0.0001$) (Figure 1). Sixteen percent of the sites were high affinity ($IC_{50} = 62.5 \pm 0.4$ pM) and 84% of the sites were low affinity ($IC_{50} = 1.9 \pm 0.13$ μM). These results are consistent with those from past studies of this receptor type (Olson and Derby, 1995).

Inhibition by structural analogues of taurine

Two structural analogues of taurine, β-alanine and hypotaurine, are expected to act as competitors for the taurine receptors since they act as agonists of taurine receptors in physiological studies (Cromarty and Derby, 1997). The inhibition data for β-alanine and hypotaurine were fit best by one-site inhibition curves ($r^2 = 0.95$, $P < 0.05$ and $r^2 = 0.85$, $P < 0.05$ respectively). The data did not fit two-site curves, as did taurine, because β-alanine and hypotaurine were tested only at concentrations between 10^{-7} and 10^{-3} M, thus revealing only the low-affinity receptor. The curves for both β-alanine and hypotaurine were shifted slightly from the low-affinity site for taurine (taurine $IC_{50} = 1.85 \pm 0.13$ μM; β-alanine $IC_{50} = 11.2 \pm$ μM; hypotaurine $IC_{50} = 12.7 \pm 1.6$ μM). Therefore, based on these binding curves, both β-alanine and hypotaurine appear to be true competitors for the low-affinity taurine receptors (Figure 1A,B). However, when β-alanine and hypotaurine were combined in a mixture, at low concentrations the inhibition by this mixture was greater than that for unlabeled taurine, β-alanine or hypotaurine alone at the concentration of the mixture (Figure 1C). This may indicate that when β-alanine and hypotaurine are mixed, they have inhibitory effects on taurine binding in addition to their individual competitive effects, resulting in enhanced inhibition of taurine binding.

Inhibition of taurine binding by single odorants that are structurally unrelated to taurine

Our results with the six single odorants that are structurally dissimilar to taurine (Table 1) show that AMP, L-cysteine, ammonium, DL-succinate and L-glutamate apparently inhibit taurine binding through non-competitive inhibition, as indicated by inhibition binding curves that do not fit either one- or two-site competition regressions (data not shown). Betaine appears to be a highly effective competitive inhibitor of taurine binding and can completely inhibit taurine binding at concentrations near 1 mM (data not shown). Since these results confirm those of Olson and Derby (1995), responses are shown only to selected concentrations (Table 2).

Inhibition of taurine binding by mixtures of odorants structurally unrelated to taurine

The effects on taurine binding of select concentration of nine mixtures of structurally dissimilar compounds are given in Table 2. These mixtures include some (AG, CG,

Table 2 Inhibition of [^{3}H]taurine binding by mixtures of structurally unrelated compounds

Mixture	Regression with best and significant fit ($P < 0.05$)	Max. inhibition value for mixture at 1 mM (in %)	Inhibition value for mixture and components at 1 μM (in %)							Inhibition value for mixture at $n \times 1$ μM (in %)							Type of binding interaction among components
			Mix	A	B	C	G	N	S	Mix	A	B	C	G	N	S	
AG	linear	35	14	12			20			12	13			16			non-additive
CG	linear	23	0			10[a]	20[a]			8			19[a]	16[a]			complex
ACG	one-site non-linear	40	8	12		10	20[a]			10	13	20		16			complex
CGN	none	50	18			10	20	12		11			20	16	18		non-additive
ACGN	linear	27	5	12		10	20[a]	12		4	13		20[a]	17[a]	18[a]		complex
BCGN	one-site non-linear	100	31		36	10[a]	20[a]	12		36		59[a]	20	17[a]	18[a]		complex
BCGNS	one-site non-linear	100	24		36[a]	10[a]	20	12	28	36		64[a]	20[a]	17[a]	18[a]	37	complex
ABCGN	one-site non-linear	100	29	12[a]	36	10[a]	20	12		52	13[a]	64	20[a]	17[a]	18[a]		non-additive
ABCGNS	one-site non-linear	100	5	12	36[a]	10	20[a]	12	28	33	23[a]	64[a]	20[a]	17[a]	18[a]	37	complex

'Inhibition value for mixture' represents mean % inhibition for the mixture and that mixture's individual components. n = number of components in the mixture; for example, for two-component mixture, $n = 2$. For 'Type of binding interaction among components', 'complex' means that inhibition by the mixture was less than that of the most effective component at its concentration in the mixture, and 'non-additive' means that inhibition by each was not different from the inhibition by their components, although each mixture's inhibition was not the sum of its components' effects. The data for each mixture and the single compounds are from at least three binding experiments each performed in triplicate. The results for the single compounds are combined data from our study, from Olson and Derby (1995) and from Burgess and Derby (1997).

