PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 86 (2003) 133–141

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Binding of the pheromonal steroid, 5α-androst-16-en-3-one, to membrane-enriched fractions of boar and rat olfactory epithelium; preliminary evidence for binding protein being a glycoprotein

M.A. Kraevskaya [a], M.J.P. Higgins [a,1], M.T. Wilson [b], D.B. Gower [c,*]

[a] *Division of Biochemistry and Molecular Biology, United Medical and Dental Schools, Guy's Hospital, London SE1 9RT, UK*
[b] *Department of Biological Sciences, University of Essex, Central Campus, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK*
[c] *Department of Forensic Science and Drug Monitoring, Franklin-Wilkins Building, King's College London, 150 Stamford Street, London SE1 9NN, UK*

Accepted 17 May 2003

In memoriam: Dr. Maria A. Kraevskaya (Dr. Maria A. Kraevskaya died at the tragically early age of 36 years on 8 August 1993)

Abstract

A high degree of binding of 5α-[^{3}H]-androstenone was recorded in membrane-enriched fractions of porcine olfactory tissue. The specific (i.e. high affinity, low capacity) binding had a mean K_a approximately $2 \times 10^8\ M^{-1}$. A Hill plot of the data showed a Hill coefficient of approximately 2, possibly suggesting co-operativity of binding, with binding constants increasing from 8×10^7 to $1.6 \times 10^9\ M^{-1}$ with increasing substrate concentration. The level of specific binding of 5α-[^{3}H]-androstenone was nearly 10-fold higher than in corresponding respiratory tissue preparations and was markedly reduced in the presence of excess (approximately 1 μM) unlabelled 5α-androstenone. Corresponding fractions derived from rat olfactory tissue showed only 25% of the binding recorded for the pig.

After incubation of 5α-[^{3}H]-androstenone with solubilised olfactory cilial tissue (porcine), gel filtration and chromatography on a typical "glycoprotein" column (Concanavalin A-Sepharose B) were performed. Specific binding was recorded only in fractions corresponding to glycoproteins with M_r of approximately 70–90 kDa.

In a third series of experiments, fractions containing high concentrations of cilia, some still attached to the dendritic endings (as shown by electron microscopy) were obtained by a novel method involving stripping them off the nasal epithelium. The basal adenylate cyclase (AC) activity was very significantly ($P < 0.01$) higher in olfactory, compared with respiratory, cilia; storage at −70 °C for 3 weeks greatly reduced AC activity. When fresh male and female porcine olfactory cilia preparations were incubated with 5α-androstenone plus GTP, AC activity was increased fourfold ($P < 0.01$). However, responses of porcine respiratory cilia were not significant statistically, neither were changes in basal levels of AC activities in rat olfactory cilia.

Keywords: Boar; Rat; Olfaction; Cilia; 5α-Androst-16-en-3-one; Steroid binding

1. Introduction

During the past 30 years, considerable advances have been made in the elucidation of the transduction mechanisms involved in olfaction and in the nature of the molecules involved in odorant binding [1]. In earlier work, experiments were generally performed with whole, or crude homogenates of, olfactory epithelium. The separation of olfactory cilia by, for example, the Ca^{2+}-shock method [2,3], however, has enabled researchers to show that the olfactory cilia membranes carry the odorant binding proteins as well as the proteins involved in the transduction processes. The former appear to be transmembrane glycoproteins, e.g. [4], while mediation of the latter processes requires second messengers, e.g. c-AMP, inositol trisphosphate, Ca^{2+} ions [5–7]. Many of the proteins involved, such as the specific olfactory G-protein (G_{olf}) and ion-channel proteins, have been characterised and cloned [1,8,9].

Odorants are detected by approximately 1000 different G-protein-coupled odorant receptors (ORs), which are encoded by a multigene family [10,11]. The extraordinary discriminatory capacity of an olfactory system, which can

* Corresponding author. Tel.: +44-20-7848-4848; fax: +44-20-7848-4980.
E-mail address: dbgower@the-brents.freeserve.co.uk (D.B. Gower).
[1] Present address: St. Mary Redcliffe and Temple School, Somerset Square, Redcliffe, Bristol BS1 6RT, UK.

0960-0760/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00264-4

distinguish between thousands of odors, has been subjected to research scrutiny in recent years [12–14]. It now appears that combinatorial receptor codes occur, in that one OR recognises multiple odorants and that one odorant is recognised by multiple ORs. However, different odorants are recognised by different combinations of ORs [15,16].

In several laboratories, including our own, a search has been made for a specific protein(s) which might bind the odorous steroid, 5α-androst-16-en-3-one (5α-androstenone). Although this compound was isolated from boar testis 60 years ago [17], it was not until 1971 that its importance as a releaser pheromone in the pig was realised [18] and later as a primer pheromone [19]. These effects are now known [20] to be mediated via the olfactory system and not via the vomeronasal organ. Since the mid-1980s, the importance of 5α-androstenone and the related steroids, 4,16-androstadien-3-one and 5α-androstenol, as human pheromones has been explored [21,22].

The extreme hydrophobicity of 5α-androstenone has hindered progress, to some extent, in studies of specific binding proteins. It has been difficult to characterise small degrees of specific (i.e. saturable and displacable) binding in the presence of very high non-specific binding [23]. In the present work therefore, we wished to achieve the following goals:

(a) Utilise membrane-enriched olfactory preparations, obtained from the pig and, for comparison, the rat, in order to study the specific binding of 5α-androstenone in more detail.
(b) Prepare cilia-rich fractions (Boekhoff et al. [24], modified), which have been used for binding studies and for partial characterisation of the proteins involved as glycoproteins of approximately 70–90 kDa.
(c) Explore the possibility of c-AMP being involved in transduction mechanisms for 5α-androstenone. For these studies, highly purified olfactory cilia preparations have been obtained by a method utilising the adhesive properties of charged cellulose and other filter membranes.

