Odorous chemical perturbations of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities

Effects on native and lipid-substituted preparations from individual turbinals from dog olfactory tissue

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Individual turbinals from the right and left sides of dog olfactory tissue were removed and nerve-ending-particle preparations were prepared. ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities of the individual preparations, and the effect of several odorous compounds [including (+)- and (–)-carvone] on the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities, were determined. The maximally stimulatory odorant concentration in the reaction mixture for the majority of odorants was found to be 1.0 mM. Matched pairs of left/right turbinals showed a lack of bilateral symmetry of response. ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities of various dog brain nerve-ending particle preparations responded only slightly to 1.0 mM odorants. The role of phospholipids in the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity was found to be critical. Partial replacement of endogenous lipid with either synthetic phospholipids or extracted lipids resulted in changes in stimulation obtained with endogenous lipids alone.

Although the molecular basis for perception of odours has yet to be established, electrophysiological, psychophysical and biochemical approaches for example, have attempted to support a number of different theories on the initial events of odour perception. All theories must account for certain properties of the odour-sensing process, such as differences in perceived odours between optical isomers and the ability to sense only changes in odour concentration.

The psychophysical approach has provided data in areas such as perceptions of intensity (Engen, 1971), behavioural responses (Schneider, 1974; Eppler, 1974; Steiner, 1976) and 'primary odours' (Dravnieks, 1974; Laffort *et al.*, 1974). However, these types of studies cannot directly measure events occurring at the molecular level.

Electrophysiological studies on the effects of odorant stimuli on single receptor cells of the migratory locust (Kafka, 1970) showed significantly different stimulatory and inhibitory responses from different receptor cells to a common stimulus. The electrophysiological response of the majority of neurons of frog nasal tissue to odorants was inhibition, although some excitation responses were recorded (Gesteland *et al.*, 1965). It has been

proposed that olfactory receptors are proteins, since a variety of protein thiol-specific reagents could alter or abolish the electrophysiological response (Getchell & Gesteland, 1972; Getchell & Getchell, 1974; Frazier & Heitz, 1975). Prior and/or concomitant treatment of the olfactory tissue with an odorant and a thiol-specific reagent led to the protection of the tissue's ability to respond to the particular odorant and to odorants of similar size and structure but not for dissimilar molecules (Getchell & Gesteland, 1972; Getchell & Getchell, 1974).

Biochemical studies indicating a possible enzyme-odorant interaction have been published previously (Koch, 1969; Koch, 1971/1972; Koch & Desai, 1974; Koch & Gilliland, 1977; Rossi & Koch, 1981). This enzyme-perturbation hypothesis states that the stimulation and/or inhibition of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity from nerve-ending particles of olfactory bipolar sensing cells is in some way responsible for, or associated with, changes in electrophysiological response (Koch & Gilliland, 1977; Rossi & Koch, 1981). Perturbation of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity from olfactory nerve-ending particles by a series of straight-chain odorous alcohols and ketones has been demonstrated in a number of different animals, including the rat (Rossi & Koch, 1981), mouse R. B. Koch & J. E. Smith, unpublished work), dog (Koch & Gilliland, 1977), cow (R. B. Koch &

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J. E. Smith, unpublished work) and rabbit (Koch & Desai, 1974). Rossi & Koch (1981) have demonstrated that the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity of matched left/right pairs of rat olfactory nerve-ending particles exhibited a marked lack of bilateral symmetry response. Studies by Koch & Gilliland (1977) showed various responses (stimulation and inhibition) to odorants by $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity from individual turbinals from the right side of the dog. Differences in response patterns were observed for a number of odorants, including the optical isomers (+)- and (-)-carvone.

The present work extends the dog olfactory studies (Koch & Gilliland, 1977), and reports initial experiments to elucidate the role of phospholipids in the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase lipoprotein complex in determining the activity response to odorous chemicals.

Materials and methods

The synthetically prepared optical isomers (+)- and (-)-carvone (odours of caraway and spearmint respectively) were a gift from Dr. J. E. Amore, who received them from the Koch-Light Laboratories, Colnbrook, Bucks., U.K. The following odorants were obtained from the Mississippi State Chemical Laboratory and purity was determined by gas chromatography: n-octanol (>99%); octan-2-one (>99%); and nonan-2-one (>97%). Odorants were stored and utilized as 1.0M-solutions in ethanol. ATP, NADH, pyruvate kinase/lactate dehydrogenase, NaCl, KCl, MgCl_2 , synthetic dioleoylglycerophosphocholine and synthetic dipalmitoylglycerophosphocholine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Lubrol WX was from I.C.I., Providence, RI, U.S.A. Other chemicals used were reagent-grade.

