EVIDENCE FOR THE SPECIFIC INVOLVEMENT OF CYCLIC AMP IN THE OLFACTORY TRANSDUCTION MECHANISM

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Summary

The effects of phosphodiesterase inhibitors and membrane-permeable derivatives of cyclic-AMP on the summated receptor potentials (electro-olfactograms) from frog primary olfactory neurones have been studied. Both classes of compounds bring about a reversible reduction in the amplitude of both the peak and plateau components of the electro-olfactograms, whereas control compounds have no effect. The effects were observed with odorants from several different families including floral, fruity, putrid, camphoraceous, minty and musky odorants. No effects were found with the corresponding cyclic-GMP derivatives. The results demonstrate the specific involvement of cyclic-AMP in the transduction step in the primary olfactory neurones.

There are two main questions concerning the mechanism of the vertebrate peripheral olfactory system. Firstly, there is the question of the olfactory code and the molecular basis of the quality discrimination mechanism in the primary olfactory neurones. The most plausible explanation for this problem is the occurrence of olfactory receptor proteins on the plasma membrane of the neurones (1 - 4). Secondly, there is the question of the transduction step, i.e. , the nature of the molecular mechanisms responsible for translating the odorant-membrane interaction into a generator potential. A variety of mechanisms have been proposed for this key step in the olfactory process,(5), but experimental evidence to support these mechanisms is lacking. Since adenylate cyclase fulfills a transduction role in several types of cells including neurones (6, 7) , we have looked for the possibility of this enzyme being the basis of the olfactory transduction mechanism. The olfactory mucosa is known to contain an adenylate cyclase (8, 9), but to establish that odorants act through the second messenger cAMP, it is necessary to fulfill all the criteria for cyclic nucleotide involvement in a physiological process which were laid down by Sutherland (10). For the olfactory system , these criteria are as follows:
(1) the receptor cells should contain adenylate cyclase and the odorant should

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alter the intracellular cAMP levels in these cells; (2) odorants should modulate the activity of adenylate cyclase in a plasma membrane preparation from the receptor cells; (3) inhibitors of the phosphodiesterase which destroys the cAMP should affect the action of the odorants on the olfactory mucosa; and (4) cAMP or its derivatives should be able to stimulate the action of odorants on the tissue.

The experiments reported in this paper investigate the third and fourth of the Sutherland criteria and provide evidence for the specific involvement of cyclic AMP in the generation of the olfactory receptor potentials.

MATERIALS AND METHODS

The square wave pulses of the odorant vapours were applied to the olfactory mucosa using an olfactometer of our design (11, 12). The electro-olfactogram (EOG) was measured by conventional methods as described previously (13). The frogs Rana Temporaria which were under urethane anaesthesia or were pithed, had a strong circulation and were maintained in a moist atmosphere at room temperature. The inhibitor dissolved in Ringer's solution for frogs, pH 7.0 , was added gently to the mucosa so as to minimize mechanical stimulation. Drainage of the fluid from the olfactory cavity was prevented by stopping up the buccal aperture with a Teflon strip. The solution of the inhibitor was left in contact with the mucosa for 1 minute and was then removed by gentle aspiration, avoiding any damage to the tissue. The recording electrode was raised during the application of the drug solution. The test puffs of odorant (10 secs in duration) were then applied with an interval of two minutes between the puffs. In order to minimize damage to the tissue we did not wash out the inhibitor after each application and the effect of the drugs on the EOG was carried out by titrating the mucosa with increasing concentrations of the drug.

An experiment was concluded by washing out the drug from the tissue, using Ringer's solution. This usually resulted in the EOG peak regaining its original amplitude (± 10%) and results are reported only for experiments in which this reversibility of the effect was achieved. The phosphodiesterase inhibition SQ20009 (1-ethyl-4-(isopropyl-indenehydrazino)-1H-Pyrazolo-3,4)-pyridine-5-carboxylic acid, ethyl ester) was a gift from Squibb Pharmaceutical Co.Ltd., and the inhibitor RO 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidionone) was a gift from Hoffman-la-Roche Inc. All other chemicals used in these experiments were obtained from the Sigma Chemical Company and were of the highest purity available.