2. Materials and methods

2.1. Materials and chemicals

Regenerated cellulose and 'Zeta Probe' filter membranes were obtained from Sartorius, GmbH, Göttingen and Biorad, UK, respectively. Concanavalin A-Sepharose 4B and α-methyl-D-mannopyranoside were purchased from Sigma Chemical Co., Fancy Road, Poole, Dorset, UK. This company also provided theophyllin, GTP, dithiothreitol, Triton X-100, CHAPS ([(3-chloroamidopropyl) dimethyl amoniol]-1-propane sulphonate) and HEPES (*N*′-2-hydroxyl-piperazine-*N*′-2-ethane-sulphonic acid). Minimal essential medium (MEM) came from Gibco, UK and 5α-[4,5-^{3}H]-androstenone (specific radioactivity 50 Ci/mmole) was obtained from Isocommerz, Dresden, Germany. Molecular weight markers for chromatography were purchased from Bio-Rad Laboratories Ltd., Maylands Avenue, Hemel Hempstead, Herts, UK.

2.2. Animals

Mature pigs (1–2 years) were housed at the Agriculture and Food Research Institute, Babraham, Cambridge, UK and adult (approximately 1 year old) grey rats in the Animal House facilities at United Medical and Dental Schools (UMDS, Guy's Campus). Immediately after slaughter, olfactory tissue was taken from the ethmoturbinate of the nasal cavity and septum of pigs [25]. Respiratory tissue was taken from the mid-part of the nasal passage, at 4–10 cm distance from the entry to the nasal cavity. Olfactory tissues of the rats were taken similarly. Membrane-enriched fractions were prepared as below.

2.3. Preparation of olfactory and respiratory membrane-enriched fractions and cilia

2.3.1. Membrane-enriched fractions

The olfactory (O) and respiratory (R) porcine epithelium was cut into small pieces, placed in buffer (A) (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA and 1 mM PMSF) and homogenised at 0–4 °C using a Polytron homogeniser. The homogenate was centrifuged twice at 1500 × *g* for 10 min and the supernatant then centrifuged at 100,000 × *g* for 40 min to obtain a membrane-enriched fraction (O1, R1) and cytosolic fraction (O2, R2). The membrane pellet was suspended in buffer A and either used immediately or stored at −70 °C. Protein content of membrane-enriched fraction and cytosols was determined according to Bradford [26]. These fractions were used in binding studies (Section 2.4).

2.3.2. Cilia

Cilia-enriched fractions were obtained by a modification of the method of Boekhoff et al. [24]. Porcine olfactory and respiratory tissues were cut into small portions and bathed for 3 h in Ringer solution (2 mM HEPES, 112 mM NaCl, 3.4 mM KCl, 2.4 mM $NaHCO_3$, 1.2 mM $MgS0_4$, pH 7.4), washed twice with Ringer solution containing 2 mM EDTA to dissolve the mucus. The medium was then replaced with ice-cold Ringer solution supplemented with 10 mM $CaCl_2$, and tissues were gently stirred for 20 min. The deciliated epithelia were removed by centrifugation for 10 min at 5000 × *g*. The supernatant was collected and the tissue was incubated again with a solution containing 10 mM $CaCl_2$ for 20 min. The deciliated epithelium was precipitated by centrifugation and the supernatants containing the detached cilia were combined and layered on top of aqueous sucrose (45%, w/v). After centrifugation for 1 h at 100,000 × *g* at 4 °C, the white band of purified cilia at the interphase was collected. The cilia were diluted with Ringer solution and

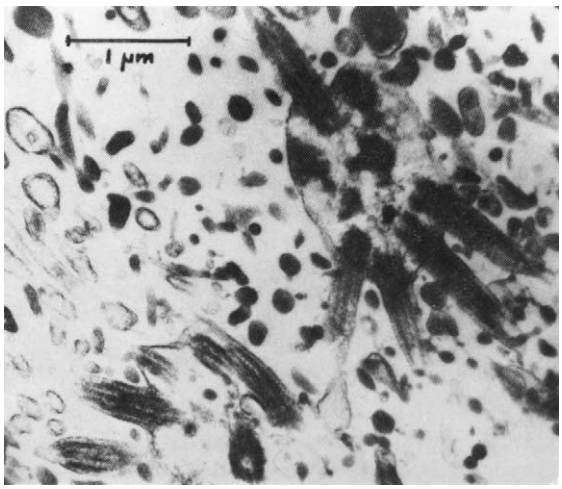

Fig. 1. Electron micrograph of material stripped from the surface of porcine olfactory epithelium with regenerated cellulose acetate, showing numerous cilia, some attached to dendritic knobs. Reproduced with permission from Henderson et al. [27], 1992, *Biochemical Society Transactions*, 20, 352S, copyright The Biochemical Society.

pelleted by centrifugation at 100,000 × *g* for 20 min. This procedure was repeated to wash sucrose from the cilia, then the pellet was suspended in Ringer solution and kept at −70 °C.

2.3.3. Highly purified cilia preparation

Fractions containing high concentrations of cilia, some still attached to dendritic endings (as shown by electron microscopy), were prepared by a novel method. Pieces of regenerated cellulose 'Zeta Probe' filter membranes were pressed gently on to the exposed surfaces of olfactory and respiratory mucosae of freshly killed rat and pig, then stripped away, as we have described earlier [27]. Filters were washed briefly in culture medium (MEM), buffered at pH 7.2 with HEPES, to remove debris. The resulting preparations were shown to contain a high concentration of identifiable olfactory cilia, often still attached to dendritic endings (Fig. 1). In preliminary assays [27], α-tubulin was shown to be present in olfactory cilia preparations consistent with the cilia being of neural origin (data not given). These 'Zeta Probe' preparations were then used for adenylate cyclase (AC) estimation.

2.4. Binding of 5α-[4,5-^{3}H]-androstenone to olfactory and respiratory membrane fractions

Binding to membrane-enriched and cytosolic fractions O1, O2 and R1, R2 (prepared as in Sections 2.3.1 and 2.3.2) was measured basically as described earlier [16,19,20]. 5α-[^{3}H]-Androstenone (0–25 nM) was incubated at 0–4 C for 1 h with the tissue sample (100 μg protein per sample), with or without excess (1 μM) of unlabelled 5α-androstenone. The total volume of samples for incubation was 0.5 ml. Non-specific binding was determined as the amount of radioactivity remaining in the supernatant or membrane-enriched fractions in the presence of excess (approximately 1 μM) unlabelled 5α-androstenone. The value chosen was nearly 1000 times greater than the average K_a of binding (see Section 3.1). Specific binding was determined as the difference in counts bound in presence (non-specific binding) and absence (total binding) of excess unlabelled 5α-androstenone, as above. Bound and free [^{3}H]-androstenone in membrane-enriched fractions O1, R1, were separated with Whatman GF/B glass fibre filter papers. Filters were previously stored in 20 mM Tris–HCl buffer, pH 7.5 with 1% BSA and 150 mM NaCl for 30 min and washed after filtration (10 ml/min) with 20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20. For cytosolic fractions O2 and R2, bound and free 5α-[^{3}H]-androstenone were separated by the charcoal adsorption method as previously described [23,25,28].