Dogs were killed in the Veterinary Medicine Laboratory by intramuscular injection of succinylcholine. Exsanguination was performed by severing the brachial artery to drain as much blood from the nasal area as possible. The nasal olfactory turbinal area was exposed with an electric bone saw on both sides of the nasal septum to facilitate matching of turbinals from opposite sides. Individual turbinals were removed from the superior to the inferior positions of alternating sides and designated right (R) or left (L) from the dog's point of view. Various regions of the dog brain [right and left frontal lobe (RFL, LFL), right and left occipital lobe (ROL, LOL), right and left olfactory bulb (ROB, LOB), caudate nucleus (CN), thalamus (T), hippocampus (H) and paleopallium (P)] were also removed for comparison of response to odorants. Sample homogenization and fractionation was that of Koch (1969). The B fraction, containing nerve-ending

particles and mitochondria, was assayed for $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase and Mg^{2+} -dependent ATPase activities at 27°C by the continuous method of Pullman *et al.* (1960), as reported by Koch & Gilliland (1977). The stimulation of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity from the crude B fraction was shown to be similar to the response by the enzyme in the nerve-ending-particle membrane fraction (Koch, 1971/1972). All odorants were tested at 1.0mM concentration in ethanol unless otherwise noted. Ethanol (10 μl or less) had no effect on $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity or the coupling enzymes in the reaction mixture. The lipids from the cortex of a dog brain and cow olfactory tissue were extracted by the method of Folch *et al.* (1957) and ethanol added to 50%.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard. $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase specific activities are reported as μmol of P_i/mg of protein per h using $6.22\text{mm}^{-1} \cdot \text{cm}^{-1}$ as the absorption coefficient for NADH at 340nm.

Replacement of endogenous lipids by lipid extracts and synthetic phospholipids utilizing the method of Warren *et al.* (1974a,b) was carried out with one modification. Lubrol WX was the detergent utilized instead of cholate or deoxycholate. Briefly, 1.0ml of enzyme sample (0.7–1.0mg of protein) in 0.32M-sucrose/10mM-imidazole buffer (pH 7.5)/1mM-EDTA (called 'sucrose solution') was mixed with 200 μl of 8% Lubrol WX and held at 4°C for 15 min. An appropriate lipid preparation (10mg in 200 μl of 8% Lubrol WX) was added (final Lubrol WX concentration 2.6%) and held at 4°C for 30 min. A 1.0ml portion of the enzyme, Lubrol WX and exogenous lipid mixture was layered on a 1.0ml cushion of 20% sucrose/10mM-imidazole buffer (pH 7.5)/1mM-EDTA in a 2ml centrifuge tube. The sample was centrifuged in a Beckman L2-62B ultracentrifuge using an SW36 rotor with 2ml tube adaptors for 17h at 4°C and 193 000g. Pellets were resuspended in 1.0ml of sucrose solution and immediately tested for $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity response to odorants. Lipids were then extracted (Folch *et al.*, 1957) for chromatographic analysis. Chromatography of lipids was conducted using silica-gel G plates. The developing solvent was chloroform/methanol/acetic acid/water (85:15:10:4, by vol.). The plates were air-dried and spots visually detected by spraying with 50% H_2SO_4 and charring.

Results

The effects of different concentrations of odorants [nonan-2-one, (+)-carvone and (-)-carvone] on the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activities of the first three turbinals from each side of the nasal

septum are shown in Table 1. Differences in specific activities greater than 0.15 are significant at $P < 0.05$. The activities that responded to the odorants with stimulation usually showed the greatest stimulation at 1.0 mM concentration. Some turbinal ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities responded to increasing odorant concentrations with increasing inhibition [e.g., preparation R1 to all odorants and preparation L3 to (–)-carvone]. ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities in response to odorant concentrations greater than 1.0 mM usually exhibited decreased stimulation or increased inhibition. At the highest concentration of odorant (3.0 mM) all turbinal ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities tested either exhibited decreased stimulation, or, in some cases, a switch occurred from stimulation to inhibition. Since the majority of maximal stimulatory responses were observed at 1.0 mM odorant concentration, in agreement with the results of Koch & Gilliland (1977), a comparison of odorant responses from left/right matched pairs of turbinas was carried out at 1.0 mM odorant. The high concentration of odorants is recognized by the authors; however, conditions used in these studies would not be expected to duplicate intact animal sensitivity. The enzyme was no longer positioned in the plasma membrane of an intact nerve cell. Also, the odorants were dispersed in a liquid medium, which cannot be directly correlated with the conditions of the olfactory epithelium tissue.