[a]The % inhibition value for the individual component is significantly different from the % inhibition value for the mixture.

ACG, CGN, ACGN) whose components individually are non-competitive inhibitors of taurine binding and other mixtures (BCGN, BCGNS, ABCGN, ABCGNS) that contain one component, betaine, that is a competitive inhibitor of taurine binding and other components that are non-competitive inhibitors of taurine binding.

The maximal inhibition values were 100% for all mixtures containing betaine and 23–50% for mixtures not containing betaine. When tested at 1 μM (i.e. equimolar to [^{3}H]taurine), mixtures had inhibition values that ranged from 0 (CG) to 31% (BCGN) and were <20% for most mixtures (CG, ACGN, ABCGNS, ACG, AG, CGN). Mixtures containing betaine were highly inhibitory because betaine alone was a highly effective competitive inhibitor of taurine binding (current data; Olson and Derby, 1995).

To determine if the inhibitory effect of a mixture is predictable from the data on inhibition by the mixture's components and the principles of either competitive or non-competitive binding, we compared the inhibitory effects of the mixtures and the components at equimolar concentrations. Since our protocol did not allow us to test all mixtures and components at equimolar concentrations, regression analyses of the inhibition data were used to estimate the amount of inhibition caused by any concentration of each mixture and by each individual component in a mixture. Our regression analysis showed that all four mixtures containing betaine were best fit by a one-site non-linear regression. This reflects the dominating effect of betaine in the mixture, since betaine itself is the most inhibitory single component whose inhibition data are best fit by a one-site curve. For mixtures not containing betaine, all except one were fit by linear regression or no regression at all. The mixture CGN is shown as an example (Figure 2A). The one exception, ACG, was fit by a one-site curve, even though none of its components had one-site inhibition curves (Figure 2A). The one-site curve for the mixture ACG probably does not mean that the components A, C and G are competing for taurine binding sites.

From these regressions, inhibitory values for most odorant concentrations could be determined. As an example of this procedure, inhibition by a three-component mixture can be compared with inhibition by each of the three components in the mixture at equimolar concentrations, even though each component was tested at 0.1, 1 and 10 μM and the mixture was tested at 0.3, 3 and 30 μM. Regressions allowed us to estimate the inhibition by each component at 3 μM and the mixture at 1 μM, allowing for equimolar comparisons at both 1 and 3 μM.

Table 2 shows a comparison for inhibition of taurine binding by mixtures and individual components. If all components in a mixture non-competitively inhibit taurine binding by interacting with different and independent sites

