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Full Papers

Evidence for an olfactory receptor which responds to nicotine – nicotine as an odorant

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Summary. The tobacco alkaloid (S)(−)-nicotine, when applied as a vapour to an in vitro head preparation, stimulates the olfactory epithelium in three strains of rats and to a lesser extent in two strains of mice. The electro-olfactogram (EOG) generated by nicotine has similar characteristics to the EOGs produced by known odorants. The nicotine EOG increases with increasing concentration of nicotine vapour (1–100 nM) applied to the olfactory epithelium.
Differential reduction of the nicotine EOG by the lectin concanavalin A is seen in Wistar and Lister Hooded rats. The reduction of the nicotine EOG by concanavalin A is prevented by adding alpha-methyl-D-mannoside to the lectin superfusion medium. This suggests that there is a glyco-moiety associated with at least one olfactory receptor responding to nicotine.
Our results suggest that rat olfactory epithelium has receptor sites for nicotine. Nicotine is an unusual compound because it shows both odorant and pharmacological properties.
Key words. Nicotine; odorant; electro-olfactogram; olfaction; receptor; rat.

Introduction

The biochemical properties of nicotine and in particular its effects as an agonist for the nicotinic acetylcholine receptor, have been extensively reviewed in the literature[2,3]. Specific binding of nicotine to human[4], mouse[5,6] and rat brain[7–9], to rat liver[10] and to human leucocyte membranes[11] has been demonstrated. Nicotine is known to be the primary satisfaction factor for tobacco and to influence the flavour of the smoke considerably[12]. It occurs at high levels in tobacco products, typically 1.8 mg of nicotine per cigarette[3]. Considering the latter and the amount of work carried out with nicotine, it is surprising that there are no detailed studies reported on nicotine as a stimulant for the olfactory epithelium.
We calculate the saturated vapour phase concentration above pure nicotine to be 3.6 mM at 20 °C, which is likely to produce perceivable concentrations in the olfactory mucosa.

It has been noted that nicotine evaporates appreciably when exposed to room air[13].

The aim of this work is to describe the action of nicotine on olfactory epithelium. The results are compared with the stimulatory properties of some known odorants. We have also used our established chemical modification methods for olfactory epithelium[14, 15] to modify the response of the olfactory receptor(s) for nicotine.

Materials and methods

i-Pentyl acetate (i-amyl acetate), 97%, (Aldrich Chemical Co., UK), i-pentanoic acid, 98%, (Fluka AG, W. Germ.) and cineole, 99%, (BDH Chemicals Ltd, UK), were used in the experiments without further purification. (S)(−)-Nicotine, concanavalin A type IV and alpha-methyl-D-mannoside grade III were from Sigma Chemical Co., UK. Male Wistar, Sprague Dawley and Lister Hooded rats (200–250 g) and male Balb/c and MF1/Ola mice (7 weeks old) were from Harlan Olac Ltd, UK, All other reagents used were of analytical grade.

The nicotine was redistilled under reduced pressure to 99.9% purity (major impurity < 0.1%) and stored under nitrogen at −20 °C. After 15 months storage under these conditions, the nicotine had discoloured but was still 99.7% pure. The purity was determined by capillary gas liquid chromatography using a Carlo-Erba Fractovap 2450 set to the splitter mode; column type OV-1 (internal diameter 0.3 mm, length 13 m), column flow-rate 1–2 ml/min (helium carrier gas), at an oven temperature of 142 °C and injector temperature 200 °C.

Redistilled nicotine was diluted to 20% (v/v) in paraffin (Fisons water white liquid paraffin, specific gravity 0.83–0.86) and stored under nitrogen at room temperature before use. A fresh dilution was made every 5 days, or earlier if the solution had discoloured. Dilutions in paraffin of i-pentyl acetate, i-pentanoic acid and of cineole were also used in the experiments. Passage of clean dry air across the surface of 2 ml of this odorant solution produced vapour which was diluted with filtered, humidified air and allowed to equilibrate in the apparatus before use. The 2-ml aliquots of diluted odorant were kept at constant temperature (15 °C) and were used for 24 h, after which the solutions were changed. In the case of nicotine, the odorant container and stock solutions were replaced at the first sign of discolouration. Preliminary experiments indicated that the 2-ml aliquot was sufficient for a 24-h period. Regular checks were made to determine whether or not any vapour from the paraffin alone elicited a response on the rat half head preparation. On the few occasions that a response was detected, the paraffin and all stock solutions were replaced.