Figs. 1 and 2 show that ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities from most turbinas were stimulated by 1.0 mM odorant. In general, nonan-2-one

and n-octanol elicited the greatest stimulation. Responses to (+)- and (–)-carvone were primarily inhibition of enzyme activity. When the responses from matched left/right turbinas were compared, a marked lack of bilateral symmetry was observed. In some cases (preparations L3 and R3; L8 and R8) the pattern of responses to the five odorants was qualitatively similar, although quantitatively significantly different ($P < 0.05$). Other pairs (L12 and R12; L1 and R1) were completely different in their response patterns.

The enzyme activity from the individual turbinas showed a remarkable ability to distinguish between the optical isomers (+)- and (–)-carvone. Figs. 1 and 2 show that preparation L1 in response to (–)-carvone showed strong stimulation (212%) whereas (+)-carvone elicited inhibition (–16%) with respect to the control. Preparations R2 and L7 responded to (–)-carvone with stimulation but did not respond to (+)-carvone. The enzyme response was opposite; i.e., no response to (–)-carvone and stimulation to (+)-carvone for preparations R3, L3 and L8.

Nerve-ending-particle fractions from specific brain regions were prepared to test the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity response to the odorants utilized in the present study to compare the response of the brain enzyme with that of the olfactory enzyme. The ten brain regions (LFL, RFL, LOL, ROL, LOB, ROB, T, H, P and CN) were assayed for ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities and their response to nonan-2-one, n-octanol, and (+)- and (–)-carvone. The ($\text{Na}^+ + \text{K}^+$)-dependent ATPase

Table 1. *Effect of various concentrations of odorants on ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities from individual turbinal preparations*

Specific activity values are averages of at least three replications. Differences in specific activities between control and odorant greater than 0.15 are significant at $P < 0.05$.

Sample	Control specific activity ($\mu\text{mol of P}_i/\text{mg}$ of protein per h)	Odorant	Odorant concn. (mM) ...	Specific activity ($\mu\text{mol of P}_i/\text{mg of protein per h}$)			
				0.5	1	2	3
L1	0.96	Nonan-2-one		1.27	1.59	1.15	0.86
L2	1.30	Nonan-2-one		1.30	1.89	1.72	1.51
		(+)-Carvone		1.51	1.69	1.17	0.87
		(–)-Carvone		1.30	1.87	1.72	1.30
L3	1.28	Nonan-2-one		1.25	1.79	1.69	1.54
		(+)-Carvone		1.41	1.54	1.22	1.15
		(–)-Carvone		1.28	1.15	1.09	0.77
R1	0.69	Nonan-2-one		0.69	0.69	0.59	0.55
		(+)-Carvone		0.69	0.69	0.55	0.48
		(–)-Carvone		0.60	0.49	0.44	0.33
R2	1.51	Nonan-2-one		1.69	1.87	1.90	1.39
		(+)-Carvone		1.51	1.57	1.62	1.51
		(–)-Carvone		1.63	1.72	1.54	1.36
R3	1.11	Nonan-2-one		1.33	1.61	1.24	1.00
		(+)-Carvone		1.47	2.02	1.61	1.11
		(–)-Carvone		1.21	1.22	1.35	1.20