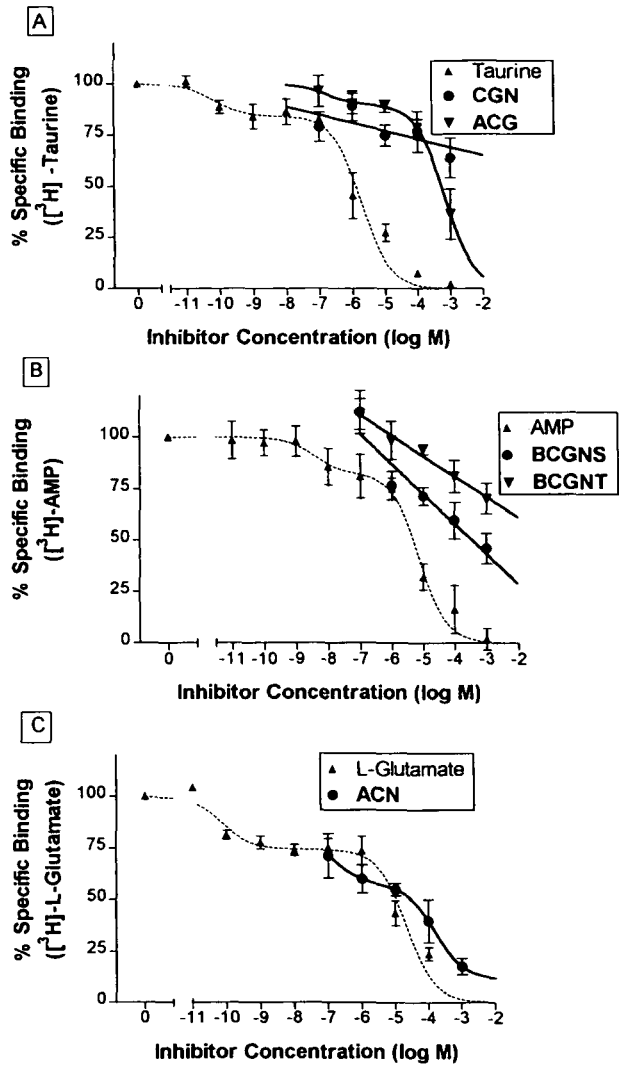

Figure 2 Inhibition of binding of 1 μM tritiated odorants by mixtures of unlabeled structurally unrelated odorants. (A) Binding of 1 μM [^{3}H]taurine in the presence of the mixtures CGN and ACG (bold lines). The inhibition curve for unlabeled taurine (dashed line, from Figure 1) is shown for comparison. (B) Binding of 1 μM [^{3}H]AMP in the presence of the mixtures BCGNS and BCGNT (bold lines). The inhibition curve for unlabeled AMP (dashed line, from Figure 3) is shown for comparison. (C) Binding of 1 μM [^{3}H]L-glutamate in the presence of the mixture ACN (bold line). The inhibition curve for unlabeled L-glutamate (dashed line, from Figure 4) is shown for comparison.

on the taurine receptor, then the inhibition value for the mixture is expected to be greater than the inhibition value for each of the mixture's components at its concentration in the mixture, and would approximate the sum of the inhibition values for the components. If the components in a mixture competitively inhibit taurine binding by interacting with identical sites, then the inhibition by the mixture should not be greater than the inhibition caused by any component in the mixture at its concentration in the mixture.

The most common result for mixtures was a 'complex binding interaction', in which the inhibition of taurine binding by the mixture was less than that of the most effective component at its concentration in that mixture. For example, for the mixture CG (Table 2), both C and G at 1 μM were more inhibitory (10 and 20% inhibition, respectively) than CG at either 1 (0%) or 2 μM (8%). Thus, the inhibitory effect of both C and G on taurine binding was reduced upon mixing them. In a second example of complex binding interactions, for ACGN, 1 μM G was more inhibitory (20%) than ACGN at either 1 (5%) or 4 μM (4%). This type of complex binding interaction also occurred for ACG, ACGN, BCGN, BCGNS and ABCGNS.

There were no examples of a mixture whose inhibitory effect on taurine binding was the sum of the inhibitory effects of its components, and thus it is unlikely that the components are non-competitively inhibiting taurine binding by each interacting with sites completely independent of the other components.

For the mixtures AG and CGN, the inhibition was not different from the inhibition by their components, although each mixture's inhibition was not the sum of its components' effects. For example, for AG, taurine binding was inhibited by 12% in 1 μM A, 20% in 1 μM G, 14% in 1 μM AG and 12% in 2 μM AG, all of which are not significantly different. Furthermore, the inhibition values for 2 μM AG (12%), 2 μM A (13%) and 2 μM G (16%) were not significantly different, showing that AG does not inhibit the binding of taurine any greater than either one of the individual components. This indicates that there may be competitive interactions between A and G when combined to form the mixture AG, such that A and G are competing with taurine and/or each other for binding sites.

The composition of a mixture was sometimes more important than its number of components in determining the mixture's inhibitory effect on binding. For example, the two-component mixture AG caused greater inhibition of taurine binding than either ACGN or ABCGNS at 1 μM (Table 2).

AMP receptors

Inhibition by unlabeled AMP

Two affinity sites exist for the AMP receptors between 10^{-11} and 10^{-3} M ($r^2 = 0.81$, $P < 0.05$), with an IC_{50} for the high-affinity site (19% of all sites) of 3.55 ± 0.95 nM and for the low-affinity site (81% of all sites) 7.52 ± 0.16 μM (Figure 3). Previous inhibition binding studies of AMP receptors demonstrated the existence of one affinity site for AMP receptors although there was a suggestion that two sites may be present (Olson *et al.*, 1992; Olson and Derby 1995).