The odorant application system was essentially as used previously[14, 15] and as described in detail elsewhere[16].

Recording electro-olfactograms (EOGs). The procedures for recording EOGs from the olfactory epithelium and for concanavalin A modification were essentially as described elsewhere[14, 15].

The animal was stunned and killed by cervical dislocation. Following decapitation, the head was cut in sagittal section and the exposed septum was immediately removed, taking care not to touch the underlying olfactory turbinates. The half head was mounted on the cooled head stage (the head temperature was kept below 17 °C) for a 15-min superfusion with oxygenated Locke's Ringer solution, (flow rate 2 ml/min) after which the Ringer solution was removed by aspiration. EOGs were recorded after a 15-min rest period and after a steady baseline had been attained.

For the dose-response relationship and concanavalin A experiments, the odorants were presented to the epithelium as a 1-s vapour pulse, followed by a 1-min recovery period. Duplicate presentations of each test odorant were made to the epithelium during any one recording period. Recordings were taken from the third turbinate, from a region denoted by the circle on T3 of figure 1.

A standard odorant, i-pentyl acetate, at a fixed concentration, was presented to the epithelium at regular intervals (every 3rd or 4th application). i-Pentyl acetate has been widely used as a standard or reference odorant by workers in olfaction[14, 15, 17, 18].

The response measured in these experiments was the amplitude of the initial EOG peak. The normalised EOG response was obtained on dividing the test odour EOG at time x by the standard odour EOG, also at time x (calculated by interpolating from neighbouring presentations of the standard odorant). The mean of this value for each presentation of the same odorant is the value A. Use of the A value enabled us to take account of variation in EOG amplitude between rats.

Concanavalin A treatment. After the initial odorant sequence had been applied to the epithelium, the electrode was lifted from the tissue. This was followed by a 5-min rinse of the epithelium with concanavalin A (0.5 mg/ml, 2 ml/min) in oxygenated Ringer solution. A 10-min Ringer-only rinse washed unreacted concanavalin A from the tissue, after which excess liquid was aspirated from the tissue as before. The electrode was then lowered on to the tissue at the same position and a rest period of at least 15 min was allowed before an identical sequence of the test odorants was applied. In control experiments concanavalin A was absent from the procedure.

A measure of the EOG survival, L, was determined by dividing the mean EOG (in mV) for the standard odorant after treatment, by the mean standard odorant EOG before treatment. Thus, 100% survival of the EOG for the standard odorant during the experiment gave an L value of 1. Results were not used from 2 rats which showed unusually low L values of < 0.5, which indicated either a poor preparation or an excessive dose of concanavalin A.

The parameter R, necessary for describing modification of the response by a reagent, is defined as the normalised response after treatment divided by the normalised response from the same odorant before treatment. Thus, an EOG which was unaffected by the treatment had R = 1, an odorant whose response was diminished by 50% had R = 0.5, under conditions such that the response to the standard odorant was unaffected.

Summary of parameters used to analyse EOGs:

$$L = \frac{\text{mean EOG for standard odorant after treatment}}{\text{mean EOG for standard odorant before treatment}}$$

$$A = \text{mean}\,\frac{\text{EOG test odorant}}{\text{EOG standard odorant}}$$

$$R = \frac{\text{A after treatment}}{\text{A before treatment}}\ \text{(for the same odour)}$$

Effect of alpha-methyl-D-mannoside (mannoside). In these experiments, the lectin was dissolved in Ringer solution with mannoside at a final concentration of 20 mM. This was applied to the epithelium as described above.

Calculations. The vapour concentration of odorant presented to the epithelium was calculated from the vapour pressure for the odorant at 15 °C, interpolated from standard tables[19].

