Characterization of pigment aggregating α_2 -adrenoceptors of fish melanophores by use of different agonists after partial irreversible receptor inactivation

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1 The affinity for, and the intrinsic efficacy on, postsynaptic melanosome aggregating α_2 -adrenoceptors of fish melanophores was studied for B-HT 920, clonidine, medetomidine, noradrenaline, phenylephrine and UK-14,304. Investigations were carried out by evaluating the effects of progressive, irreversible inactivation of the α_2 -adrenoceptors by benextramine.

2 The double reciprocal plots of equieffective concentrations of B-HT 920, clonidine, noradrenaline and phenylephrine were linear, which indicated that these compounds exerted their effects, mainly, through interaction with one receptor site.

3 The affinity for the α_2 -adrenoceptor selective agonist B-HT 920, was found to be about 1000 times higher than the affinity for the α_1 -adrenoceptor selective agonist phenylephrine.

4 The corresponding plot of equieffective concentrations of medetomidine was not linear, which may indicate that this imidazole compound exerted its effect through more than one receptor site. However, when phenoxybenzamine was used in place of the more selective benextramine, a linear relationship was obtained.

Introduction

Spectacular colour changes of lower vertebrates are mainly due to motile activities of specialized integumentary pigment cells, the chromatophores. These cells are highly branched, and, upon appropriate stimulation, they either aggregate pigment granules centrally or disperse them throughout the cells. Among different types of chromatophores, the melanophores, which contain melanin granules called melanosomes, have been most widely studied due to the conspicuousness of the pigment and its permanence after preparation.

It was originally suggested (Fujii, 1961) that the sympathetic nerve-to-melanophore transmission in teleosts was mediated by α -adrenoceptors, since dibenamine, antagonized effectively the melanosome aggregating effect of adrenaline and noradrenaline, as well as those of neural stimuli.

Adrenoceptors have been divided into α_1 - and α_2 -subtypes on a functional basis (Langer, 1974) and on the basis of the potency order of a number of selective drugs (Berthelsen & Pettinger, 1977; Wikberg,

1978). Recently, we have suggested that the postjunctional α -adrenoceptors mediating pigment migration in the melanophores from several fish species, could be characterized as being of the α_2 subtype (Andersson *et al.*, 1984; Karlsson *et al.*, 1985; 1987; 1988). This suggestion is mainly based on the observation that the α_2 -adrenoceptor selective antagonist, yohimbine, strongly inhibited both the nerve-induced and noradrenaline-induced pigment aggregation, whereas the α_1 -selective antagonist, prazosin, failed to or only weakly inhibited these responses. The presence of α_2 -adrenoceptors as mediators of the melanosome aggregation in teleosts has recently been confirmed by Morishita (1987).

To characterize further the postjunctional α -adrenoceptors mediating pigment migration in the melanophores of the cuckoo wrasse (*Labrus ossifagus*), we have used a procedure developed by Furchgott (1966). This procedure uses the effects of progressive alkylation of the melanophore α -adrenoceptors to determine the dissociation constants and the efficacy of different α -adrenoceptor agonists.

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Methods

Isolation of scales

Scales from the cuckoo wrasse (*Labrus ossifagus* L.) were removed from the dark areas of the dermis and immediately transferred to a saline buffer solution containing (mm): NaCl 150, KCl 5.2, CaCl₂ 2.9, MgSO₄ 1.8, Na₂HPO₄ 2.4, NaHCO₃ 17.9 and glucose 5.6 (Nilsson, 1971; Andersson *et al.*, 1984). The saline buffer was equilibrated with 5% CO₂, 95% O₂ and kept at 20°C at a pH of 7.3.

Evaluation of the α -adrenoceptor-induced pigment aggregation

The scales were placed with the dermal side down on glass microscope slides and suspended in 50 μ l of the saline buffer solution. The effects of different drugs on the aggregating response of the melanophores were studied through a light microscope (Leitz SM Lux) using a magnification of $\times 100$. The effects were evaluated according to the melanophore index (Hogben & Slome, 1931; Andersson *et al.*, 1984; Karlsson *et al.*, 1987). All drugs used in the experiments were dissolved in the saline buffer solution.

