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OCTOPAMINE RECEPTORS IN LOCUST NERVOUS TISSUE

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Abstract—The octopamine binding site in the nervous tissue of the migratory locust *Locusta migratoria* is identified as an octopamine receptor of class 2. The binding of octopamine to the binding site is saturable, reversible, stereospecific and shows a pharmacological profile typical for octopamine receptors. Saturation analysis results in a single class of non-interacting binding sites ($K_D = 7.9 \pm 0.9$ nM; $B_{max} = 160$ fmol/mg). The pharmacological analysis shows that the phenyliminoimidazolidines NC 7 and NC 5 ($K_i = 0.29$ and 0.87 nM, respectively) are the most potent agonists, and that mianserin ($K_i = 1.20$ nM) is the most potent antagonist ever reported for octopamine receptors in direct binding studies.

Octopamine, the monohydroxyphenolic analogue of noradrenaline, can act as a neurotransmitter, neurohormone or neuromodulator in the invertebrate nervous tissue [1–3]. The effect of octopamine on different invertebrate tissues has been studied in detail by Evans [4]. It seems that octopamine is important for the generation of rhythmic behaviours in invertebrates, e.g. flying and walking in locusts [5], swimming and chewing in crustaceans [6–8], and also for the circadian rhythms in arthropods [9].

Whereas the octopamine receptors of class one which modulate the myogenic rhythm in the locust extensor-tibiae-muscle [10] are only found in this tissue, all other investigated octopamine receptors are thought to belong to class two. The octopamine receptors of this class mediate the physiological effects of octopamine by means of stimulating the adenylate-cyclase system [11].

These receptors are found only in invertebrate tissues and have pharmacological similarities to adrenergic receptors [10, 12]. Investigations of the octopamine binding site using a radio-receptor-assay were performed with *Drosophila* head homogenates [13, 14] and firefly light organs [15]. These investigations demonstrated octopamine binding sites in the two different tissues which show similarities to octopamine receptors.

In the present investigation octopamine binding sites in locust nervous tissue are described exhibiting all properties required for a specific interaction as postulated by Laduron [16] and may thus be considered as an octopamine receptor.

MATERIALS AND METHODS

Animals. For all experiments migratory locusts (*Locusta migratoria* L.) of both sexes, 20–40 days after imaginal moult, were used. The locusts were reared at *ca.* 35° (light–dark cycle, 12 hr–12 hr) and fed with a diet of bran and fresh wheat.

Tissue preparation. The brain, the suboesophageal and the thoracic ganglia were dissected and used for all experiments. The ganglia were homogenized in a buffer containing: 50 mM Tris base and 10 mM $MgSO_4$ adjusted to pH 7.6 with acetic acid. The buffer contained the following proteinase inhibitors: 200 μM PMSF, 5 mg/L Trypsin inhibitor (soybean), 2 mg/L Pepstatin A, and 2 mg/L Leupeptin. The homogenization was performed with 10 strokes (low speed) of a glass/teflon homogenizer followed by 10 strokes (low speed) with a glass/glass homogenizer. After homogenization the extract was centrifuged for 20 min (30,000 *g*), the supernatant discarded and the pellet resuspended in the original volume and centrifuged again. This procedure was repeated twice. The last preparation was distributed into aliquots, centrifuged (20 min, 30,000 *g*), the supernatant discarded, and the pellets stored frozen until use. All mentioned procedures were performed at 2°.

Incubation and assay. The incubation was performed at 21° in the homogenization buffer. The total incubation volume was 100 μL, containing 10 nM [^{3}H]octopamine, and the appropriate concentrations of the tested substances. For control experiments the incubation medium contained 10 μM unlabeled octopamine. Binding under these conditions is interpreted as unspecific binding (normally 15–25% of the total binding). The incubation was started by addition of the homogenate (freshly rehomogenized in incubation buffer, *ca.* 100–150 μg/mL protein) and terminated after 45 min incubation by filtration through prewetted Whatman GF/C glassfiber filters subsequently washed with 12 mL of ice-cold wash-buffer (50 mM Tris base, 10 mM $MgSO_4$, pH 7.6 adjusted with acetic acid). The filtration was performed with low pressure in a simple multichannel filtration device. Radioactivity of the filters was measured in a scintillation-counter (40% efficiency). Protein determination was performed according to Lowry *et al.* [17].