Inhibition by structural analogues of AMP

Two physiologically competitive agonists for the AMP receptors, 6-chloropurine and XMP, were tested between 10^{-7} and 10^{-3} M (Figure 3). The inhibition data for both

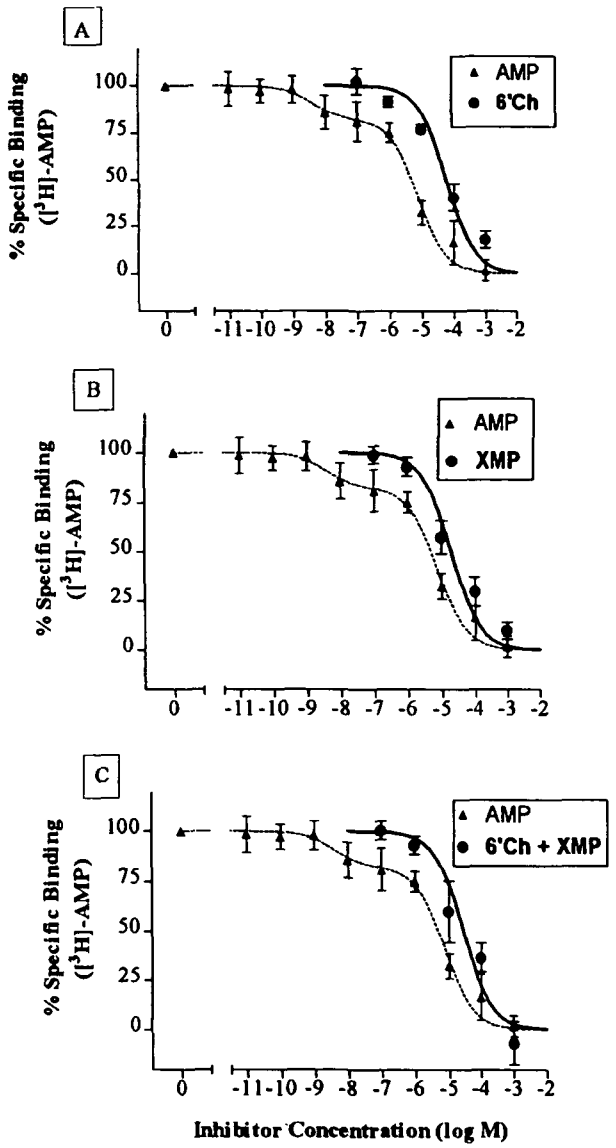

Figure 3 Inhibition of binding of 1 μM [^{3}H]AMP by unlabeled AMP (dashed line) and unlabeled structural analogues of AMP (bold lines). P2 was incubated with 1 μM [^{3}H]AMP + varying concentrations of unlabeled 6-chloropurine (A), XMP (B) or a mixture of 6-chloropurine + XMP (C). Values are means ± SEM for two experiments run in triplicate.

6-chloropurine and XMP were best fit by one-site competition curves ($r^2 = 0.86$, $P < 0.05$ and $r^2 = 0.80$, $P < 0.001$ respectively). The IC_{50} value for 6-chloropurine was 58.7 ± 0.98 μM and the maximum inhibition was 88% (Figure 3A). The IC_{50} value for XMP was 19.3 μM ± 1.1 μM and the maximum inhibition was 95% (Figure 3B). When 6-chloropurine and XMP were tested in a mixture as an inhibitor, the maximum inhibition approached 100% at the highest concentration (10^{-3} M), although at all other concentrations of inhibitor the amount of inhibition by the mixture was no greater than the inhibition by either individual component (Figure 3C). These data suggest that the structural analogues, 6-chloropurine and XMP, were competitive inhibitors of the low-affinity AMP binding sites.

Inhibition of AMP binding by single odorants that are structurally unrelated to AMP

The single odorants ammonium, betaine, L-cysteine, L-glutamate, DL-succinate and taurine apparently inhibited AMP through non-competitive mechanisms, as indicated by the fact that only linear regressions could be fitted to their inhibition data. Table 3 shows inhibitory effects of selected concentrations of these compounds.

Inhibition of AMP binding by mixtures of odorants structurally unrelated to AMP

The effects of the 12 mixtures tested as inhibitors of AMP binding are listed in Table 3. All of the components of these mixtures when tested individually were non-competitive inhibitors of AMP binding (see above section). The maximum inhibition values for the mixtures ranged from 12 (for CGNS) to 70% (CGT), but were usually (i.e. for 8/12 mixtures) >36%. At a concentration equal to that of [^{3}H]taurine (i.e. 1 μM), the mixtures had inhibition values that ranged from 0 (for BC, CG, CGNT, BCGNT) to 31% (for CGT) but were >10% for all but two mixtures (CGT and BCGN).

