Identification of a specific olfactory receptor for 2-isobutyl-3-methoxypyrazine

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2-Isobutyl-3-methoxypyrazine, a potent bell-pepper odourant, binds to cow olfactory mucosa homogenate. The receptor is saturable in the micromolar range and is competitively inhibited by other bell-pepper odourants, but not by other pyrazines of different odours. Other tissues do not bind 2-isobutyl-3-methoxypyrazine at a significant extent. We suggest that this receptor is involved in odour discrimination.

The first event in odour perception is the recognition of the odourant molecules by receptors present in the olfactory mucosa. According to a model proposed by Amoore (1977) and on the basis of observations of specific anosmias, there should exist some 30 of such specific receptors, each one related to a so called 'primary odour'.

Very few papers have been published on the biochemical characterization of these receptors and in most cases there is poor agreement among them. Gennings *et al.* (1977) studied an olfactory receptor for the boar pheromone 5α-androst-16-en-3-one, but their data could not be reproduced by other researchers (Pelosi *et al.*, 1978; Persaud, 1980; Persaud *et al.*, 1980). Price (1978) described the purification of an anisole-binding protein from dog olfactory epithelium, but did not report any characteristics of such protein. The work of Fesenko *et al.* (1979*a,b*) deals with the characterization of a camphor olfactory receptor in the frog and in the rat. Cagan & Zeiger (1978) measured the binding constants of amino acids to rainbow-trout olfactory mucosa and found a good correlation with electrophysiological responses measured *in vivo*. Rhein & Cagan (1980) then provided evidence for the localization of such receptors on the olfactory cilia of the same fish. Finally, Novoselov *et al.* (1980) reported some characteristics of an alanine receptor in the skate olfactory epithelium, but their data markedly differ from those of Cagan & Zeiger (1978) for the trout. In this paper, evidence is provided for the presence in the cow olfactory mucosa of a specific receptor that binds 2-isobutyl-3-methoxypyrazine. This compound, which exhibits an extremely intense odour of bell peppers (Seifert *et al.*, 1970), has been selected on the basis of its exceptionally low olfactory threshold, a measure of strong binding to the specific receptor.

Moreover, the great amount of olfactory data (odour descriptions and olfactory thresholds) available in the literature for compounds of similar structures provide a good basis for a biochemical approach to the specificity of this receptor site.

Materials and methods

Materials

[³H]2-Isobutyl-3-methoxypyrazine was obtained by isotopic exchange in the laboratories of The Radiochemical Centre, Amersham, Bucks., U.K., as described previously (Pelosi *et al.*, 1981). The specific radioactivity was 2.08 Ci/mmol. 2-Methylpyrazine, 2-methyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine were obtained from Samples Chemical Company, Alpharetta, GA, U.S.A. 2-Isobutyl-3-methoxypyrazine was prepared from leucine, via the intermediate 2-chloro-3-isobutylpyrazine, by the method of Karmas & Spoerri (1952) and Klein *et al.* (1964). 4-Butyl-5-propylthiazole was synthesized by the procedure described by Kurkjy & Brown (1952). All other chemicals were of reagent grade.

Preparation of homogenates

Cow olfactory mucosa (8–10 g from animals 4–6 months old) was taken within 15 min after death, finely cut and homogenized at 4°C in a 0.05 M Tris/HCl buffer, pH 7.4, containing 0.001 M-EDTA, in a Polytron homogenizer at maximum speed for 15 s, to give a 10% (w/v) homogenate. All other tissues were treated in the same conditions, but with a Potter homogenizer with a motor-driven Teflon pestle. The homogenates were centrifuged at 4°C and 20000 g for 10 min and the supernatant (crude extract) was used for the experiments, within the same day.

Measurement of bound [^3H]2-isobutyl-3-methoxy-pyrazine

Crude extract (0.100 ml) was incubated with the appropriate amount of [^3H]2-isobutyl-3-methoxypyrazine, the competing ligand, when needed, and buffer to a final volume of 0.150 ml, for 5 min at 4 °C. The incubated mixture was then applied to a Sephadex G-25 column (0.7 cm × 15 cm) and eluted with 0.01 M-Tris/HCl buffer, pH 7.4, at 0.5 ml/min. Fractions (0.25 ml) were collected directly into mini-vials containing 2 ml of Hydro-Luma scintillator fluid (Lumac, Basel, Switzerland) and counted for radioactivity. The area under the first peak, usually occurring in fractions 9–12, was evaluated and taken as a measure of the bound [^3H]2-isobutyl-3-methoxypyrazine. Non-specific binding was measured under the same conditions, but in the presence of unlabelled 1 mM-2-isobutyl-3-methoxypyrazine.