2.4.1. Analysis of binding data (Hill plots)

The binding curve in Fig. 2, curve 3 was transformed using a Hill plot. This requires the values of bound steroid (pmol/mg) to be estimated at saturating concentration of free steroid. This was achieved from a reciprocal plot (i.e. 1/[bound steroid] versus 1/[free steroid]) and extrapolating to the ordinate where the value 1/[bound steroid]$_{max}$ may be found. Hence, the value of [bound steroid]$_{max}$ is calculated. Using this value the fractional saturation, Y, (Y = [bound steroid]/[bound steroid]$_{max}$) was calculated as a function of free steroid and this in turn used to construct a Hill Plot, $\log(Y/(1-Y))$ versus log[free steroid] (Fig. 3).

Hill plots are particularly useful in the analysis of binding data. Simple binding processes yield straight lines of slope (Hill coefficient, h) equal to unity and where a slope, h, greater than unity is observed this provides unequivocal evidence for positive co-operativity of binding.

The mechanism of co-operativity is generally framed in terms of a two state model (R and T states) in which the equilibrium between the states is shifted during a ligand titration from almost all T state in favor of R state by virtue of the higher affinity of the R state for the ligand (here a steroid). This model predicts that data collected from binding studies on a co-operative system, when transformed by a Hill plot, follows a trajectory that, at low concentrations of ligand, is asymptotic to the T state binding curve (linear with $h = 1$) and at high concentrations of ligand is asymptotic to the R state curve (also linear with $h = 1$). In Fig. 3 the T state asymptote has been estimated by extrapolation of the data while the R state asymptote has been estimated from the symmetry of the plot.

The value of log[free steroid] when $\log(Y/(1-Y)) = 0$ gives the value of $\log K$, where K is the dissociation equilibrium constant. This property has been used to estimate these values for the R and T states and to give a mean value for the receptor.

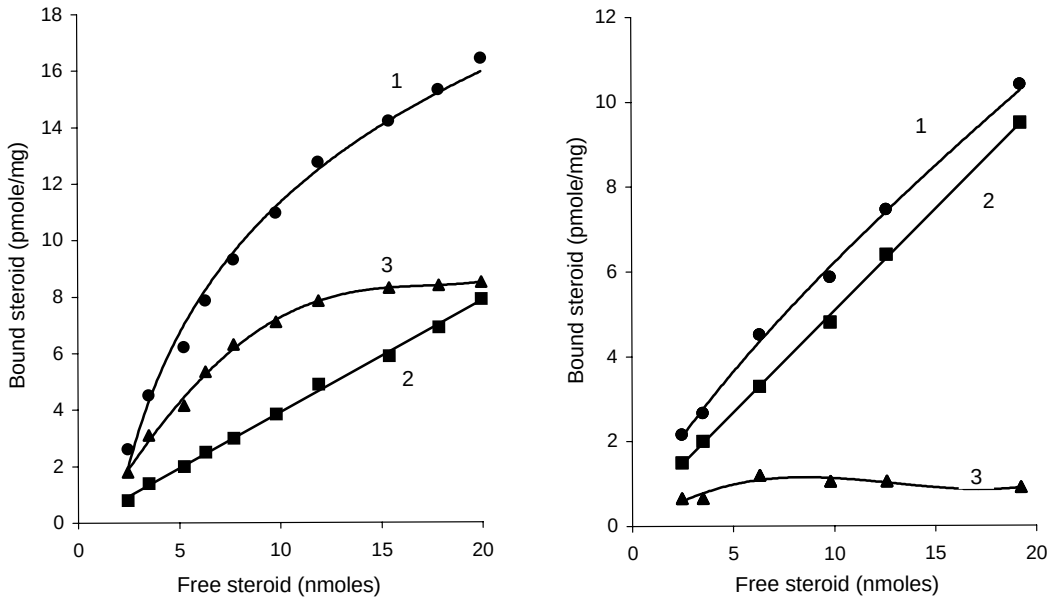

Fig. 2. Binding of 5α-[4,5-^{3}H]-androst-16-en-3-one to porcine nasal tissue fractions. Porcine olfactory O1 (left) and respiratory (R1) (right) membrane-enriched fractions were prepared as described in Section 2.3.1. Solubilised tissues were incubated with 5α-[^{3}H]-androstenone (0–25 nM) with/without a 250-fold excess of unlabelled 5α-androstenone at 0–4 °C for 60 min; 100 μg protein per sample. (1) Total binding (●); (2) non-specific binding (■), determined as the amount of radioactivity remaining in the supernatant (after charcoal treatment and centrifugation) in presence of excess (1 μM) of unlabelled steroid; (3) specific binding (▲) (determined as the difference in radioactivity found in presence and absence of excess unlabelled steroid).

2.5. Gel chromatography

In order to investigate which proteins bound to 5α-[^{3}H]-androstenone in the incubations described above, gel exclusion chromatography was carried out on a column (30 cm × 1 cm) of Superose 12 (Pharmacia, Sweden) at 0–4 °C. These were equilibrated with buffer A, containing Triton X-100 (1%, w/v). Cilia-enriched fractions were used as it was expected that high concentrations of proteins that could be involved in binding would be present [3,4] and