Irreversible blockade of postsynaptic α -adrenoceptors by benextramine

Concentration-response curves for different α -adrenoceptor agonists (i.e. B-HT 920, clonidine, medetomidine, noradrenaline, phenylephrine and UK-14,304) were performed after pretreatment with benextramine (Melchiorre, 1981) for 30 min. The scales were then washed 3 times in saline buffer solution for 20 min to remove non-reacted benextramine. Control scales were run in parallel. In some experiments, phenoxybenzamine, was used in place of benextramine.

Statistical and mathematical evaluation

Furchgott (1966) has developed the following equation to describe the relationship between concentration-response curves of agonists obtained in the absence, and after partial irreversible receptor inactivation

$$1/[A] = 1/q[A'] + (1 - q)/qK_A$$

where $[A]$ and $[A']$ are corresponding equieffective concentrations of agonist before and after partial irreversible receptor inactivation; K_A is the equilibrium dissociation constant of the agonist and q is the fraction of receptors not alkylated by the treatment (see Furchgott, 1966; Furchgott & Bursztyn, 1967; Ruffolo, 1982). If $[A]$ and $[A']$ are assumed to

be equal to the concentration of agonist in the bathing medium, plotting the reciprocals of the concentrations of agonist before receptor inactivation (i.e. $1/[A]$) against the reciprocals of equieffective concentrations of agonists after partial receptor inactivation (i.e. $1/[A']$), will yield a straight line, from which the slope and the intercept may be used to calculate the dissociation constant of the agonist (K_A). The fraction of receptors not alkylated by the treatment, q , is obtained as the reciprocal of the slope (i.e. $1/\text{slope}$). Concentration-response curves were evaluated by fitting the experimental data to the logistic function by non-linear regression (Parker & Waud, 1971) using a computer and digital plotter. All straight lines were fitted by linear regression.

All values are given as mean $\pm$ s.e. mean of 5 experiments, which are indicated by vertical lines on the figures.

Drugs

The following drugs were used: benextramine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), B-HT 920 (5-allyl-2-amino-5,6,7,8-tetrahydrothiazolo = [4,5-d]azepine-dihydrochloride; Thomae, Biberach, Germany), clonidine hydrochloride (Boehringer-Ingelheim, Ingelheim, Germany), cocaine chloride (ACO, Solna, Sweden), medetomidine hydrochloride (Farnos Group Ltd, Turku, Finland), (–)-noradrenaline bitartrate (Sigma Chemical Co.), phenoxybenzamine hydrochloride (Smith Kline & French Research Ltd, Welwyn, Hertfordshire), (–)-phenylephrine hydrochloride (Sigma Chemical Co.) and UK 14,304 ((5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline)tartrate; Pfizer Central Research, Sandwich, Kent). Stock solutions were prepared daily in demineralized water and kept refrigerated. Shortly before use, they were diluted in the saline buffer solution.

Results

Effects of benextramine on the melanophore-aggregating response induced by different α -adrenoceptor agonists

Concentration-response curves for the pigment aggregating effects of B-HT 920 (Figure 1a), clonidine (Figure 1b), medetomidine (Figure 1c), noradrenaline (Figure 1d) and phenylephrine (Figure 1e), are presented in Figure 1. In order to eliminate neuronal uptake of noradrenaline and phenylephrine, 10^{-5} M cocaine was included. The α_2 -adrenoceptor selective agonist, medetomidine, was by far the most potent, whereas the α_1 -adrenoceptor selective agonist, phenylephrine,

Figure 1. Concentration-response curves for the melanosome aggregating effects of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e), following pretreatment with various concentrations of benextramine (1×10^{-6} – 1×10^{-5} M; ●, ▲). Each point is the mean of observations on 5 different scales. Vertical lines indicate s.e.mean. The horizontal lines connecting controls (○) and the benextramine-treated curves represent the equieffective concentrations of the different agonists used in the double reciprocal analyses presented in Figure 2.

was a very weak agonist. Clonidine, in accordance with the result of a previous study (Andersson *et al.*, 1984), was found to be a partial agonist. Figure 1 also illustrates the progressive alkylation of α -adrenoceptors. Treatment with benextramine produces the characteristic pattern of irreversible receptor alkylation (i.e., nonparallel, rightward displacements of the concentration-response curves, with progressive reduction in the maximal effect).

The clonidine analogue UK-14,304 was found to be completely without stimulant effect on the *L. ossifagus* melanophores.