Evaluation. Calculation of the dissociation constants for [^{3}H]octopamine and the tested ligands were performed using the "ligand"-program [18].

Chemicals. The [^{3}H]octopamine (44 Ci/mmol) was purchased from Amersham-Buchler

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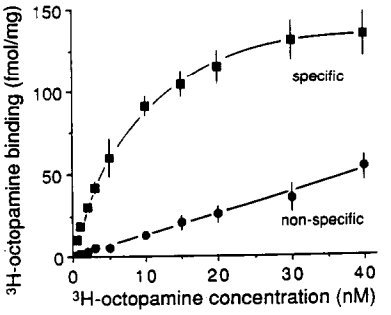

Fig. 1. Binding of [^{3}H]octopamine to crude locust nervous tissue preparations in dependence of [^{3}H]octopamine concentration. Whereas the specific binding (total binding minus non-specific binding) is saturated at *ca.* 20–30 nM [^{3}H]octopamine, the non-specific binding shows a linear increase. Each value was determined three to five times in triplicate (±SD).

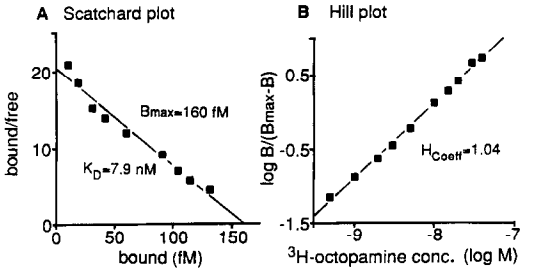

Fig. 2. Scatchard plot and Hill plot of the [^{3}H]octopamine saturation data. (A) The Scatchard analysis, using a computerized approach ("ligand"-program; [18]) inferred the existence of a single binding site ($K_D = 7.9 \pm 0.9$ nM, $B_{max} = 160$ fmol/mg). (B) Hill plot analysis results in a Hill-coefficient of 1.04 also indicating a single binding site. *B*, specific binding; B_{max}, maximal concentration of binding sites.

(Braunschweig, F.R.G.), adrenaline, clonidine HCl, naphazoline HCl, noradrenaline HCl, (−)noradrenaline HCl, propranolol HCl, octopamine HCl, synephrine HCl, tolazoline HCl and tyramine HCl were from Sigma (Deisenhofen, F.R.G.). Chlordimeform HCl from Serva (Heidelberg, F.R.G.), mianserin HCl from Research Biochem. Inc. (Wangen, F.R.G.), phentolamine and demetylchlordimeform were generous gifts of Ciba-Geigy (Basel, Switzerland), the phenyliminoimidazolidines NC 5 and NC 7 of Boehringer (Ingelheim, F.R.G.).

All chemicals were p.A. quality.

RESULTS

It is shown in this investigation that the specific binding of octopamine to its binding site is proportional to the protein concentration in the tested range (50–400 μg/mL). The specific binding is approximately constant over the pH range tested (6.8–8.4), showing a slight maximum between pH 7.4 and 7.8.

Saturability of the binding

The specific binding of octopamine to its binding site could be saturated in the nanomolar range. Saturation is achieved at a concentration of *ca.* 30–40 nM. The non-specific binding is, within the investigated range, proportional to the octopamine concentration (Fig. 1). Presentation of the saturation data using the Scatchard-plot results in a linear decrease of the curve (Fig. 2A). Therefore, it is likely that octopamine is bound to only one homogeneous binding site, representing the octopamine receptor ($K_D = 7.9 \pm 0.9$ nM, $B_{max} = 160$ fmol/mg). The Hill-plot analysis (Fig. 2B) results in a Hill-coefficient of 1.04, also indicative for the existence of a single class of binding sites.