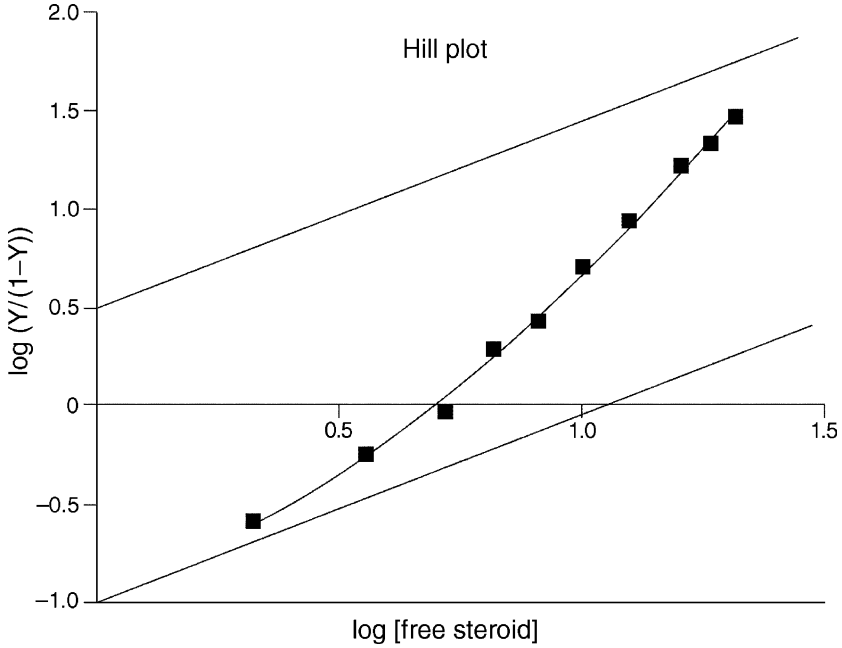

Fig. 3. Hill plot of the data from specific binding curve 3 of Fig. 2. The asymptotes to each end of the data curve are estimated and give binding constants of 8×10^7 and 1.6×10^9 M^{-1}. Y is defined as the 'fractional saturation', i.e. fraction of total binding sites bound with steroid at a given steroid concentration. The point at which the data curve intersects the log[steroid] axis provides a value for the mean K_a of binding, 2×10^8 M^{-1}.

hence give a better chance in isolation and purification studies. Accordingly, fractions (2 ml each) were applied to the columns and eluted with 30 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and Triton X-100 (1%, w/v) at a flow rate of 0.5 ml/min; fractions (1 ml each) were collected. Portions (200 μl) of each fraction were used for ^{3}H-content and protein determination while the proteins in the remainders were monitored on SDS-polyacrylamide gels (10 or 5–15% gradient) [29], with detection of protein bands using Coomassie Blue.

2.6. Separation and detection of glycoproteins

After solubilisation of cilia-rich fractions with Triton X-100 (0.5%, w/v) in 10 mM phosphate buffer for 40 min at 0–4 °C, followed by centrifugation at 100,000 × *g* for 20 min, supernatants were diluted to contain 0.1% of Triton X-100. They were then loaded on to 'glycoprotein' columns (5 ml) of Concanavalin A-Sepharose 4B, previously equilibrated with 10 mM phosphate buffer containing Triton X-100 (0.1%, w/v). Elution was effected with 5 volumes of 0.25 M α-methyl-D-mannopyranoside in phosphate buffer containing Triton X-100 (0.1%, w/v) to provide a fraction containing glycoproteins. These glycoprotein fractions were dialysed against 10 mM phosphate buffer containing Triton X-100 (0.1%, w/v), freeze-dried, diluted with 1 ml of the same buffer and centrifuged at 20,000 × *g* for 15 min. Supernatants were incubated with 20 mM 5α-[^{3}H]-androstenone with or without a 250-fold excess of unlabelled 5α-androstenone for 60 min at 0–4 °C as above. Each fraction was subjected to gel-filtration chromatography on Superose-12 which had been equilibrated in the same buffer, fractions (1 ml) being collected.

2.7. Measurement of adenylate cyclase: mediation of c-AMP in 5α-androstenone effect

Pieces of 'Zeta Probe' paper with blotted, purified cilia (Section 2.3.3) were incubated in 100 μl of incubation medium (50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM theophyllin, 1 mM ATP), containing 0.1 mM GTP, for 10 min at 37 °C in absence (basal level) and presence of 5 μM 5α-androst-16-en-3-one. Reactions were stopped by addition of 100 μl of ethanol and 200 μl of chloroform, vortex-mixed, stored in ice for 10 min, and centrifuged for 15 min at 2000 rpm. Adenylate cyclase activity in 20 μl water/ethanol fraction (after evaporation) was determined by the method of concurrent binding by c-AMP-binding protein (c-AMP-KIT, Amersham). The rest of the medium was evaporated in a Speed Vacuum Concentrator (Savant, Hicksville, USA) and proteins solubilised using 3% CHAPS (40 min), and dialysed against water (16 h). Samples were freeze-dried and used for protein assay [26].

3. Results

3.1. Binding of 5α-[^{3}H]-androstenone to fractions of pig and rat nasal tissues

Table 1 shows that highest levels of specific binding of 5α-[^{3}H]-androstenone were found in fractions of porcine olfactory tissue enriched with plasma membranes (O1). The level of binding (7986 fmol/mg) in fraction O1 (pig) was almost 10-fold higher (834 fmol/mg) than in the corresponding respiratory fraction (R1). Membrane-enriched fraction O1 from the rat also showed some specific binding but this was only 25% that of O1 obtained from the pig. It is also worth noting the appreciable binding recorded in porcine cytosol (O2, R2).

In one experiment, the encouraging initial results for porcine olfactory and respiratory tissues (O1, O2) were analysed in more detail (Fig. 2) in order to study the influence of increasing the concentration of free steroid present. Curve 3 clearly shows a plateau for bound steroid with increasing free steroid concentration, entirely consistent with saturation of receptors, i.e. specific binding of low capacity. No such plateau is seen for total or non-specific binding (curves 1 and 2). Moreover, the values of bound steroid for saturation are identical to those found in our initial experiments (Table 1). Fig. 2B (curve3) shows similar results for porcine respiratory tissue, but with saturation values being almost 10 times less than in Fig. 2A, as anticipated for non-neural tissue (Table 1). As curves 1 and 2 were clearly linear, with no indications of plateau even at high concentrations of free steroid, we believed that it was unnecessary to study these in any more detail.

A Hill plot (Fig. 3), obtained from these data (see Section 2.4.1) exhibited a Hill coefficient (*h*) of approximately 2, possibly suggesting co-operativity of binding. The binding affinity increased from $8 \times 10^{7}\ M^{-1}$ (T state) to $1.6 \times 10^{9}\ M^{-1}$ (R state) as substrate concentration increased.