Calculation of dissociation constants

The double reciprocal plots of equieffective concentrations of B-HT 920 (Figure 2a), clonidine (Figure 2b), noradrenaline (Figure 2d) and phenylephrine (Figure 2e) seem to be linear. In the case of medetomidine (Figure 2c), a non-linear relationship was obtained, which may indicate that medetomidine acts through more than one binding site. However, a linear relationship was obtained after partial receptor inactivation with the alkylating compound, phenoxybenzamine (3×10^{-6} – 1×10^{-5} M; not shown). Table 1 presents the calculated dissociation

constants. In the table, and in the following, the dissociation constant of medetomidine is approximated to the value obtained by calculating equieffective concentrations before and after partial inactivation with phenoxybenzamine. It must be stressed that this value is only an approximation, since the treatment with benextramine revealed a non-linear relationship. Nevertheless, it is evident from this table, that the α_2 -adrenoceptor selective agonists B-HT 920, clonidine and medetomidine have a high affinity to the binding sites, whereas the α_1 -adrenoceptor selective agonist phenylephrine has a low affinity.

Calculation of efficacies

The intrinsic efficacies were calculated from the slopes of the double reciprocal plots of the equieffective agonist concentrations after treatment with different concentrations of benextramine (or phenoxybenzamine). From the analyses of progressive inactivation of the α -adrenoceptor pool with benextramine it was possible to plot the effect of a given agonist as a function of the fraction of α -adrenoceptors remaining intact (Figure 3). In accordance with Furchgott & Bursztyn (1967), the relative efficacies of a series of agonists on a given response

Figure 2 The reciprocals of equieffective concentrations of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e) regarding the aggregating effects on untreated and benextramine-treated melanophores. Each point is the mean of observations on 5 different scales. The particular equieffective concentrations used in the reciprocals are indicated by horizontal lines in Figure 1. The slope and the intercept are used to calculate the dissociation constant of the agonists (K_A) (see Methods). The fraction of receptors not alkylated by benextramine (q) is obtained as the reciprocal of the slope (i.e. $1/\text{slope}$).

could be estimated. In order to do this, the ratios between the receptors that must be occupied by the agonist with the highest efficacy and the fraction of receptors that must be occupied by another agonist

Table 1 Dissociation constants (K_A) and relative efficacy for different α -adrenoceptor agonists in respect to their melanosome aggregating effect within the melanophores of the cuckoo wrasse

Agonists	K_A (M) ^a	Relative efficacy ^b
B-HT 920	5.1×10^{-6}	0.04
Clonidine	2.7×10^{-5}	0.09
Medetomidine ^c	3.0×10^{-6}	1.00
Noradrenaline	4.8×10^{-6}	0.28
Phenylephrine	2.9×10^{-3}	0.08

^a The dissociation constants (K_A) are determined, in accordance with the method of Furchgott (1966), from the double reciprocal plots of equieffective concentrations of the different agonists on untreated and benextramine-treated melanophore-containing scales

^b Half maximal response expressed as a fraction of the half maximal response obtained with medetomidine

^c K_A is determined after receptor inactivation with phenoxybenzamine

for the same response, need to be determined. From the data shown in Figure 3, it was possible to determine the fraction of receptors needed to produce half-maximal response to the different agonists. For the compounds in Figure 3, relative efficacies are given in Table 1.

Discussion

Upon appropriate stimulation, the mammalian α_2 -adrenoceptors are known to lower the cyclic AMP levels via an inhibiting action on adenylate cyclase (Ui, 1984; Limbird, 1988). In previous investigations, we have shown that the α_2 -adrenoceptors of the teleost melanophores also act via a negative control of adenylate cyclase.

The present investigation reveals that the α -adrenoceptors within the melanophores of the cuckoo wrasse have a high affinity for certain α_2 -adrenoceptor agonists ($K_A \approx 10^{-6}$ M), whereas they have a low affinity for the α_1 -adrenoceptor agonist phenylephrine ($K_A \approx 10^{-3}$ M). This is in agreement with our previous findings concerning the ability of selective α_1 - and α_2 -adrenoceptor antagonists (prazosin and yohimbine) to inhibit nerve-induced and noradrenaline-induced melanosome aggregation. Although the potency of phenylephrine

Figure 3 Relationship between maximum melanosome aggregating effects of B-HT 920 (a), clonidine (b), medetomidine (c), noradrenaline (d) and phenylephrine (e), and the fraction of receptors not alkylated by the irreversible α -adrenoceptor antagonist benextramine (in the case of medetomidine (c), phenoxybenzamine was used).

was only about 1/1000 of that observed for the α_2 -adrenoceptor selective agonists, it functioned as a full agonist with a comparatively high intrinsic efficacy. Therefore, the low potency of phenylephrine seems to depend solely on its very low affinity for the α_2 -adrenoceptor.