Binding interactions between the components of mixtures for AMP binding were analyzed based on regression analysis as done previously for taurine binding (Table 3). The inhibition curves for 10 of the mixtures were significantly fit only by linear regressions. Examples are shown for BCGNS and BCGNT (Figure 2B). One mixture (BCGNST) could not be significantly fit by any regression equation, and one mixture (CGT) was fit best by a two-site non-linear regression. It is unclear why CNT was best fit by a two-site non-linear regression when its components were fit by linear regressions.

All mixtures except one (CGT) showed complex binding interactions for AMP binding sites, where the inhibition value for the mixture was significantly less than that for at least one of the components at its concentration in the mixture. The mixture CG is an example of complex binding interactions (Table 3). At 1 and 2 μM, CG inhibited AMP binding by 0 and 13% respectively. These values are significantly less than either C (26 and 30% at 1 and 2 μM respectively) or G (27 and 28% at 1 and 2 μM respectively). Another example is BCGNST, where the components C, G and S were all more inhibitory at 1 μM (26, 27 and 20% respectively) than BCGNST at 6 μM (3%) (Table 3). These results suggest that complex binding interactions occur for most mixtures containing AMP.

The lone case in which complex binding interactions were not observed for AMP binding is the mixture CGT (Table 3). C, G and T at 1 μM were less effective inhibitors alone (26, 27 and 7%) than mixed (41%) and thus demonstrate at least partial summation of their effects.

Table 3 Inhibition of [^{3}H]AMP binding by mixtures of structurally unrelated compounds

Mixture	Regression with best and significant fit ($P < 0.05$)	Max. inhibition value for mixture at 1 mM (in %)	Inhibition value for mixture and components at 1 μM (in %)							Inhibition value for mixture at $n \times 1$ μM (in %)							Type of binding interaction among components
			Mix	B	C	G	N	S	T	Mix	B	C	G	N	S	T	
BC	linear	22	0	5	26[a]					0	10	30[a]					complex
CG	linear	35	0		26[a]	27[a]				13		30[a]	28[a]				complex
GT	linear	28	9			27[a]			7	21			28			10	complex
BCG	linear	29	9	5	26[a]	27[a]				13	10	30[a]	29[a]				complex
CGN	linear	49	6		26[a]	27[a]	2			8		30[a]	29[a]	9			complex
CGT	two-site non-linear	70	31		26	27			7[a]	41		30[a]	29[a]			11[a]	non-additive
CGNT	linear	43	0		26[a]	27[a]	2		7	0		31[a]	30[a]	9		12	complex
CGNS	linear	12	4		26[a]	27[a]	2	20[a]		9		31[a]	30[a]	9	21		complex
BCGN	linear	51	17	5	26	27	2			17	11	31[a]	30[a]	9			complex
BCGNT	linear	30	0	5	26[a]	27[a]	2		7	1	12	32[a]	30[a]	9		13	
BCGNS	linear	24	5	5	26[a]	27[a]	2	20[a]		23	12	32	30	9	21		complex
BCGNST	none	15	3	5	26[a]	27[a]	2	20[a]	7	3	12	32[a]	31[a]	9	21[a]	13	complex

See legend of Table 2 for description.

An example of the importance of the specific composition of a mixture, rather than the number of components, in determining its inhibitory effect is that CGT and BCGN each inhibited AMP binding more than BCGNST (inhibition values at 1 μM are 31, 17 and 3% respectively; Table 3).

L-Glutamate receptors

Inhibition by unlabeled L-glutamate

Inhibition data for L-glutamate between 10^{-11} and 10^{-3} M were best fit by a two-site competition curve ($r^2 = 0.86$, $P < 0.05$) (Figure 4). The IC_{50} values for the high- and low-affinity sites were 70.9 ± 3.5 pM (25% of all binding sites) and 21.6 ± 1.1 μM (75% of all binding sites) respectively. These data are consistent with previous studies of L-glutamate binding (Burgess and Derby, 1997).

Inhibition by a structural analogue of L-glutamate

NMDA was the only structural analogue that was tested as an inhibitor of L-glutamate binding (Figure 4). The data for NMDA inhibition of L-glutamate binding were best fit by a one-site competition curve ($r^2 = 0.95$, $P < 0.001$) since the NMDA concentrations tested were between 10^{-7} and 10^{-3} M, which are too high to reveal the high-affinity site (Burgess and Derby, 1997). The IC_{50} for the one site was 26.2 ± 0.91 μM. Comparison of this value with the IC_{50} for L-glutamate, which was 21.6 ± 1.1 μM, shows that NMDA is an excellent competitor for this low-affinity L-glutamate binding site.