Kinetic analysis

At the incubation temperature (21°) the specific binding of octopamine (10 nM) to its binding site reached equilibrum after *ca.* 20 min (Fig. 3) and was

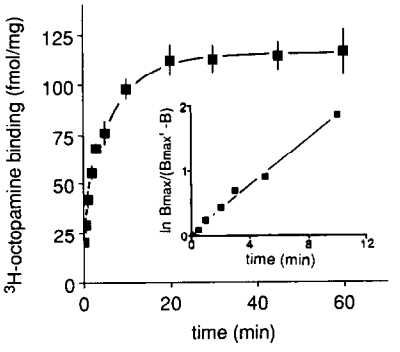

Fig. 3. Association kinetics of the specific binding of [^{3}H]octopamine (10 nM, 21°) to the receptor binding site. Each value was determined three times in triplicate (±SD). Analysis of these data results in a $K_{obs} = 0.182$ min^{-1} (inset). The constant of the forward reaction K_{+1} ($K_{+1} = (K_{obs} - K_{-1})/C_{oct}$) is 9.5×10^6 min^{-1} mol^{-1}. B, specific binding; B_{max}', equilibrium specific binding with 10 nM [^{3}H]octopamine.

stable over the time investigated (up to 60 min). The evaluation of the association data resulted in a kinetic constant K_{obs} of 0.182 min^{-1} (Fig. 3, inset). When, after reaching equilibrum (45 min association), 10 μM of unlabeled octopamine was added, the specific binding decreased after 20–30 min to a stable minimum (*ca.* 20% of the initial specific binding; Fig. 4). That demonstrates the reversibility of the binding of octopamine to its binding site. Calculation of the constant of the backward reaction K_{-1} results in a value of 0.0869 min^{-1} (Fig. 4, inset). With these results, the kinetic constant of the forward reaction K_{+1} could be determined ($K_{+1} = 9.5 \times 10^6$ min^{-1} mol^{-1}). The calculation of the dissociation constant K_D using the kinetic constants ($K_D = K_{-1}/K_{+1}$) results in a K_D of 9.15 nM.

Stereospecificity

The binding of octopamine to its receptor could be inhibited stereospecifically (Table 1) by noradrenaline, the 3-hydroxylated analogue of octo-

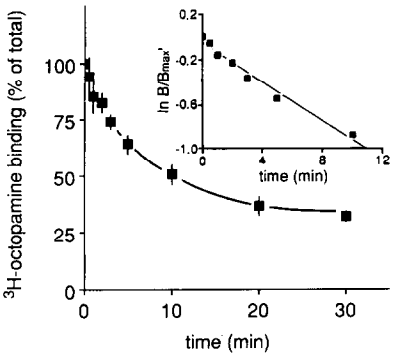

Fig. 4. Dissociation kinetics of the binding of [^{3}H]octopamine (10 nM, 21°) to the binding site. After reaching equilibrium (45 min association) 10 μM octopamine were added and the binding determined after the appropriate times (each value is determined three times in triplicate, ±SD). The analysis of these data results in a constant of the backward reaction K_{-1} of 0.0896 min^{-1} (inset). Evaluation of the kinetic constants for the determination of the dissociation constant K_D ($K_D = K_{-1}/K_{+1}$) results in a value of 9.15 nM. (For abbreviations see Figs 2 and 3.)

Table 1. Ligands of the octopamine receptor from locust nervous tissue and their binding constants

Tested ligands	A K_i (nM)	B K_{iOc}/K_{ix}
Biogenic amines		
Synephrine	3.38	1.96
Octopamine	6.62	1.00
Tyramine	51.6	0.13
Adrenaline	416	0.016
(−)Noradrenaline	475	0.014
(±)Noradrenaline	908	0.0073
Formamidines		
Demethylchlordimeform	1.82	3.64
Chlordimeform	124	0.053
2-Benzylamidazolines		
Naphazoline	3.03	2.18
Tolazoline	18.5	0.36
Phenyliminoimidazolidines		
NC 7	0.29	22.8
NC 5	0.87	7.61
Clonidine	47.4	0.14
Antagonists		
Mianserin	1.20	5.52
Phentolamine	19	0.35
Propranolol	29,200	0.000023

Data are from displacement experiments (see Fig. 5). The K_i values (A) of the listed substances were determined using the "ligand" program with the assumption that only a single binding site exists [18]. In (B) the relative affinity compared with octopamine is given.

pamine. Comparing the (−)isomer of noradrenaline with the (±)racemate resulted in K_i values of 475 and 908 nM, respectively. The (−)isomer is, therefore, as active as the double amount of the (±)racemate, containing only 50% of the (−)isomer. This indicates the (+)isomer to be the inactive one.