Fig.1. Response of $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activities of individual turbinals 1–6 to various odorants. Values are means $\pm$ S.D. (represented by the bars) of at least three determinations. A 1.0ml reaction mixture contains: 5mM-ATP, 135mM-imidazole/HCl buffer, pH 7.5, 0.2M-NADH, 0.6mM-phosphoenolpyruvate, 0.02% bovine serum albumin, 5mM-MgCl₂, 100mM-NaCl, 20mM-KCl, approx. 9 units of pyruvate kinase and 12 units of lactate dehydrogenase, plus or minus 1.0mM-ouabain. $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity (ΔA_{340}) is total activity minus activity with ouabain. Odorants (1 μ l; 1.0M) were added from ethanol solutions. Key to the Figure: C, control; 1, nonan-2-one; 2, n-octanol; 3, octan-2-one; 4, (–)-carvone; 5, (+)-carvone; R, right turbinals; L, left turbinals. Non-shaded columns are not significantly different from control values at $P < 0.05$.

activities exhibited only small responses at 1.0mM concentration of odorants. The largest increase in activity was 10% stimulation of the LFL by nonan-2-one (significant at $P < 0.05$) and the largest decrease 7% inhibition of LOL by n-octanol (significant at $p < 0.05$). Activities in the presence of all other odorants were not significantly ($P < 0.05$) different from control $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activities from the ten brain regions.

Lipid-replacement studies

Replacement of endogenous lipids of the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase lipoprotein complex was carried out to test the role of the lipoprotein structure in the enzyme-perturbation hypothesis (Koch, 1971/1972; Koch & Gilliland, 1977; Rossi & Koch, 1981). Lipid replacement was performed on olfactory preparations R7, R8, L11 and a dog brain nerve-ending-particle preparation for comparison. Folch-extracted lipids of dog brain ('dog brain lipids') and cow olfactory tissue as well as the synthetic phospholipids dioleoylglycerophosphocholine and dipalmitoylglycerophosphocholine, were used to replace endogenous dog olfactory lipids.

Odorant concentration in all tests was 1.0mM. The solubilized olfactory $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase preparations from turbinals R7, R8 and L11 did not respond to odorants (results not shown). Dog brain $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activities did not respond to the odorants either with endogenous lipids or when substituted with dioleoylglycerophosphocholine, dipalmitoylglycerophosphocholine or cow olfactory lipids (Table 2).

Dog-brain-lipid substitution of preparation L11 greatly reduced the stimulation or inhibition by nonan-2-one or (+)-carvone respectively, of the $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity compared with endogenous lipids (Table 2). Dioleoylglycerophosphocholine substitution caused mixed responses to the odorants tested for the R7, R8 and L11 olfactory-tissue $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase preparations. Cow-olfactory-tissue substitution caused the greatest percentage change over endogenous lipids (Table 2). In both cow-olfactory-tissue- and dioleoylglycerophosphocholine-substituted preparations of R7, R8 and L11, $(\text{Na}^+ + \text{K}^+)$ -dependent ATPase activity responses were quite similar (Table 2).

Fig. 2. Response of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities of individual turbinals 7–12 to various odorants. Values are means $\pm$ s.d. (represented by the bars) of at least three determinations. Key to the Figure: C, control; 1, nonan-2-one; 2, n-octanol; 3, octan-2-one; 4, (–)-carvone; 5, (+)-carvone; R, right turbinals; L, left turbinals. Non-shaded bars are not significantly different from control values at $P < 0.05$.

Table 2. Effect of lipid substitution on ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities from individual dog turbinals and a dog brain preparation

All odorants were tested at 1.0 mM concentration. Abbreviations used: COL, cow olfactory lipids; DOL, dioleoyl-glycerophosphocholine; DPL, dipalmitoylglycerophosphocholine; DBL, dog brain lipids. Values are averages of at least three replications and indicate the change compared with the control activity. Differences of changes of 10% or more are significant at $P < 0.05$.

	Change in enzyme activity (%)				
	Endogenous	COL	DOL	DPL	DBL
R7					
Nonan-2-one	10	110	52	32	
n-Octanol	47	32	25	20	
R8					
Nonan-2-one	75	128	55		
(–)-Carvone	27	37	47		
(+)-Carvone	44	45	24		
L11					
Nonan-2-one	20	102	66		5
n-Octanol	3	66			
(–)-Carvone	0	46	20		0
(+)-Carvone	–25	13	–12		0
Dog brain					
Nonan-2-one	5	0	0		
n-Octanol	0	0	0	0	
(–)-Carvone	0	0	8		
(+)-Carvone	–10	0	0		

Lipids extracted from lipid-substituted ($\text{Na}^+ + \text{K}^+$)-dependent ATPase preparations were chromatographed on silica-gel G plates. Estimation from

the size and intensity of the exogenous lipid spot on developed plates of the substituted olfactory tissue indicated an approx. 50% substitution of exogenous

lipids for endogenous lipids and a small amount of bound Lubrol WX remained, which ran with the solvent front (results not shown).