Two distinct classes of chemical compounds have been shown to interact with presynaptic α -adrenoceptors (Starke *et al.*, 1975); namely, imidazolines and phenylethylamines. Ruffolo *et al.* (1977) suggested that imidazolines and phenylethylamines may interact at different sites on the α -adrenoceptors, based on the lack of cross-desensitization between these two classes of compounds.

Although a relatively novel class of azepine derivatives, such as B-HT 920, is not comprised of phenylethylamines or imidazolines, pharmacologically, the activity of the derivatives parallels that of imidazolines (Ruffolo, 1984). The two classes of α -adrenoceptor agonists seem to bind to and/or activate α -adrenoceptors in a different manner, regardless of the subtype they activate.

According to Furchgott (1966), a linear relationship of the double reciprocal plot of equieffective concentrations of an agonist, before and after a partial and irreversible inactivation of a receptor, is only obtained when the irreversible antagonist occupies the same active site of the receptor as does the agonist. Thus, a non-linear relationship of the double reciprocal plot indicates more than one binding site. The novel imidazole compound mede-

tomidine, which has been shown to possess a very high α_2 -adrenoceptor selectivity and potency (Savola *et al.*, 1986), possibly interacts with two, kinetically different binding sites in the melanophores. The non-linear relationship of the double reciprocal plot of equieffective concentrations of medetomidine, before and after receptor inactivation with benextramine, probably reflects different sensitivities of the binding sites to benextramine. In accordance with previous findings (Mottram, 1983a,b; Vizi *et al.*, 1983; Mottram & Thaker, 1984), it is proposed that pharmacologically, these sites could be classified as being of an imidazoline-binding type and of a phenylethylamine-binding type.

In contrast to the effect of benextramine on the medetomidine-induced melanosome aggregation, it was found that phenoxybenzamine resulted in a linear relationship. This difference presumably reflects a non-selective action of phenoxybenzamine (Furchgott, 1966).

Benextramine-inactivation of the responses elicited by B-HT 920, clonidine, noradrenaline and phenylephrine, resulted in linear relationships, indicating that these compounds interact mainly with a single receptor site, although not necessarily with the same site. We suggest that in the case of B-HT 920 and clonidine this binding site is of an imidazoline type. Furthermore, it is tempting to assume that noradrenaline and phenylephrine interact with a phenylethylamine-binding site within the melanophores. Medetomidine presumably interacts with

both of these two sites, which may explain both the high potency and the very high intrinsic efficiency of that particular imidazole compound.

Although a great degree of uncertainty concerning the affinity of medetomidine exists, it is interesting to compare the affinity and the intrinsic efficacy of medetomidine to those of the other α_2 -adrenoceptor agonist B-HT 920. Such a comparison reveals that the high potency of medetomidine, is, to a great extent, due to the extremely high intrinsic efficacy of this particular compound. Medetomidine has to occupy only 2% of the receptors to elicit a 50% aggregation response of the melanophores, whereas B-HT 920 must occupy 50% of the receptors for a comparable response. The response of B-HT 920 seems to be directly proportional to the stimulus generated by the drug-receptor complex.

It has been suggested that α -adrenoceptor agonists with high intrinsic efficacy activate several concurrent signalling mechanisms, whereas α -adrenoceptor agonists with low intrinsic efficacy activate only a single signalling mechanism (Nichols & Ruffolo, 1988). A number of physiological findings suggest that attenuation of cyclic AMP production is not

sufficient to serve as the only signal for eliciting certain α_2 -adrenoceptor-mediated effects (Limbird, 1988). Investigations are under way at our laboratory to test the possibility that α_2 -adrenoceptor agonists with high intrinsic efficacy, in addition to inhibiting the adenylate cyclase of the melanophores, also activate other concurrent signalling mechanisms.

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