The concentration of odorant which reached the olfactory receptors could not be determined. Further information on the composition, production and volume of the mucus and on odorant removal from the mucus are required before such calculations would be possible. Some properties of the mucus

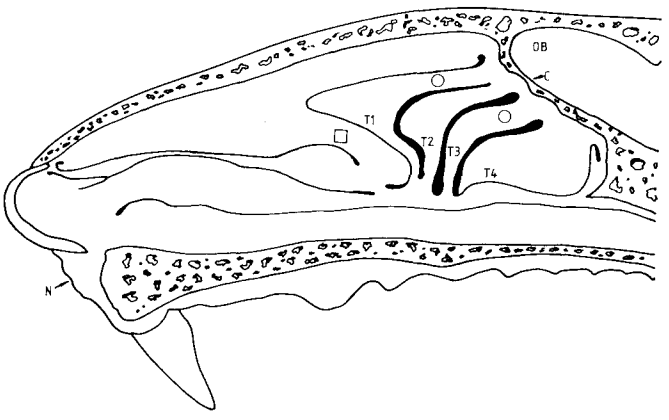

Figure 1. Schematic diagram of a rat head following sagittal sectioning and removal of the septum. OB, olfactory bulb; C, cribriform plate; N, naris; T1,T2,T3,T4 = exposed surfaces of the olfactory turbinates. The regions from which recordings were taken are denoted by circles on the olfactory turbinates and by a square on nasal respiratory epithelium.

have been discussed elsewhere[20]. For sparingly soluble odorants it was possible to estimate the concentration of odorant in the mucus from the water to air partition coefficient. An odorant with an air/water partition coefficient of > 50,000 would not equilibrate between air and mucus, when presented to the epithelium as a vapour pulse of one second. The estimated mucus concentration in this case was taken as the vapour concentration of odorant presented to the epithelium multiplied by 50,000, since the volume of odorized air passing over the mucus was approximately 50,000 times the volume of the mucus[16]. The vapour concentrations of the odorants used in the experiments and estimates of the corresponding mucus concentrations of the odorants are shown in the table.

Results

Nicotine stimulates the olfactory epithelium. If nicotine is to be accepted as an odorant it must stimulate the olfactory epithelium and produce an EOG comparable to the EOGs from known odorants.

Figure 2(a) shows the EOG recorded from the third turbinate following a 10-s pulse of nicotine vapour presented to the Wistar rat olfactory epithelium. The trace shows an initial rapid change in potential of the epithelium on stimulation followed by a reduction in amplitude to a plateau on continued stimulation. When the nicotine vapour was removed, the potential fell immediately towards the baseline value.

The EOG response to a 10-s presentation of nicotine vapour shown in figure 2(a) is comparable to the response of the same preparation to a 10-s i-pentyl acetate pulse shown in figure 2(b). The removal of odorant from the olfactory epithelium and thus the decay of the EOG response will depend on the solubility of the odorant in the mucus layer and on the pKa of the odorant and hence its charge at physiological pH. i-Pentyl acetate was removed from the site of the olfactory response faster than nicotine, as is apparent from the rate of return of the respective EOG traces to the baseline value.

The nasal epithelium of the same preparation was then superfused with Ringer solution for 3 min and the electrode moved to a position anterior to the olfactory turbinates as shown by the square in figure 1. Similar 10-s presentations of nicotine and i-pentyl acetate vapour were made to the preparation, the results of which are shown in figures 2(c) and 2(d) respectively. Neither odorant produced an EOG-like response at this position. The preparation was then superfused with Ringer solution, after which the electrode was placed back onto the third turbinate. EOG responses comparable to those recorded earlier were obtained, indicating that the preparation was still responding to odorants. The preparation was then removed from the head stage and examined under a binocular microscope with incident illumination. The surface of the epithelium in the region denoted by the square in figure 1 had a perceptible motion, suggesting the presence of active respiratory cilia. No movement could be observed on the surface of the epithelium on the third turbinate, from which EOGs to nicotine vapour and i-pentyl acetate vapour were recorded. These observations were made over 100 min after the initial dissection to expose the nasal epithelium of the preparation.

We also recorded this EOG response to nicotine vapour from Lister Hooded and Sprague Dawley rats and from Balb/c and MF1/Ola mice (data not shown).

Nicotine EOG dose-response relationship. Figure 3(a) shows the EOGs to nicotine at various concentrations, recorded from the olfactory epithelium of a Wistar rat in the region denoted by the circle drawn on T3 of figure 1. Saturation of the response to nicotine, as determined by the A value, was not seen over the range of vapour concentrations tested. The results from a group of such experiments are shown in figure 3(b), measured from the third turbinate in the region denoted by the circle drawn on T3 of figure 1.

In some preparations, near saturation of the response to nicotine was seen. An example of this is shown in figure 3(c). Recordings were taken in this case from the region denoted by the circle drawn on T1 of figure 1. Results from two out of the 8 cases used in figure 3(b) also showed this pattern of response. It was difficult to determine whether this was best explained by biochemical or experimental factors.