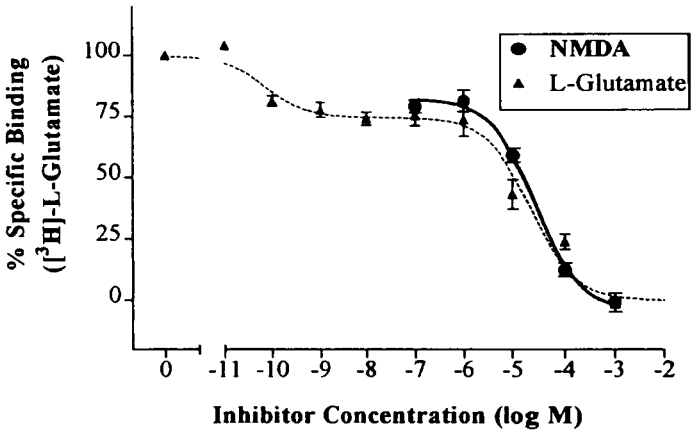

Figure 4 Inhibition of binding of 1 μM [^{3}H]L-glutamate by unlabeled L-glutamate (dashed line) and unlabeled NMDA (bold line), a structural analogue of L-glutamate. P2 was incubated with 1 μM [^{3}H]L-glutamate + varying concentrations of unlabeled NMDA. Values are means ± SEM for two experiments run in triplicate.

Inhibition of L-glutamate binding by single odorants that are structurally unrelated to L-glutamate

The odorants AMP, betaine, succinate, taurine and ammonium had inhibitory effects on L-glutamate binding that were apparently non-competitive. The one exception was L-cysteine, which was a strong competitive inhibitor of L-glutamate binding, as was seen earlier by Burgess and Derby (1997).

Inhibition of L-glutamate binding by mixtures of odorants structurally unrelated to L-glutamate

The effects of the 15 mixtures that were tested as inhibitors

Table 4 Inhibition of [^{3}H]glutamate binding by mixtures of structurally unrelated compounds

Mixture	Regression with best and significant fit ($P < 0.05$)	Max. inhibition value for mixture at 1 mM (in %)	Inhibition value for mixture and components at 1 μM (in %)							Inhibition value for mixture at $n \times 1$ μM (in %)							Type of binding interaction among components
			Mix	A	B	C	N	S	T	Mix	A	B	C	N	S	T	
AC	one-site non-linear	100	1	45[a]		31a				14	40[a]		47[a]				complex
AT	one-site non-linear	77	17	45[a]					29[a]	23	40[a]					31[a]	complex
CT	one-site non-linear	82	20			31			29	17			47[a]			31	complex
ABC	two-site non-linear	85	25	45[a]	40[a]	31				26	42	41	50[a]				complex
ACN	two-site non-linear	76	38	45		31	32			40	42		50	33			non-additive
ACT	linear	100	35	45[a]		31			29	43	42		50			31	non-additive
CNT	two-site non-linear	82	24			31	32		29	25			50[a]	33		31	non-additive
ABCN	linear	95	35	45[a]	40	31	32			36	42	42	53[a]	33			complex
ACNS	two-site non-linear	100	43	45		31	32	27		47	42		53	33[a]	28[a]		complex
ACNT	two-site non-linear	100	10	45[a]		31[a]	32[a]		29[a]	14	42[a]		53[a]	33[a]		31[a]	complex
BCNT	two-site non-linear	100	34		40	31	32		29	37		42	53	33		31	non-additive
ABCNS	two-site non-linear	100	35	45[a]	40	31	32	27		41	42	43	54	33	28		non-additive
ABCNT	two-site non-linear	99	39	45	40	31	32		29	38	42	43	54	33		31	non-additive
BCNST	two-site non-linear	85	8		40[a]	31[a]	32[a]	27	29[a]	20		43[a]	54[a]	33	28	31	complex
ABCNST	two-site non-linear	100	21	45[a]	40[a]	31	32	27	29	31	42	44	56[a]	34	29	31	complex

See legend of Table 2 for description.

of L-glutamate binding are shown in Table 4. All but one mixture (AT) contained L-cysteine and thus had both non-competitive and competitive inhibitors of L-glutamate binding. All mixtures were highly inhibitory of L-glutamate binding. The maximal inhibition values of glutamate binding ranged from 76 to 100%. When tested at 1 μM (i.e. equimolar to L-glutamate), the mixtures had inhibition values ranging from 1 (for AC) to 43% (for ACNS).