Table 1
Specific binding of 5α-[4,5-^{3}H]-androst-16-en-3-one by membrane-enriched fractions of pig and rat olfactory (O) and pig respiratory (R) tissues

Fraction	Specific binding (fmol/mg protein) ($n = 3$)[a]
O1, membrane-enriched, pig	7986 ± 618
O2, cytosol, pig	2080 ± 98
R1, membrane-enriched, pig	834 ± 78
R2, cytosol, pig	2080 ± 95
O1, membrane-enriched, rat	2016 ± 106

Fractions were obtained as described in Section 2.3.1. After solubilisation where necessary, fractions were incubated with 5α-[^{3}H]-androstenone (20 nmol/l) with/without a 250-fold excess of unlabelled 5α-androstenone. Specific binding is the difference in binding radioactivity in the absence or presence of the excess unlabelled steroid. Bound and free 5α-[^{3}H]-androstenone were separated by charcoal (for soluble fractions) or with Whatman GF/B glass fibre filters for membrane fractions. For further details, see Section 2.4.

[a] The values are mean ± S.D.

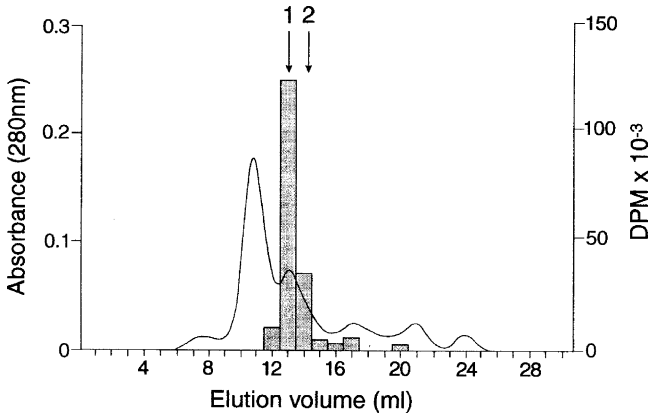

Fig. 4. Analytical gel chromatography of porcine olfactory cilia-enriched fractions. Cilia fractions were prepared as described in Section 2.3.2. Solubilised extracts (Triton X-100, 1%, w/v) were incubated with 5α-[^{3}H]-androstenone (see Section 2.5) before being subjected to column chromatography on Superose 12. Fractions (1 ml) eluted with 30 mM Tris–HCl, pH 8.0, containing 150 mM NaCl and Triton X-100 (1%, w/v), were collected and portions analysed for protein and radioactivity (bold line). (1) Elution position of bovine serum albumin (M_r 69 kDa); (2) ovalbumin (M_r 46 kDa).

3.2. Fractionation of proteins involved in binding 5α-androstenone

In pilot studies, solubilised cilia-enriched fractions (prepared as in Section 2.3.2) and previously incubated with ^{3}H-androstenone, were subjected to gel filtration on Superose 12 columns. Two major protein peaks were obtained which bound with the labelled steroid (M_r approximately 45–90 kDa) (Fig. 4). In attempts to increase the yield of protein, and to determine if this was glycoprotein, further solubilised cilia-enriched fractions were prepared. After incubation with 5α-[^{3}H]-androstenone, the solubilised 'cilia' fractions, were subjected to column chromatography on Concanavalin A-Sepharose B, providing fractions which contained glycoproteins (see Section 2). Gel-filtration of these on Superose 12 provided fractions containing glycoproteins, high levels of specific (displaceable) binding of 5α-[^{3}H]-androstenone being found only in fractions 12.0–12.5 ml. These corresponded to olfactory cilia glycoproteins of M_r approximately 70–90 kDa (Fig. 5).

3.3. Adenylate cyclase activity of nasal fractions

Basal levels of AC activity (Table 2) were some threefold higher ($P < 0.01$) in porcine olfactory, than in respiratory, purified cilia prepared by the 'Zeta Probe' method (Section 2.3.3); the sex of the animal made no significant difference to the results. Storage of olfactory cilia samples at −70 °C for 3 weeks resulted in approximately 50% ($P < 0.01$) reduction in activity. The AC activity of olfactory cilia of female rats was approximately twofold higher ($P < 0.05$) than in porcine olfactory cilia.

Table 2 also summarises the changes in AC activity in response to 5α-androstenone (5×10^{-6} mol/l) and GTP (10^{-10} mol/l). Fresh male and female pig olfactory cilia showed substantial increases ($P < 0.01$) in AC activity; in contrast, there was no significant response with respiratory cilia. The responses with stored olfactory cilia preparations (3 weeks at −70 °C) were reduced but still highly significant ($P < 0.01$), bearing in mind the reduced basal levels. Fresh male rat olfactory cilia had an AC activity almost twice ($P < 0.05$) that of corresponding porcine preparations, although rat respiratory cilia had only half the activity; there was no significant response with either

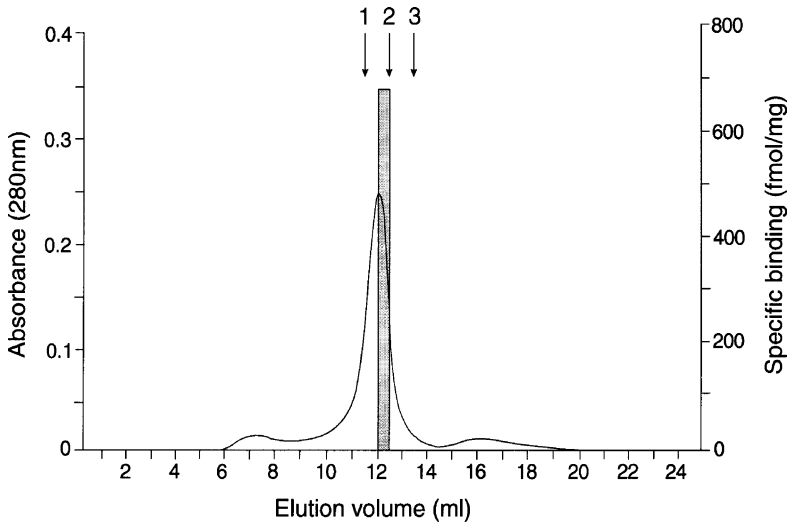

Fig. 5. Chromatographic purification of the glycoprotein fraction. After using column chromatography on Superose 12 (Fig. 4), the major fractions binding [^{3}H]-androstenone were subjected to chromatography on a 'glycoprotein' column of Concanavalin A-Sepharose 4B and the glycoprotein fraction subjected to gel filtration on Superose 12, yielding various glycoproteins. Specific binding of radioactivity was noted only in fractions 12.0–12.5 ml corresponding to glycoproteins of M_r approximately 70–90 kDa. For further details, see Sections 2.5 and 2.6. (1) Elution position of pre-stained β-galactosidase (M_r 133 kDa); (2) bovine serum albumin (M_r 69 kDa); (3) ovalbumin (M_r 46 kDa).