A study on Lister Hooded rats (n = 5) showed a dose-response relationship to nicotine vapour similar to that seen in the Wistar rat, over the same concentration range (data not shown). Since a large number of variables (such as the unknown concentration of the odorant in the mucus) were involved in this experiment, we did not attempt to calculate a binding constant from the dose-response relationship data.

Concanavalin A inhibition of the nicotine EOG. A concanavalin A modification study was carried out on the rat olfactory response to vapour from nicotine (33 nM), cineole (155 nM), i-pentanoic acid (134 nM) and i-pentyl acetate (75 nM).

The results of a concanavalin A study on the Wistar and Lister Hooded rat are shown in figure 4. The EOGs from cineole, nicotine and i-pentanoic acid were reduced by conca-

Concentrations of odorants. Experiments to which these concentrations apply; * 10-s pulse of odorant, ** response versus concentration of nicotine and *** concanavalin A treatment. The water/air partition coefficient, W/A, is expressed for all forms of the odorant at pH 7.0 and 15 °C. VC-vapour concentration and EMC-estimated mucus concentration of odorant. a) after a 1-s and b) after a 10-s continuous presentation of vapour

Odorant	W/A	-log VC	-log EMC	Experiment
i-Pentyl acetate	81	7.12	5.22	***
		7.11	5.20	**
		6.96	5.06	*
Cineole	250	6.81	4.41	***
i-Pentanoic acid	4.0×10^6	6.87	2.17	***
Nicotine	2.3×10^6	8.74	4.05	**
		8.09	3.39	**
		7.78	3.09	**
		7.48	2.78	***
		7.34	2.64[a]	*
		7.34	1.64[b]	*
		7.31	2.61	**
		7.01	2.31	**

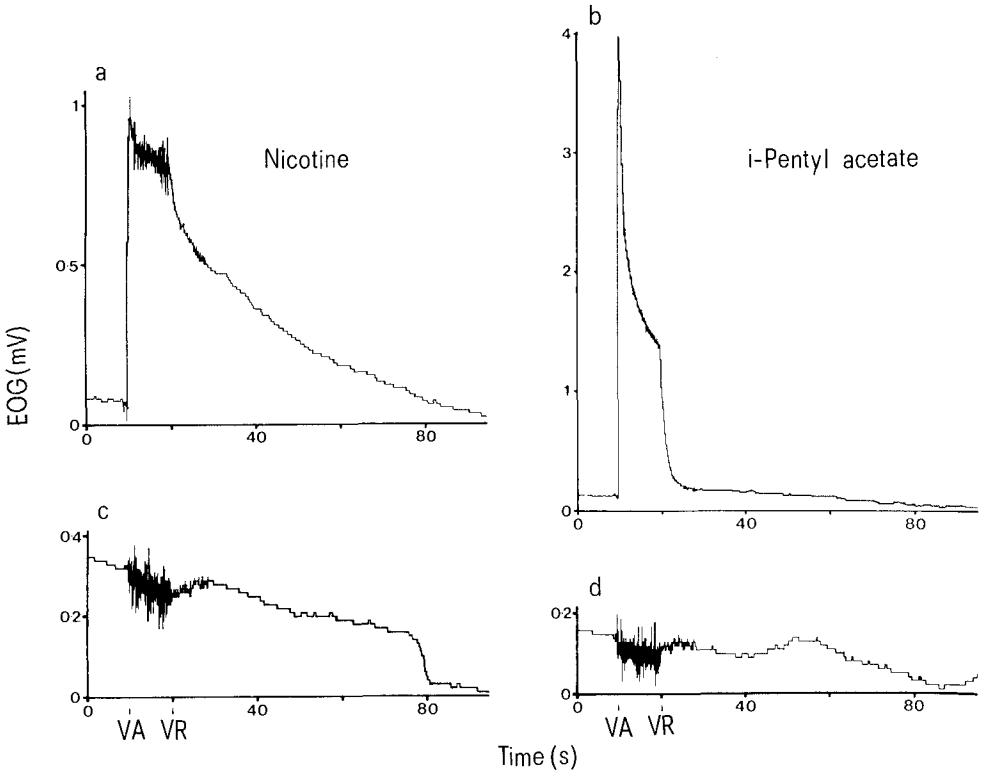

Figure 2. EOG response to a 10-s presentation of vapour from (a) and (c) nicotine and (b) and (d) i-pentyl acetate, recorded from the same rat. Traces (a) and (b) were obtained from olfactory epithelium and traces (c) and (d) from nasal respiratory epithelium. The concentrations of the odorants are shown in the table. The time of vapour application, VA, and of vapour removal, VR, is marked on the x-axis.

navalin A in both strains of rat. The R value for cineole was reduced by 31% and 23%, the nicotine R value was reduced by 47% and 30% and the i-pentanoic acid R value by 81% and 48% after concanavalin A treatment, in the Wistar and Lister Hooded rats respectively.