Protein determination

Total protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

Fig. 1 shows the binding curve obtained with cow olfactory mucosa homogenate and [^3H]2-isobutyl-3-methoxypyrazine. Saturation occurs in the micromolar range and the Scatchard plot (inset) in-
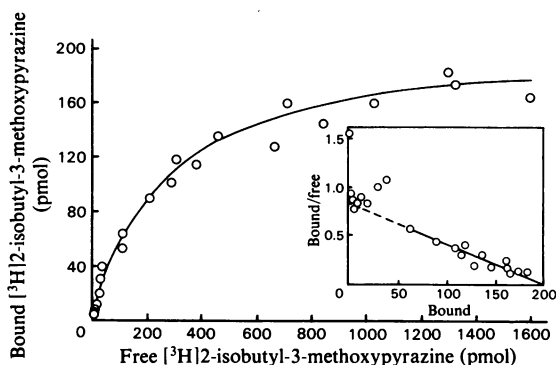

Fig. 1. *Binding of [^3H]2-isobutyl-3-methyoxypyrazine to cow olfactory mucosa homogenate*

Crude extract (0.100 ml) was incubated with the ligand and buffer to a final volume of 0.150 ml for 5 min at 4 °C to reach equilibrium. Bound [^3H]2-isobutyl-3-methoxypyrazine was separated by chromatography on Sephadex G-25. Non-specific binding measured in the presence of 1 mM-2-isobutyl-3-methoxypyrazine, has been subtracted. The Scatchard plot is shown as an inset.

dicates a single type of site at this concentration of the ligand.

The total number of binding sites has been estimated to be 2000 pmol/ml of homogenate, corresponding to 270 pmol/mg of protein. The dissociation constant, K_D, as calculated from this curve, was 1.2 μM.

The few data available in the literature, concerning dissociation constants and number of binding sites of olfactory receptors, fall into two main groups, although they have been measured with different ligands, animals and techniques. Values of K_D in the nanomolar range and a low number of binding sites (n) are reported by Gennings *et al.* (1977) ($K_D = 1.2$ nM, $n = 3.3$ pmol of 5α-androst-16-en-3-one/mg of protein in the sow) and by Fesenko *et al.* (1979a,b) ($K_D = 0.13$ nM, $n = 0.18$ pmol of L-alanine/mg of protein in the skate; $K_D = 1$ nM for camphor in frog and rat). Values of K_D in the micromolar range and a high number of binding sites were found by Pelosi *et al.* (1978) ($K_D = 60$ nM, $n = 32$ pmol of 5α-androstan-3-one/mg of protein in cow and rabbit), by Persaud (1980) ($K_D = 40$ nM, $n = 28$ pmol of 5α-androstan-3-one/mg of protein in the sow) and by Cagan & Zeiger (1978) ($K_D = 5.6$ μM, $n = 440$ pmol of L-alanine/mg of protein in the trout). These data suggest the existence of two classes of binding sites in olfactory receptors. In our case, the identification of a receptor with a K_D in the micromolar range does not exclude the presence of another site binding the same pyrazine with a much lower K_D. This may explain the non-linearity of the Scatchard plot observed in the nanomolar range, but this aspect still deserves further investigation.

Fig. 2 summarizes the results of competitive binding experiments, performed with cow olfactory mucosa homogenate (0.100 ml) incubated with a constant amount (15 pmol) of [^3H]2-isobutyl-3-methoxypyrazine and a 10-, 100- or 1000-fold excess of the appropriate competitor.