Table 2
Adenylate cyclase (AC) activity in porcine and rat olfactory and respiratory cilia-enriched fractions

Sample	AC activity (pmol/min/mg protein) ($n = 3$)[a]		P-value
	Basal Level	Level in presence of 5α-androstenone	
Fresh, porcine olfactory (male)	935 ± 237	2670 ± 287	<0.01**
Fresh, porcine olfactory (female)	860 ± 280	3110 ± 980	0.01**
Porcine, olfactory (female), 3 weeks, −70 °C	440 ± 46	1548 ± 210	0.01**
Fresh, porcine respiratory (female)	317 ± 71	298 ± 61	<0.01**
Fresh, rat olfactory (male)	1470 ± 256	1264 ± 246	N.S., 0.46
Fresh, rat respiratory (male)	424 ± 126	413 ± 76	N.S., 0.8

AC activity of porcine cilia-enriched fractions (prepared by the 'Zeta Probe' method) was measured as described in Sections 2.3.3 and 2.7. (**) Highly significant; (*) significant; (N.S.) not significant. Statistical comparison (two tailed t-test) were for fresh porcine olfactory, male vs. female pig basal levels: N.S., $P > 0.5$ and in presence of 5α-androsterone: N.S., $P > 0.5$. Fresh vs. frozen female basal; ** $P \leq 0.01$ and in presence of 5α-androsterone; ** $P < 0.01$. Basal levels: pig vs. rat olfactory; * $P = 0.04$, and respiratory; * $P = 0.02$.

[a] The values are mean ± S.D.

olfactory or respiratory cilia preparations of rats to the presence of 5α-androstenone.

4. Discussion

4.1. Binding of 5α-androstenone to porcine and rat nasal membrane-enriched fractions

In the present work, the use of membrane-enriched fractions has resulted in steroid binding results which have been far more consistent than those obtained using crude tissue homogenates [23]. Previously, large amounts of non-specific binding of 5α-androstenone have been recorded, partly because of the non-purified homogenate used and partly because of the extreme hydrophobicity of 5α-androstenone. This characteristic of the steroid is clearly shown by the marked linear increase in binding, with no suggestion of saturation (Fig. 2, curve 2). The porcine membrane-enriched olfactory fraction (O1) (Table 1), showed specific binding and Fig. 2, curve 3 was consistent with this. It is unfortunate that, through constraints of time, this latter experiment could only be performed once. Nonetheless, it is gratifying that the plateau (saturation values for specific binding, Fig. 2A and B, curve 3) are virtually identical to the corresponding specific binding data in Table 1. Furthermore, analysis of the data (Fig. 2, curve 3) by Hill plot (Fig. 3) yielded a mean K_a of $2 \times 10^8\ M^{-1}$, which compares well with K_a of $8.4 \times 10^8\ M^{-1}$ recorded earlier [29] and indicates that our data are of good quality. For 5α-androstan-3-one, a saturated analogue of 5α-androstenone which also has a potent "urinous" odor, a K_a of approximately $10^9\ M^{-1}$ was obtained using the rough membrane fraction of porcine olfactory tissue [30]. With crude homogenates of sheep olfactory tissue, a K_a of $7 \times 10^8\ M^{-1}$ was recorded for the saturated analogue [31]. In both these studies [30,31], 5α-androstenone effectively displaced 5α-androstanone from binding sites. In 1997 [32], we showed that the K_a for 5α-androstenone binding to human testicular cytosols was $8 \times 10^9\ M^{-1}$, but this degree of binding is less tight than that shown by 5α-dihydrotestosterone (5α-DHT) for androgen-target tissues, e.g. rat prostate, when the K_a was found to be $3 \times 10^{10}\ M^{-1}$ [33].

The finding that the Hill coefficient approached a value of 2 (Fig. 3) suggests the possibility of positive co-operativity of binding, the K_a increasing from 8×10^7 to $1.6 \times 10^9\ M^{-1}$ with increasing substrate concentration (see Section 2.4.1). This phenomenon has not been noted before for 5α-androstenone, to our knowledge, but the effect is consistent with the known pheromonal actions of 5α-androstenone [18,19], when small increases in steroid concentration can result in large increases in physiological effects. The perception of the odor of this steroid by humans can be learned by repeated exposure (unpublished results from this laboratory). The cellular and molecular basis of odor adaptation have also been studied recently [34].

Table 1 also records some specific binding of 5α-androstenone in cytosolic fractions obtained from porcine olfactory (O2) and respiratory (R2) tissue, a phenomenon which has been noted earlier [34]. It is difficult to explain this phenomenon unless the steroid is binding to cytosolic/microsomal enzymes present in these fractions. In earlier work [35,36], our group recorded the presence of 3α(β)-hydroxysteroid dehydrogenases (3α(β)-HSDs), which can catalyse the reduction of 5α-androstenone to the corresponding alcohols, 5α-androst-16-en-3α(β)-ol, the 3β-isomer having a less potent odor than the 3α-alcohol or the initial 3-oxo compound. In addition, the existence and activity of cytochrome P-450-dependent oxidases [37,38] may also bind to 5α-androstenone, resulting in its metabolism, thus giving a diminution of the olfactory signal (as is known to be the case for other odorants [39]). Whether the cytosolic fractions could have been contaminated by very small fragments of membrane-rich material as a result of the homogenisation/centrifugation process may provide another explanation.

It is also significant that the binding of 5α-androstenone to rat membrane-enriched fraction (O1) is appreciable but only approximately 25% of the corresponding porcine (O1) value (2016 compared with 7986 fmol/mg tissue) (Table 1). Although the primer and releaser pheromonal effects of

5α-androstenone in the pig are well documented [18,19], there is no evidence for similar effects in the rat, to our knowledge, despite the fact that rat testis does contain small quantities of 5α-androstenone and other 16-androstenes [40]. Whether rats can actually perceive the odor of 5α-androstenone is unknown, to our knowledge.