Figure 4 also shows the results from the Wistar rat when mannoside was added to the concanavalin A superfusion medium. The results show that the sugar prevented concanavalin A inhibition of the olfactory response to all three test odorants.

The L values shown in figure 4(b) represent the 'survival' of the EOG response to the standard odorant after each treatment. An analysis of variance revealed that there was no significant difference in L between concanavalin A, control, mannoside plus concanavalin A, and strain of rat ($p > 0.05$). L reflects effects which the reagent has on all EOGs and any specific effects which it may have on the receptors stimulated by the reference odour. Thus, any non-specific effects on the EOGs were not significantly different between treatments.

Discussion

Nicotine stimulation of the olfactory epithelium. The EOG, a summated receptor potential, is a useful measure of the initial events of odorant interaction with the olfactory epithelium[21]. One of the properties of an odorant is the ability to stimulate the olfactory epithelium to produce an EOG.

The results shown in figure 2 suggest that nicotine vapour can stimulate the in vitro olfactory preparation to produce an EOG, and as with the known odorant i-pentyl acetate, is unable to stimulate an EOG-like response from nasal respiratory epithelium. These findings suggest the presence of at least one olfactory receptor which responds to nicotine, in the olfactory epithelium of the three strains of rats and two strains of mice tested so far.

Nicotine is an unusual odorant in that it has ionizable groups (pKa = 7.9) and will become predominantly charged at physiological pH (90–80% nicotinium ion at pH 7.0–7.4)[3]. We assume that the nicotine vapour which reaches the olfactory epithelium will partition into the mucus layer and from it's water/air partition coefficient, we estimate that the nicotine will be concentrated in the mucus by a factor of two-million-fold at equilibrium. However, during the course of a typical nicotine vapour pulse in our experiments, equilibrium in the mucus will not be reached. The greater time taken for a soluble odorant (nicotine) to disperse from the olfactory mucus as opposed to a sparingly soluble odorant (i-pentyl acetate), may account for the delay in the potential returning to baseline in figure 2(a) when compared with figure 2(b).

The uncharged nicotine molecule will pass through the membranes of the epithelial cells and as has been suggested for other odorants, may accumulate in the cytoplasm[20]. Nicotine is more likely to remain inside the cells than other odorants due to its charge. It is possible that this may produce toxic and other effects which we have not measured. In addition, there are several enzyme systems present in the nasal mucosa which could metabolise nicotine[22]. For example, nasal cytochrome P-450-dependent monooxygenases have been shown to metabolise nicotine to produce formaldehyde[23]. It is possible that metabolites of nicotine may also affect the olfactory system in some way. This is supported by experimental evidence showing accumulation of nicotine or metabolites in the olfactory mucosa[24].

Concanavalin A inhibition of nicotine EOGs. Chemical modification of the olfactory epithelium leads to altered EOG responses and is a possible method for identifying classes of olfactory receptors. We have previously observed concanavalin A inhibition of the EOG from cineole and especially from i-pentanoic acid in the Wistar rat[15,17] and here show a similar effect on nicotine EOGs (figure 4).