Discussion

The results showed that the olfactory-tissue ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity had specificity of response to different odorous chemicals. However, ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activities from the various brain-region preparations essentially failed to respond significantly at 1.0 mM odorant concentration. The responses of enzyme activities from turbinal preparations from the right side of the olfactory epithelium were in general agreement with the findings of Koch & Gilliland (1977) for similar preparations from dog olfactory tissue.

The lack of bilateral symmetry of enzyme activity responses of matched left/right pairs of turbinals was also in agreement with the findings of Rossi & Koch (1981) for the responses from matched rat olfactory-tissue turbinals. This lack of bilateral response could allow a greater differentiation of perceived odour quality by supplying the olfactory bulbs a greater diversity of electrical stimuli for possible pattern recognition by the brain.

The question remains as to how an odorant elicits a response from an olfactory bipolar nerve cell. The ($\text{Na}^+ + \text{K}^+$)-dependent ATPase enzyme is a lipoprotein complex of two protein subunits [an approx. 100 000-mol.wt. catalytic subunit and an approx. 50 000-mol.wt. glycoprotein subunit (Kyte, 1972; Hokin *et al.*, 1973; Dixon & Hokin, 1974; Craig & Kyte, 1980)]. It has been reported that the electric organ of the dogfish shark and kidney membranes contain an Na^+ -specific ionophore (Shamoo & Albers, 1973) that may be the glycoprotein subunit of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase enzyme (Shamoo *et al.*, 1975). A Ca^{2+} -specific ionophore has been shown to be a part of the Ca^{2+} -dependent ATPase enzyme complex of sarcoplasmic reticulum (Stewart *et al.*, 1976). Jean and his co-workers (Jean *et al.*, 1975; Jean & Albers, 1977) demonstrated that the glycoprotein subunit was an integral part of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase enzyme by use of antibody inhibition studies. Churchill & Hokin (1976) showed that the glycoprotein subunit was less susceptible to digestion with trypsin in the presence of Na^+ or K^+ . It was suggested that the decreased susceptibility was due to conformational changes that may function in ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity. Conversely, it has been shown that concanavalin A binding to, or sialic acid removal (50%) from, the glycoprotein subunit had no effect on the activity of purified ($\text{Na}^+ + \text{K}^+$)-dependent ATPase, suggesting that the glycoprotein does not play a catalytic role in the enzyme (Marshall, 1976).

The above findings could be interpreted as indicating that the glycoprotein subunit, although not active in the catalytic ion-pump action, may be associated with Na^+ channels. Perturbation of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity may be related to depolarization or hyperpolarization of the nerve membrane through an ionophoric action of the glycoprotein subunit of the enzyme.

The results of the present study suggest that the type of phospholipid in the lipoprotein complex could be an important factor affecting the odorant perturbation of ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity in olfactory-tissue preparations. In other studies it has been shown that delipidation by detergents or phospholipases reduced ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity markedly but activity could be partially or totally restored by addition of phospholipids (Simpkins & Hokin, 1973). They proposed that the loss of phospholipid resulted in a conformational change in the enzyme, indicating that the phospholipid may play an important role in maintaining the appropriate conformation of the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase protein structure for biological activity. The type of phospholipid as well as the fatty acid components of the phospholipid associated with the enzyme has been shown to be important in restoring complete activity to a delipidated enzyme preparation (Kimmelberg & Papahadjopoulos, 1974; Walker & Wheeler, 1975).

The ability of olfactory-tissue ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity to show spatial differentiation in its response to an odorant could indicate that a characteristic pattern of response is important for analysis of odour quality by the brain. These findings show agreement with the electrophysiological results obtained by Kafka (1970) in which exposure to a single odorant resulted in a characteristic pattern of stimulatory and inhibitory responses of different nerve cells from the antenna of the migratory locust. The fact that the ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity from individual turbinals demonstrated differentiation in its response to compounds so closely related in physical properties as the optical isomers (+)- and (-)-carvone indicates the perturbation of ($\text{Na}^+ + \text{K}^+$)-dependent activity has the capability of supporting the functional requirements of detection.

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