Binding interactions between the components of mixtures were analyzed for L-glutamate binding as was done for taurine and AMP binding. Regressions for the inhibition curves for the mixtures were calculated, and from these regressions inhibition values were calculated for each of the above mixtures at the concentration of the individual components in those mixtures and for the individual components at the concentration of the mixtures (Table 4). Most mixtures were significantly fit by either a one-site (AC, AT, CT) or two-site non-linear regression curve (ABC, ACN, CNT, ACNS, ACNT, BCNT, ABCNS, ABCNT, BCNST, ABCNST). The mixture ACN is shown as an example (Figure 2C). Two mixtures were fit significantly only by linear regressions (ACT, ABCN). The one- or two-site regressions for mixtures containing cysteine were expected since cysteine appeared to be a highly effective competitive inhibitor of glutamate binding (Burgess and Derby, 1997).

Many mixtures showed complex binding interactions, where the inhibition value for the mixture was significantly less than that for at least one of the components at its concentration in the mixture. The clearest examples of complex binding interactions are AC, AT, ABC, ABCN, ACNT, BCNST and ABCNST. An illustrative example is AC and ACNT (Table 4). At 1 μM the components A, B, C, N and T produced inhibition values of 45, 40, 31, 32 and 29% respectively, which are all significantly greater than the inhibition value for AC at 1 (1%) or 2 μM (14%) or for ACNT at 1 (10%) or 4 μM (14%).

A further comparison of several mixtures (AC, ACN,

ACNT, ABCNT) shows that complex interactions are dependent on the precise composition of the mixture (Table 4). At 1 μM, AC, ACN, ACNT and ABCNT inhibited L-glutamate binding by 1, 38, 10 and 39% respectively. At the higher concentrations, a similar trend occurred. Thus either a removal of one component (removal of T from ACNT to yield ACN) or addition of one component (addition of N to AC to yield ACN, or addition of B to ACNT to form ABCNT) significantly increased binding inhibition. Furthermore, BCNT, which results from replacing the A in ACNT with B, also increased binding inhibition from 10 (ACNT) to 34% (BCNT). This strongly suggests that complex interactions between the specific components in ACNT cause a reduction in the ability of some of its components to inhibit L-glutamate binding. In fact, seven of the 14 mixtures that had fewer components than ABCNST had greater inhibition of L-glutamate binding than ABCNST itself (Table 4).

For none of the mixtures tested as inhibitors of L-glutamate binding were the inhibitory effects of the mixture's components completely additive. For several mixtures (ACN, ACT, CNT, BCNT, ANCNS, ABCNT), the mixture's binding inhibition value was about equal to that of the most inhibitory component at its concentration in that mixture (Table 4).

Discussion

Single odorant compounds (e.g. taurine, AMP and L-glutamate) that are components of natural complex stimuli for the Caribbean spiny lobster *P. argus* can affect the binding of each other to their respective receptor sites in the spiny lobster's olfactory organ (Olson and Derby, 1995; Burgess and Derby, 1997). The goal of our current study was to examine the effect of complex mixtures (i.e. containing up to six components) on the binding of taurine, AMP and L-glutamate to their receptors. Our results show that a diversity of effects exists, the most prevalent being 'complex binding interactions' in which the binding inhibition by a mixture is less than that by one or more of its components. The type and intensity of binding effect is dependent more on the specific composition of the mixture than on the number of components.

Binding interactions among components of complex mixtures

Structural analogues of the radioligand showed inhibition curves best fit by one- or two-site competition regressions, indicating that they act as competitive inhibitors of the radioligand, as expected from their similarity in molecular structure. These include β-alanine and hypotaurine for taurine binding (Figure 1), 6-chloropurine and XMP for AMP binding (Figure 3), and NMDA for L-glutamate binding (Figure 4).

Most of the single odorant molecules tested in our current and previous studies (Olson and Derby, 1995; Burgess and Derby, 1997) appear to act individually as non-competitive inhibitors of binding for the taurine, AMP and L-glutamate receptor systems, as indicated by the regressions fit to the inhibition data. These include AMP, cysteine, ammonium, succinate and glutamate for taurine binding; betaine, cysteine, ammonium, glutamate, succinate and taurine for AMP binding; and AMP, betaine, ammonium, succinate and taurine for glutamate binding. The exceptions to this generalization were cases of competitive inhibition; examples include betaine's effect on taurine binding and L-cysteine's effect on L-glutamate binding. It should be noted that some compounds identified as apparent non-competitive inhibitors could in reality be competitive inhibitors. Misidentification of the competitive effect of a compound is possible when small and variable inhibition values prevent the data from being significantly fit by a competitive binding curve.