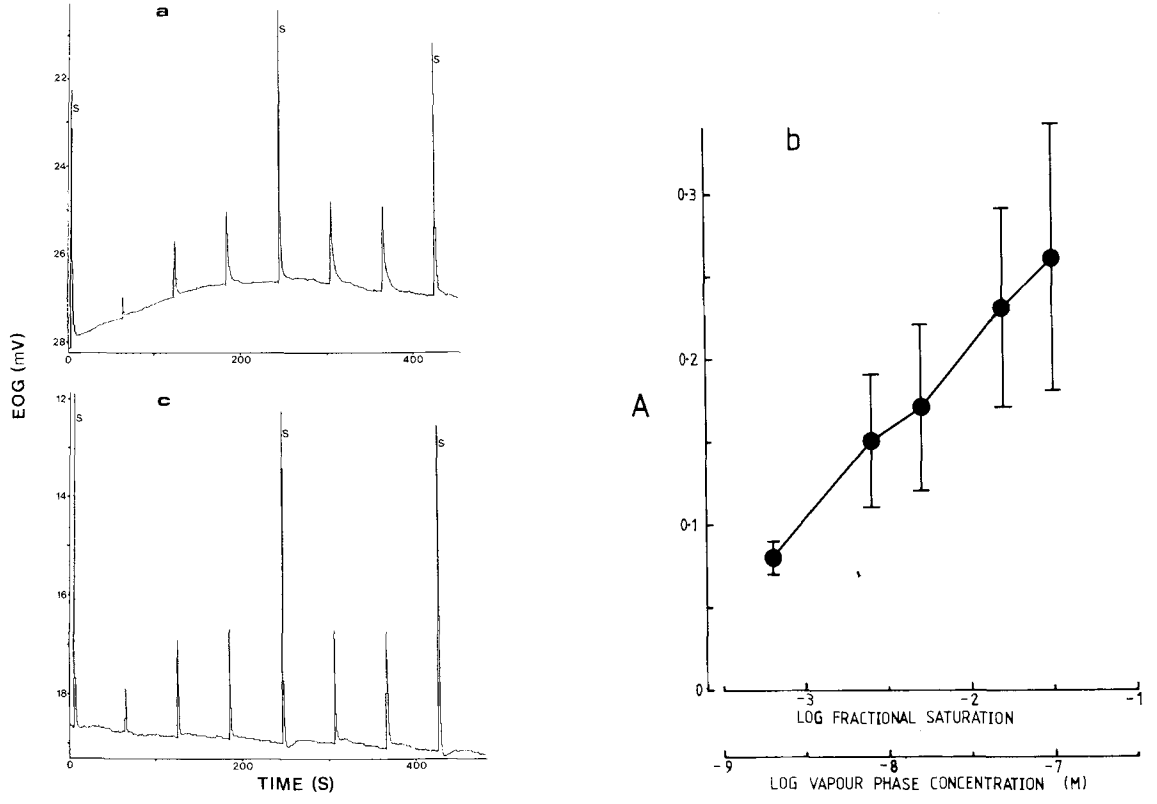

Figure 3. Relationship between nicotine concentration and response of the rat olfactory epithelium. The recordings, taken from (a) the third turbinate (T3, fig. 1) and (c) the first turbinate (T1), were the result of a 1-s presentation of odorant. The EOG response to nicotine is shown in order of increasing vapour concentration. S shows presentations of the standard odorant, i-pentyl acetate. The concentrations of odorant used are shown in the table. (b) shows a plot of mean A value versus log fractional saturation of nicotine, for a sample of studies from the third turbinate only. The error bars show the 95% confidence interval. N is 8, except at the highest nicotine concentration where n is 6. Fractional saturation is a measure of odorant saturation in air at the epithelium surface. Vapour from neat odorant, diluted by a factor of 10 before presentation, has a fractional saturation of 0.1.

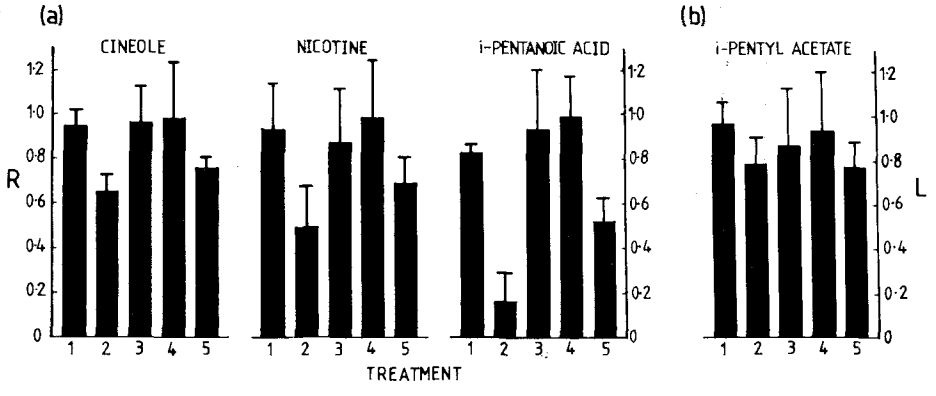

Figure 4. Effect of concanavalin A on the EOG's to vapour from (a) cineole, nicotine, i-pentanoic acid and (b) i-pentyl acetate. The y axis shows for (a) mean R value and for (b) mean L value (mean EOG survival, see text) together with the upper half of the 95% confidence interval. For the concentrations of odorant used see the table. The treatments shown are as follows: *1* control Wistar rat (n = 5 except for cineole n = 4), *2* concanavalin A Wistar rat (n = 8), *3* concanavalin A and mannoside Wistar rat (n = 5), *4* control Lister Hooded rat (n = 4), *5* concanavalin A Lister Hooded rat (n = 8).