Results and Discussion

It is not possible to ascribe a receptor function to the adenylate cyclase in the olfactory mucosa enzyme, since there are two major types of cell in the olfactory mucosae of vertebrates; the primary olfactory neurones and the supporting sustentacular cells; and the plasma membrane fraction obtained from the tissue

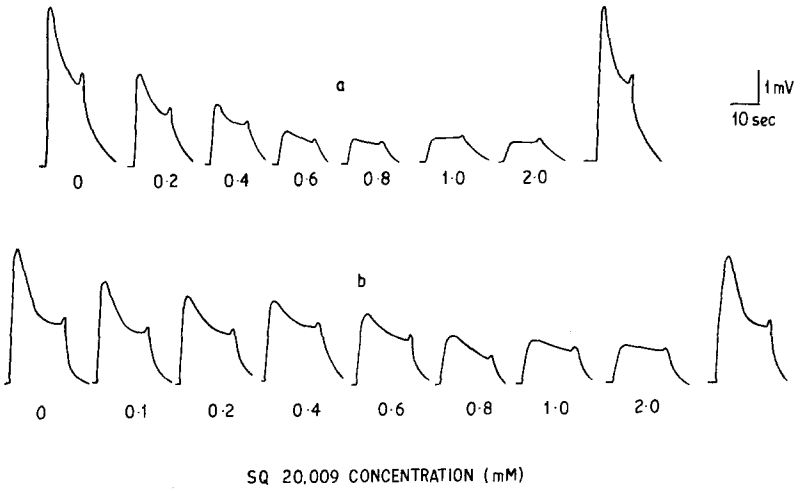

Fig.1

The effect of the phosphodiesterase inhibitor SQ 20009 on the electro-olfactogram (EOG) in the frog Rana temporaria.

a) The odorant n-amyl acetate was applied in the vapour phase for 10 seconds.

b) The odorant ethyl-n-butyrate was applied in the vapour phase for 15 seconds.

The SQ 20009 concentrations applied are shown for each EOG. The final EOG in each series was obtained after the drug had been washed out of the tissue.

contains membranes from both types of cell. We tried to resolve this point by visualizing the cellular location of the enzyme, using a histochemical method. (14) The results were equivocal and subsequently the method has been shown not to be specific for this enzyme. The adenylate cyclase in the plasma membrane fraction though stimulated by sodium fluoride , did not exhibit any specific change in activity in the presence of odorants. Presumably, as is found with several other hormone activated adenylate cyclases (6), the receptor proteins do not survive the isolation procedures. These results indicate that it is not possible to investigate the last two Sutherland criteria using a membrane preparation, since the membranes lack the functional integrity of the intact tissue. So we had to turn to the whole tissue level in the animal and to do this we measured the electro-olfactogram (EOG) from the olfactory mucosa. The EOG is believed to be the summated generator potential from several tens of primary neurones (15). The basic experiment consisted of applying square wave pulses of odorant vapour to the olfactory mucosa after the

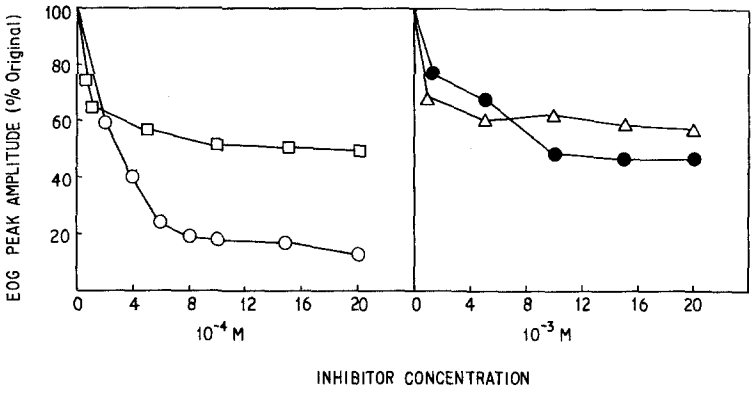

Fig.2

Effect of phosphodiesterase inhibitors on the amplitude of the EOG peak. The odorant was n-amyl acetate.
Δ Theophylline . ● Caffeine . ○ SQ 20009. □ RO 20-1724.