Receptor classification

In competition experiments, using agonists to the octopamine receptor, it could be shown that the investigated binding site resembled the characteristics of the octopamine receptor of class 2A. The ranking order of affinities (naphazoline > tolazoline > clonidine; Table 1B) is typical for this receptor subtype [10].

Inhibition by antagonists

The specific binding of octopamine to the corresponding receptor was inhibited by the three tested antagonists in the following order: mianserin ≫ phentolamine ≫ propranolol (Fig. 5B, Table 1). Phentolamine which is well known as a highly potent octopamine antagonist [15, 19] is much more active than propranolol. This is characteristic for octopamine receptors. Interestingly, mianserin, an antagonist of the mammalian serotonin and histamine receptors, is also, as previously reported for the octopamine receptors of locust flight muscle [20], the antagonist with the highest affinity to the octopamine receptor in the locust CNS (5.52 times higher affinity than octopamine).

Inhibition due to agonists

Competition experiments were performed with agonists from four different classes of compounds (Fig. 5A, Table 1). The comparison of the affinities of the investigated biogenic amines resulted in the following rank order of affinities to the binding site: synephrine > octopamine > tyramine > adrenaline > noradrenaline. The affinity of the N-methylated derivatives synephrine and adrenaline is about twice as high compared with their demethylated analogues octopamine and noradrenaline. Tyramine, the substance from which octopamine is directly synthesized by β-hydroxylation, has an affinity of 13% compared with octopamine.

The formamidine derivatives chlordimeform and demethylchlordimeform have very different affinities to the binding site (demethylchlordimeform ≫ chlordimeform), with demethylchlordimeform having a higher affinity for the octopamine receptor (3.64 times) than octopamine, another characteristic for the octopamine receptor [21]. Whereas the 2-benzylimidazoline derivative naphazoline is 2.18 times as potent as octopamine (Table 1B), the related substance tolazoline shows a much lower affinity (0.36 times compared to octopamine).

The class of compounds including the ligands with the highest affinity to the octopamine binding site are the phenyliminoimidazolidines. This class of compounds, including the well known adrenergic agonist clonidine (K_i = 47.4 nM), especially the 2.6-dimethyl-derivative (NC 5; K_i = 0.87 nM) and the 2-methyl, 4-chloro-derivative (NC 7; K_i = 0.29 nM) can displace octopamine (10 nM) nearly completely at concentrations of *ca.* 10 nM (Fig. 5A). Comparison of the affinities of NC 7 and demethylchlordimeform, which have the same substitutions at their phenolic rings, clearly demonstrates that the iminoimidazolidine structure of NC 7 leads to a higher affinity as the N=C—N—CH_3 structure of demethylchlordimeform. The two iminoimidazo-