Concanavalin A affected the EOG's from the three odorants tested in Wistar and Lister Hooded rats, to different extents. Two-way analysis of variance of the R values from concanavalin A treated rats confirmed that there was a difference between the two strains. The results of this analysis can be summarised as follows. First, there was a difference between Wistar and Lister Hooded rats with respect to overall concanavalin A effect ($p < 0.0005$). Secondly, there was a difference between the concanavalin A effect on cineole, nicotine and i-pentanoic acid ($p < 0.001$) and finally, the differences in the R values for the three odorants in the Wistar rat were not significantly different from the differences in the R values for the three odorants in the Lister Hooded rat ($p > 0.05$). The R values for all three odorants were reduced to a lesser extent following concanavalin A treatment in the Lister Hooded rat than the Wistar rat. This may be explained by

differences between the olfactory mucosa of the two strains with respect to mucus composition, mucus thickness, receptor density and receptor type. These differences may influence the ability of the odorants to stimulate the epithelium (there is no evidence to support this) and the effectiveness of the concanavalin A superfusion. Consistent with this notion is the observation that 10 times as much concanavalin A is required to selectively reduce EOG's in the frog[18]. This may be explained by the presence of a thicker layer of mucus overlying the frog olfactory epithelium than is seen in the rat[25].

Alpha-methyl-D-mannoside protection of EOGs. The results of the mannoside protection experiment seen in figure 4 show that concanavalin A modification of the olfactory response to all three odorants could be prevented by competing for the sugar residue binding site on the concanavalin A molecule with mannoside.

One-way analysis of variance showed for each odour that there was a difference in the R values for the three treatments on the Wistar rat (cineole and i-pentanoic acid $p < 0.001$, nicotine $0.01 > p > 0.001$). To identify which of the treatments contributed most to this difference, the control R values were compared with R values for concanavalin A and R values for concanavalin A and mannoside, using the Dunnett test for multiple comparisons to a control group. The results of this test showed that for each odour, only the concanavalin A treatment R values were significantly different from the control R values (cineole, nicotine and i-pentanoic acid, $p < 0.01$).

Treatment of the epithelium with mannoside after a concanavalin A superfusion does not reverse the concanavalin effect[15], but the EOGs which were protected in the concanavalin A and mannoside treatment in this study, could be reduced by subsequent treatment with concanavalin A alone ($n = 3$). These observations suggest that alpha-methyl-D-mannoside is binding to the concanavalin A molecule's sugar residue binding-site, preventing modification of the EOGs. This is evidence that the olfactory receptors which respond to cineole, nicotine and i-pentanoic acid are glycosylated and/or are close to a portion of sensory membrane which is glycosylated. Other workers have shown that there are glycoproteins unique to sensory cilia and have suggested that these proteins play a role in olfactory reception[26].

Nicotine is the key chemical found in the smoke from cigarettes and other tobacco products. We have shown that nicotine acts as an odorant in addition to its well-known role as a pharmacological agent. It is of interest that in human experiments (unpublished) subjects could smell the pure nicotine used in this work. We can speculate that other pharmacologically active odorants exist.

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Short Communications

Identification of a pre-hibernating state in myocardium from nonhibernating chipmunks

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Summary. During the hibernating season, the amplitude of the cardiac action potential plateau of nonhibernating chipmunks was reduced. Replacing external Ca by Sr inhibited the electromechanical responses of these preparations. Similar properties were observed in hibernating animal preparations, suggesting that changes in cardiac function are already triggered before hibernation begins.

Key words. Pre-hibernating state; hibernating and nonhibernating seasons; chipmunks myocardium; strontium; plateau potential.

Recently, it has been reported that the amplitude of the action potential plateau of cardiac muscle from chipmunks is reduced in the hibernating state[1]. This reduction has been shown to be due to a reduced contribution of the slow inward