The preliminary fractionation studies described here, using chromatography on Concanavalin A, followed by Superose 12, for proteins which bind with 5α-androstenone, suggest that the proteins involved are glycoproteins. Fig. 5 shows that specific binding coincides with the elution of such proteins with M_r in the range approximately 70–90 kDa. These data are consistent with those obtained for olfactory cilia in the frog [3,4] and suggest that 5α-androstenone may exert its effects on olfactory tissues (possibly cilia) in a 'classical' manner by binding to glycoproteins associated with porcine olfactory cilia, as might be anticipated from earlier analogous studies with other species, has yet to be elucidated.

4.2. Possible role of c-AMP in transduction mechanisms linked with 5α-androstenone

Our preliminary results suggest that the 'Zeta Probe' method is able to yield fairly rapidly a reasonably pure fraction of olfactory cilia as evidenced by EM studies (Fig. 1) and the presence of 'neural' proteins, such as tubulin [27]. The much higher activity of AC in olfactory (neural), compared with respiratory (non-neural), tissues is also consistent with the usefulness of the 'stripping' methodologies we have used. Such cilia preparation could be used in future experiments to explore in more detail the binding of odorous steroids, such as 5α-androstenone as well as non-steroidal odorants. In the present work, it was unfortunately not possible to proceed any further with this particular aspect of our studies owing to the tragically early and unexpected death of our group's major investigator, Dr. M.A. Kraevskaya. Despite this, however, the 'Zeta Probe' method has shown its versatility in yielding preparations of rat and pig olfactory cilia which could be utilised in exploring the role of c-AMP in transduction mechanisms linked to the action of 5α-androstenone, which has a powerful urine-like smell, as generally perceived by humans [21]. The highly significant increases in adenylate cyclase activity (Table 2) in the presence of 5α-androstenone plus theophylline and GTP are consistent with c-AMP acting as a second messenger [41,42] in mediation of the olfactory effects of this steroid. Our results suggest that 5α-androstenone may be added to the list of other odorants, including especially, fruity and floral ones, that require c-AMP as a mediator of their actions [43]. The marked response by boars is also of interest and consistent with the aggressive reaction noted when in the presence of another boar [19]. In contrast, the use of porcine respiratory cilia resulted in no significant increases in AC activity in response to 5α-androstenone, as anticipated for non-neural tissue.

Acknowledgements

We are most grateful to Drs. L.H. Bannister, R.W. Evans, N.A. Gregson, W.D. Booth and Mr. E.E. Douek, MS, FRCS for their invaluable collaboration and advice. D.B.G. thanks Mrs. S. Durie and Mrs. D.M. Gower for their help in the preparation of the various drafts of this paper and The Royal Society for an Exchange Scholarship for the late Dr. Kraevskaya (Grant No. 638056-P601/SG/KBn). Sincere thanks are given to Professor R.B. Heap, FRS for the provision of space and facilities at the AFRC Animal Research Institute, Babraham, Cambridge, to Dr. L.H. Bannister for the EM photograph (Fig. 1) and The Biochemical Society for permission to reproduce it from [27].

References

[1] R. Doty (Ed.), Handbook of Olfaction and Gustation, Marcel Dekker, New York, 1995;
G.V. Ronnett, C. Moon, G-proteins and olfactory signal transduction, Ann. Rev. Physiol 64 (2002) 189–222.
[2] R.R.H. Anholt, U. Aebi, S.H. Snyder, A partially purified preparation of isolated chemosensory cilia from the olfactory epithelium from the bull-frog, *Rana catesbriana*, J. Neurosci. 6 (1986) 1962–1969.
[3] Z. Chen, U. Pace, D. Ronen, D. Lancet, Polypeptide Sp 95: a unique glycoprotein of olfactory cilia with transmembrane receptor properties, J. Biol. Chem. 261 (1986) 1299–1305.
[4] R.R.H. Anholt, A.E. Petro, A.M. Rivers, Identification of a group of novel membrane proteins unique to chemosensory cilia of olfactory receptor cells, Biochemistry 29 (1990) 3366–3373.
[5] H. Breer, Molecular basis of signal recognition in olfaction, Pflugers Arch. 431 (6 (Suppl. 2)) (1996) R3II-2.
[6] A. Vogl, J. Noe, H. Breer, I. Boekhoff, Cross-talk between second messenger pathways, Eur. J. Biochem. 267 (2000) 4529–4535.
[7] D. Restrepo, J.H. Teeter, D. Schild, Second messenger signalling in olfactory transduction, J. Neurobiol. 30 (1996) 37–48.
[8] K. Touhara, Odor discrimination by G-protein-coupled olfactory receptors, Microsc. Res. Tech. 58 (2002) 135–141.
[9] R.S. Dhallau, K-W. Yau, K.A. Schrader, R.R. Reed, Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurones, Nature 347 (1990) 184–187.
[10] L.B. Buck, R. Axel, A novel multigene family may encode odorant receptors: a molecular basis for odor recognition, Cell 65 (1991) 175–287.
[11] H. Mutsunami, L.B. Buck, A multigene family encoding a diverse array of putative pheromone receptors in mammals, Cell 90 (1997) 775–784.
[12] P. Mombaerti, F. Wang, C. Dulae, S. Chao, A. Nemes, M. Mendelsohn, J. Edmonson, R. Axel, Visualising an olfactory sensory map, Cell 87 (1996) 675–686.
[13] D. Krautwurst, K.-W. Yau, R.R. Reed, Identification of ligands for olfactory receptors by functional expression of a receptor library, Cell 95 (1998) 917–926.
[14] H. Zhao, L. Ivic, J. Otaki, M. Hashimoto, K. Mikoshiba, S. Firestein, Functional expression of a mammalian odorant receptor, Science 279 (1998) 237–242.
[15] M. Ma, G.M. Shepherd, Functional mosaic organisation of mouse olfactory receptor neurons, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 12869–12874.
[16] B. Malnic, J. Hirano, T. Sato, L.B. Buck, Combinatorial receptor codes for odors, Cell 96 (1999) 713–723.
[17] V. Prelog, L. Ruzicka, Untersuchungen über organextrakte. 5. Mitteilung: über zwei muschusartig riechende steroide aus schweinestestes-extracten, Helv. Chim. Acta 27 (1944) 61–66.