Besides 2-isobutyl-3-methoxypyrazine itself, included as a reference, four compounds were tested. Two of them (2-methyl- and 3-methoxy-2-methyl-pyrazine) bear structural similarities to the reference pyrazine, but their odours differ in quality, described as 'nutty' for the first derivative (Pittet & Hruza, 1974) and 'roasted' for the second (Parliment & Epstein, 1973), as well as intensity, their reported olfactory thresholds being 105000 p.p.b. (Koehler *et al.*, 1971) and 4 p.p.b. (Seifert *et al.*, 1970) respectively, whereas the odour of the 2-isobutyl-3-methoxy derivative is described as bell-pepper-like, with an olfactory threshold of 0.002 p.p.b. (Seifert *et al.*, 1970). Of the other two, the former, 2-isopropyl-3-methoxypyrazine, is very similar to the reference derivative, both in structure and in odour (described as bell-pepper/raw potato), with a threshold of
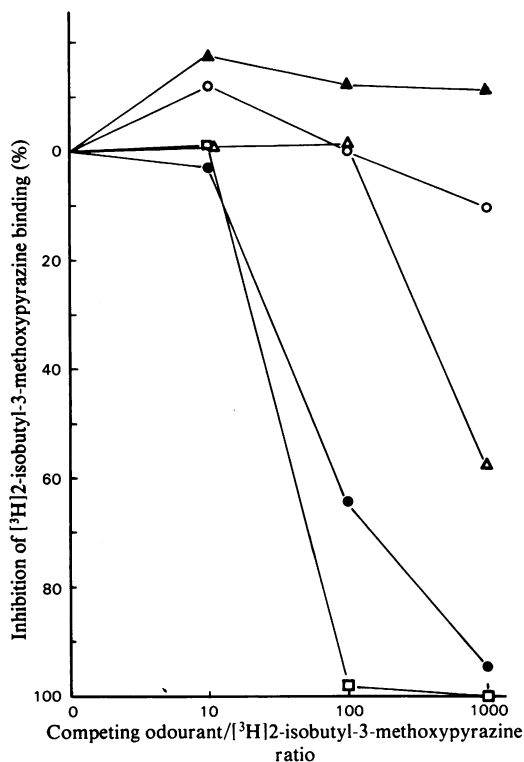
Fig. 2. *Competitive binding of various ligands to cow olfactory mucosa homogenate*
Crude extract (0.100ml) was incubated with 15pmol of [^{3}H]2-isobutyl-3-methoxypyrazine in the presence of the indicated amounts of the appropriate competitor. Bound [^{3}H]2-isobutyl-3-methoxypyrazine was evaluated as described in the text. Symbols: ▲, 2-methylpyrazine; ○, 2-methoxy-3-methylpyrazine; △, 2-isopropyl-3-methoxypyrazine; ●, 2-isobutyl-3-methoxypyrazine; □, 4-butyl-5-propylthiazole.
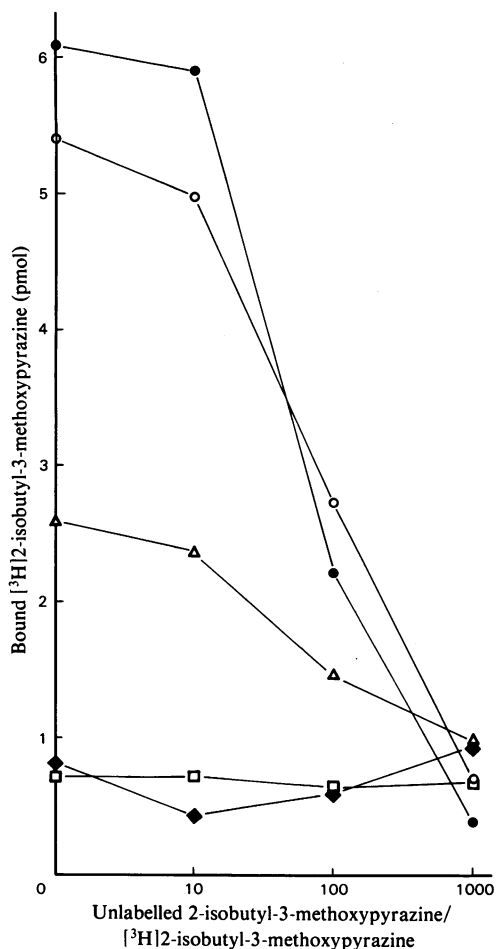

Fig. 3. *Binding of [^{3}H]2-isobutyl-3-methoxypyrazine to olfactory mucosa homogenate from various animals*
Crude extract (0.100ml) was incubated with 15pmol of [^{3}H]2-isobutyl-3-methoxypyrazine alone or diluted with the indicated amounts of unlabelled 2-isobutyl-3-methoxypyrazine. Bound [^{3}H]2-isobutyl-3-methoxypyrazine was evaluated as described in the text. Symbols: ●, cow; ○, rabbit; △, rat; ◆, trout; □, pigeon.

0.002p.p.b. (Seifert *et al.*, 1970), whereas the latter, despite being a thiazole derivative, exhibits an odour of bell pepper and an olfactory threshold of 0.003p.p.b. (Buttery *et al.*, 1976). The results show that the first two ligands do not complete appreciably with 2-isobutyl-3-methoxypyrazine for the same receptor site, whereas the others exhibit a marked effect on 2-isobutyl-3-methoxypyrazine binding.

This correlation between psychophysical data and biochemical results strongly supports the hypothesis that the identified receptor plays a role in olfaction. Several other species, known for their olfactory capacity and already used in olfaction experiments, were tested for the presence of this pyrazine receptor in the olfactory mucosa; they were wild rabbit

(*Oryctolagus cuniculus*), wistar rat (*Rattus* sp.), homing pigeon (*Columba livia*) and rainbow trout (*Salmo gairdneri*). The aim was to investigate whether this olfactory receptor was a general one, present in all animals, or confined only to certain species. The results, reported in Fig. 3, represent the working basis for a wider investigation and show that only in the three mammals the receptor is present and saturable. In the trout and in the pigeon the very low binding measured has to be regarded as non-specific, being non-saturable, even at very high concentrations of the ligand.

Finally, the binding of [3H]2-isobutyl-3-methoxypyrazine has been measured with several tissue samples from wild rabbit, used in these experiments for its more convenient handling. The tissue examined and the values of bound [3H]2-isobutyl-3-methoxypyrazine, as pmol/g of original tissue, are as follows: olfactory mucosa, 530; respiratory epithelium from nasal septum, 64; and from trachea, 30; tongue epithelium, 38; buccal mucosa, 30; cerebral cortex, 30; meninx, 42; olfactory bulb, 26; peritoneum, 27; duodenal epithelium, 31. These data clearly show that such a receptor is effectively present only in the olfactory mucosa.

The above results satisfy the main criteria for detecting the presence of a specific receptor, so it is reasonable to claim that an olfactory receptor for 2-isobutyl-3-methoxypyrazine and related odourants has been identified in the olfactory mucosa of cow and other mammals.

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