application of phosphodiesterase inhibitors and cAMP derivatives to the mucosa. Under the stimulation conditions used the EOG has a characteristic shape, exhibiting distinct peak and plateau regions (Fig.1) and resembles the generator potentials recorded from other sensory cells . Phosphodiesterase inhibitors brought about characteristic changes in the shape of the EOG as the inhibitor concentration was increased (Fig.1). The amplitude of the peak in the EOG response decreases with increasing concentration of the inhibitors but is not abolished at the highest inhibitor concentration used; instead the curves exhibit a maximum value of inhibition which is characteristic for each inhibitor (Fig.2). When the mucosa is washed with Ringer's solution after the application of the highest concentration of inhibitor, the EOG response recovers its initial value showing that the effects observed are reversible and are not due to damage to the tissue caused either by the high concentrations of drugs or by the application of a liquid stimulus. The rank order of the inhibitor concentrations required for a 50% reduction in the peak amplitude (I_{50}) is the same as that found for related effects in other tissues (16) and also for soluble phosphodiesterases (17) but the actual concentrations required with this tissue are considerably higher than those required for soluble enzymes. For the most potent inhibitor, SQ 20009, the I_{50} value was 0.51 ± 0.14 mM (mean ± S.D. of six experiments), which is

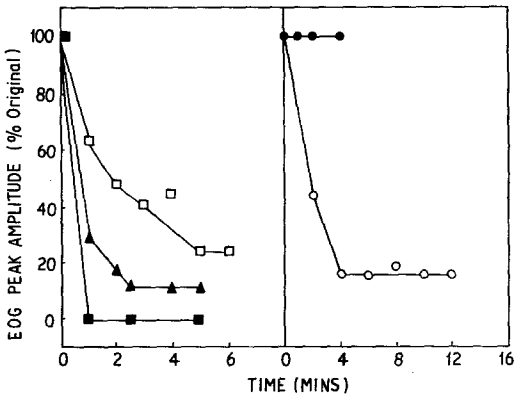

Fig.3

The time course of the inhibition of the EOG peak amplitude by cAMP derivatives and phosphodiesterase inhibitors.

Symbol	Odorant	Added Compound
□	1,8-Cineole	$N^6,O^{2'}$-dibutyryl-cAMP(1mM)
▲	1,8-Cineole	8-bromo-cAMP (1 mM)
■	1,8-Cineole	$N^6,O^{2'}$-dibutyryl-cAMP (2mM)
●	n-amyl acetate	5'-AMP (1 mM)
○	ethyl n-butyrate	SQ 20009 (1 mM)

between one to two orders of magnitude higher than the I_{50} values found for this inhibitor with a range of soluble phosphodiesterases (17). This high value probably reflects the considerable diffusion barrier imposed by the mucus layer which covers the olfactory epithelium. The reduction of the EOG was observed with odorants from several distinct odour families including n-amyl acetate (fruity), ethyl-n-butyrate (fruity), 1-8-cineole (camphoraceous), phenylacetic acid (honey/civet), 3-indolyl carbinol (faecal/floral), menthol (minty), and musk ketone (musky). Thus, the odorants binding to several different types of receptors appear to have a common transduction mechanism. The inhibition of the EOG was observed within one minute of the application of the drug (Fig.3). The cAMP derivatives $N^6,O^{2'}$-dibutyryl-cAMP and 8-bromo-cAMP, both of which are more permeable to membranes than the parent compound, brought about a rapid reversible reduction in the EOG peak amplitude and unlike the effects of the phosphodiesterase inhibitors, intracellular levels of cAMP sufficient to abolish the EOG peak were attained (Fig. 3). Only a very small (10%) reduction in the peak amplitude was obtained with $N^6,O^{2'}$-dibutyryl cGMP.

Imidazole, a phosphodiesterase activator, gave a consistent small (20%) increase in the EOG peak amplitude at a concentration of 10 mM.

The following compounds did not affect the EOG response when they were applied to the tissue at a final concentration of 1 mM: glycine, phenylalanine, glutamic acid, butyric acid, 5'-AMP, 5'-GMP, ATP, GTP. Thus, we can conclude that the observed effects are specifically associated with an adenylate cyclase system and are not a result of damage to the plasma membranes.

The complexity of the olfactory mucosa prevents us performing experiments which could directly test all the Sutherland criteria. However, our data, on balance, provides strong evidence for the specific involvement of cAMP in the production of olfactory generator potentials. A related investigation using less well-defined puffs of odorant found different effects with some of the compounds which we have used, but also concluded that cAMP is involved in the olfactory transduction process (18). We can speculate that changes in the levels of cAMP in the olfactory neurones affect the activity of kinases associated with phosphorylation of the ionophoric proteins in the plasma membrane by a mechanism essentially analogous to that postulated for neurotransmitters (7).

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