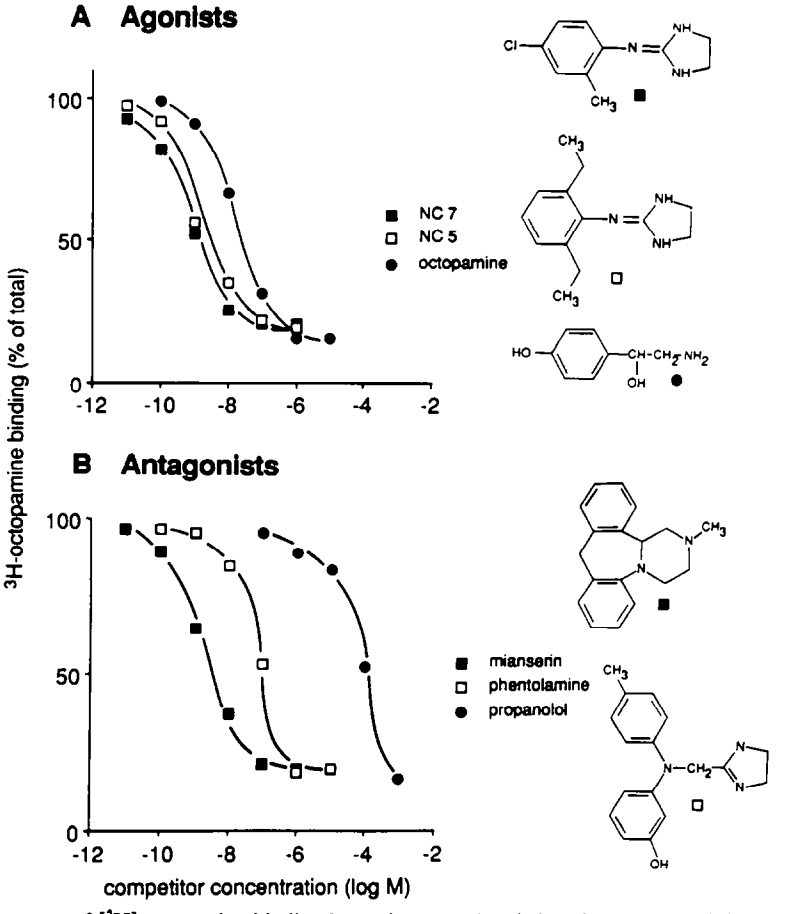

Fig. 5. Displacement of [^{3}H]octopamine binding by various agonists (A) and antagonists (B). Each of these had to compete with 10 nM [^{3}H]octopamine for the binding sites (each value is determined at least twice in triplicate).

lidines NC 5 and NC 7 have the highest affinity ever reported to the octopamine binding site (7.61 and 22.8 times higher affinities, respectively, compared with octopamine; Fig. 5A, Table 1).

DISCUSSION

The present paper shows that the investigated binding site is the octopamine receptor. All criteria [16] are fulfilled: saturability, reversibility, stereospecificity, and the conformity of the general pharmacological properties of the investigated binding site with the characterization of octopamine receptors from different tissues using different methods [19, 22].

The specific binding of octopamine is saturable and characterized by a single binding site. These results are in accordance with those of Dudai and Zvi [14], and they differ from the results of Hashemzadeh *et al.* [15] who suggested the existence of two binding sites. However, it has to be mentioned that two affinity states of the binding site could be generated by removal of Mg^{2+}-ions from the incubation medium (Roeder, in preparation). The determination of the dissociation-constant using two other independent methods resulted in values of 9.15 nM (kinetic experiments, Fig. 4) and 6.62 nM (competition experiments, Table 1). The mean of these results is 7.89 nM with a deviation of less than 20%. The maximal concentration of binding sites found in locust nervous tissue (160 fmol/mg) is higher than the B_{max} found in *Drosophila* head homogenates [14], and is as high as in the firefly lantern [15]. Therefore, the nervous tissue of locusts should be a good source for the further characterization and purification of the octopamine receptor.

The quantitative pharmacological investigations show, in the cases where D/L mixture were applied (like adrenaline, synephrine, and octopamine), a remaining uncertainty. But one major advantage of ligand binding studies is the relatively low influence of having two different isomers in a (D/L) mixture. The affinity of the active isomer, the (−)isomer in the case of octopamine and related biogenic amines, is maximally twice as high compared with the racemate. This enables us to establish a rank order of affinities using either pure substances or racemates of substances with a chirale center if they differ in their affinity more than twice.

The classification of the octopamine receptor as a

class two receptor is based on the agonist profile of the receptor. It is inferred from the relatively high affinity of naphazoline that the investigated receptor is of the subclass 2A [10]. Only mianserin, which is the antagonist with the highest affinity to the receptor, does not fit the classification. The very high affinity of mianserin is also reported from investigations measuring the elevation of the cAMP-level in locust flight muscle [20] and in the nervous tissue of *Pierplaneta* [22]. Mianserin may serve as a radioligand in future investigations of the octopamine receptor.

The extremely high affinity of the phenyliminoimidazolidines, introduced by Nathanson [12], is demonstrated here the first time at the binding site level. In contrast to Nathanson we show that in the locust nervous tissue NC 7 is more potent than NC 5. These examinations demonstrate that the 2, 4 and 6 positions of the phenolic ring are very important for the affinity. A hydroxyl or halogen group at the *para*-position, and ethyl-groups at position 2 and 6 would result in a phenyliminoimidazolidine with highest affinity. Substances like NC 5 and NC 7 may serve as tools for the further characterization and purification of the octopamine receptor, as well as, due to their high specificity for the octopamine receptor, starting points for the development of highly specific insecticides.

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