[18] H.C.B. Reed, D.R. Melrose, R.L.S. Patterson, Androgen steroids as an aid to the detection of oestrus in pig artificial insemination, Br. Vet. J. 130 (1974) 61–66.
[19] R.N. Kirkwood, P.E. Hughes, W.D. Booth, The influence of boar-related odours on puberty attainment in gilts, Anim. Prod. 36 (1983) 131–136.
[20] N.M. Dorries, E. Adkins-Regan, B.P. Halpern, Sensitivity and behavioural responses to the pheromone, androstenone, are not mediated by the vomeronasal organ in domestic pigs, Brain Behav. Evol. 49 (1997) 53–62.
[21] D.B. Gower, B.A. Ruparelia, Olfaction in humans with special reference to odorous 16-androstenes: their occurrence, perception and possible social, psychological and sexual impact, J. Endocr. 137 (1993) 167–187.
[22] D.B. Gower, Human Pheromones? in: J.S. Jellinek (Ed.), Psychological Basis of Perfumery, 4th ed., Blackie Academic, Glasgow, 1997, pp. 179–199;
B.I. Grosser, L. Monti-Bloch, C. Jennings-White, D.L. Berliner, Behavioral and electrophysiological effects of androstadienone, a human pheromone, Psychoneuroendocrinology 25 (2000) 289–299.
[23] M.R. Hancock, J.N. Gennings, D.B. Gower, On the existence of receptors to the pheromonal steroid, 5α-androst-16-en-3-one, in porcine nasal epithelium, FEBS Lett. 181 (1985) 328–334.
[24] I. Boekhoff, E. Tareilus, J. Strotman, H. Breer, Rapid activation of alternative second messengers in olfactory cilia from rats by different odorants, EMBO J. 9 (1990) 2453–2458.
[25] D.B. Gower, M.R. Hancock, L.H. Bannister, Biochemical studies on the boar pheromones, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol, and their metabolism by olfactory tissue, in: R.H. Cagan, M.R. Kare (Eds.), Biochemistry of Taste and Olfaction, Academic Press, New York, 1981, pp. 7–32.
[26] M.M. Bradford, Protein assay–dye binding, Anal. Biochem. 72 (1976) 245–255.
[27] T. Henderson, M. Kraevskaya, N. Gregson, D.B. Gower, L.H. Bannister, Biochemical and ultra-structural characterisation of olfactory receptor membranes of rat and pig, using a novel method of cilium separation, Biochem. Soc. Trans. 20 (1992) 352S.
[28] M.A. Kraevskaya, M. Kagan, E.P. Zinkevich, V.P. Shevchenko, N.F. Myasoedo, E.V. Parfenova, R.N. Etincof, Molecular mechanisms of olfaction: binding of the boar sex pheromone 5α-androst-16-en-3-one and its analogues by cytosolic fraction of porcine olfactory epithelium, Sensorye Sistemy 1 (1987) 127–136 (in Russian).
[29] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[30] J.N. Gennings, D.B. Gower, L.H. Bannister, Studies on the receptors to 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol in sow nasal mucosa, Biochim. Biophys. Acta 496 (1977) 547–556.
[31] E.P. Zinkevich, M.A. Kraevskaya, A.V. Minor, In search of molecular receptors of boar pheromone, Chem. Senses 14 (1989) 229.
[32] G.H. Dodd, K. Persaud, Biochemical mechanisms in vertebrate primary olfactory neurons, in: R.H. Cagan, M.R. Kare (Eds.), Biochemistry of Taste and Olfaction, Academic Press, New York, 1981, pp. 333–357.
[33] T.K. Kwan, M.A. Kraevskaya, H.L.J. Makin, D.J.H. Trafford, D.B. Gower, Use of gas chromatographic-mass spectrometric techniques in studies of androst-16-ene and androgen synthesis in human testis; cytosolic specific binding of 5α-androst-16-en-3-one, J. Steroid Biochem. Mol. Biol. 60 (1997) 137–146.
[34] R.J.B. King, W.I.P. Mainwaring, Steroid–Cell Interactions, Butterworths, London, 1974, p. 82.
[35] F. Zufall, T. Leinders-Zufall, The cellular and molecular basis of odor adaptation, Chem. Senses 25 (2000) 473–481.
[36] D.B. Gower, M.R. Hancock, Investigations into the metabolism and binding of the odorous steroid 5α-androst-16-en-3-one, in porcine nasal tissues, in: W. Breipohl (Ed.), Olfaction and Endocrine Regulation, IRL Press, London, 1982, pp. 267–277.
[37] M.R. Hancock, D.B. Gower, Properties of 3α-and 3β-hydroxysteroid dehydrogenases of porcine nasal epithelium: some effects of steroid hormones, Biochem. Soc. Trans 14 (1986) 1033–1034.
[38] E.B. Brittebo, J. Rafter, Steroid metabolism by rat nasal mucosa: studies on progesterone and testosterone, J. Steroid Biochem. 20 (1984) 1147–1151.
[39] E.B. Brittebo, Metabolism of progesterone by the nasal mucosa in mice and rats, Acta Pharmacol. et Toxicol. 51 (1982) 441–445.
[40] G.V. Ronnet, The molecular mechanisms of olfactory signal transduction, in: R. Doty (Ed.), Handbook of Olfaction and Gustation, Marcel Dekker, New York, 1995, pp. 127–145.
[41] T.K. Kwan, S.L. Foong, Y.T. Lim, D.B. Gower, Effects of some non-steroidal anti-inflammatory drugs (NSAIDS) on steroidogenesis in rat and porcine testis, Biochem. Mol. Biol. Intern. 31 (1993) 733–743.
[42] R.R.H. Anholt, A.M. Rivers, Olfactory transduction: cross-talk between second-messenger systems, Biochemistry 29 (1990) 4049–4054.
[43] P.B. Sklar, R.R.H. Anholt, S. Snyder, The odorant-sensitive adenylate cyclase of olfactory receptor cells, J. Biol. Chem. 261 (1986) 15538–15543.