Given the nature of the effects of these single compounds on binding of the odorants taurine, AMP and L-glutamate, one can attempt to predict the effects of mixtures from the effects of the mixtures' components. For example, for a mixture of competitive inhibitors, the inhibitory effect of the mixture should be similar to the effect of its more inhibitory components. For a mixture of components that are non-competitive inhibitors acting at different sites from each other, the mixture's effects should approximate the sum of the effects of its components. The inhibitory effects of a mixture containing both competitive and non-competitive components or of a mixture containing non-competitive components that bind to overlapping sites would depend on the intensity of the individual effects. For example, if a competitive inhibitor were highly effective, a mixture containing it might parallel the effects of that component over the other components.

Our results generally showed, as expected, competitive binding effects for mixtures of compounds structurally similar to each other and to the radioligand taurine (Figure 1) or AMP (Figure 3). An exception was the effect of low concentrations of the mixture ß-alanine + hypotaurine on taurine binding.

The effects were more variable for mixtures of naturally occurring, food-related odorant compounds that are structurally dissimilar to each other and to the radioligands taurine, AMP and L-glutamate (Tables 2–4). This is expected, since some of these mixtures included only apparently non-competitive inhibitors and other mixtures included both competitive and non-competitive inhibitors. For mixtures of odorants, there were no examples where the mixture's inhibitory effect was equal to the sum of the components' effects. In fact there were few examples where the inhibitory effect of a mixture was equal to that of an equivalent concentration of at least one of its components, and these tended to occur when one of the components was a highly effective competitive inhibitor. The most common

effect of a mixture of odorants on binding of another odorant was 'complex binding interactions', in which the magnitude of inhibition by a mixture was significantly less than inhibition by each of the components (Tables 2–4). The mechanisms responsible for such complex binding interactions may be varied and complex, and may include both competitive and non-competitive mechanisms, depending on the components. A component may have either competitive or non-competitive effects on any of the binding sites for the other components, allowing for a wide diversity of effects. A reduction in the inhibitory effect of ligand binding by other odorants, or complex binding interactions, could result from an allosteric modification of binding of other odorants in a mixture.

The magnitude of the effect of a mixture on the binding of an odorant to its receptors depends more on the specific composition of the mixture than on the number of components. Our results for all three receptor systems studied support this conclusion, by showing that mixtures with few components can cause greater binding inhibition than mixtures with more components.

The role of binding interactions in the coding of complex odorant mixtures

The coding of odorant mixtures by the olfactory system is dependent on features of the ORNs, such as the type and density of receptor proteins expressed by them (Kalinoski *et al.*, 1987; Bruch and Rulli, 1988; Ngai *et al.*, 1993; Kang and Caprio, 1995), the affinity of those receptor proteins for specific odorants (Kalinoski *et al.*, 1987; Daniel *et al.*, 1996), whether the receptor proteins are linked to active transduction pathways (Kalinoski *et al.*, 1987) and whether those active transduction pathways are inhibitory or excitatory (Dionne and Dubin, 1994; Ache and Zhainazarov, 1995). From the current study, another potentially important event in coding of complex mixtures is identified: the binding interactions between compounds in mixtures and receptor proteins that affect the receptor binding and subsequent transduction of other odorant compounds.

Mixture suppression for binary mixtures has been attributed to both binding inhibition and outward (inhibitory) ionic conductances (Michel and Ache, 1992; Dionne and Dubin, 1994; Ache and Zhainazarov, 1995), so it is possible to speculate on how both could contribute to the coding of complex odorant mixtures. Each type of receptor would bind specific components in a mixture. These specific components would activate the transduction pathways linked to that receptor. Whether the transduction cascades activated by these receptors were the same or opposing would differentially shape the net output of the ORN. In addition, as seen in this study, individual odorants affect the binding of other odorants to their receptors. Therefore, whether ORNs express one or more types of receptors or transduction cascades, the net response to a mixture would be affected by the initial binding interactions for the mixture's components, which usually includes binding inhibition.

The functional importance of such binding interactions is suggested from the demonstration that the best mathematical model for predicting the responses of spiny lobster ORNs to binary mixtures is a non-competitive model that has a term for binding inhibition (Daniel *et al.*, 1996). In the present study, it was demonstrated that mixtures of up to seven components have significant and often complex effects on the binding of an odorant to its receptors. We are currently examining the role of binding interactions in the coding of complex odorant mixtures, by applying this same mathematical model and others to the responses of ORNs to some of the two- to seven-component mixtures used in this binding study.

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