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Epinastine, a highly specific antagonist of insect neuronal octopamine receptors

Thomas Roeder *, Jörn Degen, Michael Gewecke

Universität Hamburg, Zoologisches Institut, Neurophysiologie, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany

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Abstract

The tetracyclic compound epinastine (3-amino-9, 13*b*-dihydro-1*H*-dibenz(*c*,*f*)imidazo(1,5*a*)azepine hydrochloride) that was recently introduced as a vertebrate histamine H_1 receptor antagonist has also high affinity for insect neuronal octopamine receptors. This holds true for the neuronal octopamine receptor from the locust ($K_i = 2$ Nm) as well as from the honey bee nervous system ($K_i = 1.1$ Nm). In addition to its high affinity, it has a high degree of specificity. Its affinity for other insect receptors for biogenic amines, such as 5-hydroxytryptamine, dopamine, histamine, and tyramine, is at least four orders of magnitude lower. Therefore, epinastine could serve as a highly specific antagonist of octopamine receptors that enables physiological dissection of octopaminergic neurotransmission within the nervous system of insects. To demonstrate these abilities, epinastine was used to inhibit the visually evoked activity of an identified interneuron in the visual pathway which is known to be modulated by octopamine.

Keywords: Biogenic amine; Nervous system; Descending contralateral movement detector; Neurotransmission; (Bee); (Locust)

1. Introduction

Octopamine, the monohydroxylated analogue of norepinephrine, is a major neurotransmitter in invertebrates. After its discovery in the salivary glands of Octopus, it was found in relatively high concentrations in peripheral, and central nervous tissues of most invertebrates studied (Axelrod and Saavedra, 1977; Evans, 1985). In invertebrates, octopamine functions as a neurotransmitter, -hormone, and -modulator. It modulates the activity of muscles, other peripheral organs, hemocytes, and almost all sense organs (Roeder, 1994). In the insect nervous system, octopamine seems to be essential for the generation and maintenance of most rhythmic behaviors including flight, swimming, digging, and running (Sombati and Hoyle, 1984). Even complex achievements of the nervous system such as learning and memory, or regulation of the motivational state of the animal, are influenced by this multifunctional compound (Bicker and Menzel, 1989).

Octopamine mediates its actions through a set of at least four different receptor subtypes. Evans (1981) divided octopamine receptors into three types based on physiological studies performed with a locust leg muscle. The main octopamine receptor of the insect central nervous system belongs to a peculiar receptor class, the class-3 receptor (Roeder, 1992). Detailed pharmacological studies, especially of peripheral octopamine receptors, led to the development of highly specific agonists with insecticidal activity. Supplementary, this was also done for the neuronal octopamine receptor, the putative target of octopaminergic insecticides (Roeder, 1995). In contrast to the agonists, where various high affinity substances from different classes of compounds are available, only very few antagonists are known. This is puzzling because they are of even greater importance for the physiological characterisation of octopaminergic neurotransmission (Roeder, 1990). One major drawback of these few well characterised antagonists is their lack of high specificity for octopamine receptors. This drastically reduces their usefulness for the dissection of octopaminergic neurotransmission at the physiological level.

In the current study, we characterise epinastine, an antagonist that shares a very high degree of specificity for insect neuronal octopamine receptors with exceptional affinity. It should be an important tool for the study of

* Corresponding author. Tel.: +49-40-4123-3941; fax: +49-40-4123-3937; e-mail: roeder@zoologie.uni-hamburg.de

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octopaminergic neurotransmission in the insect nervous system. To demonstrate its usefulness for the physiological dissection of octopamine action within the insect nervous system, we used epinastine to antagonise the action of endogenous octopamine on the activity of visual interneurons. Recently, it was shown that octopamine can arise the activity of the visual pathway in locusts (Bacon et al., 1995; Stern et al., 1995). Therefore, the output of the visual pathway is in the present paper taken from a giant neuron, the descending contralateral movement detector (DCMD; Rowell, 1971), to investigate the physiological function of epinastine on the neuronal octopamine receptors of the class-3.

2. Materials and methods

2.1. Animals

Experiments were done with desert locusts (*Schistocerca gregaria*) of both sexes, 7–40 days after imaginal mould. The locusts were reared at approximately 35°C (light–dark cycle 12 h–12 h) and fed with a diet of bran, and grass. Adult honey bee (*Apis mellifera*) workers were caught at the entrance of the hive.

2.2. Pharmacology

The nervous tissue (supra- and suboesophageal ganglia) of adult desert locusts or adult honey bees was carefully dissected, and stored frozen in the incubation buffer (Tris/acetic acid, 50 mM, pH 7.6, 5 mM $MgSO_4$, supplemented with 200 μM phenyl methyl sulfonyl fluoride) until use. The nervous tissue was homogenized, the homogenate centrifuged (20 000 $\times g$, 30 min, 2°C), and the pellets were resuspended in the original volume. This procedure was repeated twice to obtain a washed preparation, and the resulting pellets were stored frozen at $-70°$ until use. The incubation continued for 60 min at room temperature, and was terminated by filtration through pretreated glass fiber filters (0.3% polyethyleneimine). A total volume of 250 μl was used throughout the studies, with protein concentrations ranging from 0.5–1.5 mg/ml. Different radioligands were used for the experiments: [^{3}H]NC-5*Z* (for the octopamine receptor) at 1 nM, [^{3}H]LSD (Lysergic acid diethylamide; 5-HT receptor) at 2 nM, [^{3}H]LSD (dopamine receptor) at 2 nM, [^{3}H]mianserin (histamine receptor) at 1 nM, and [^{3}H]tyramine (tyramine receptor) at 10 nM. To study the 5-HT receptor and the dopamine receptor, incubation was performed in the presence of 10 μM dopamine or 10 μM 5-HT, respectively. Each experiment was performed at least three times in triplicate with epinastine concentrations ranging from 10^{-11} to 10^{-3} M. Further experimental details were given previously (Roeder and Nathanson, 1993; Wedemeyer et al., 1992). The competition experiments were evaluated using the LIGAND program (Munson and Rodbard, 1980).

The cAMP production of locust nervous tissue homogenates was performed according to Orchard and Lange (1986). After preparation of the homogenates, incubation proceeded for 15 min in the presence of IBMX (isobutylmethylxanthine) prior to determination of the cAMP-levels. cAMP was determined using a commercial cAMP-assay system (Amersham, Braunschweig, Germany).

2.3. Electrophysiology

To test the effect of epinastine in the nervous system, electrophysiological experiments were performed (at 21–26°C laboratory temperature) with adult *Schistocerca* of both sexes. The animals were mounted ventral-side-up on a holder. Thus extracellular recordings from the right connective between pro- and mesothoracic ganglia by a silver hook electrode were made possible. The largest action potentials measured were from a giant neuron, the DCMD (Rowell, 1971). The action potentials were controlled and magnified by an oscilloscope (Tektronix), digitized by an interface (CED 1401, Cambridge Instruments), and stored and evaluated by a personal computer. The left eye was stimulated by a small black disc on the screen of a second PC generating optical patterns (Stern and Gewecke, 1993). Temporal marks of this pattern (a 13.8s vertically, and 13.7s horizontally moved black disc on a bright background; Fig. 5, middle) were stored simultaneously with the action potentials by the first PC. During each experiment (up to 160 min duration), the DCMD-activity (impulses/test; test duration ca. 28 s) was measured every 10 min. After three tests of each experiment, locust saline (10 μl; O'Shea and Rowell, 1975) was injected into the head capsule through a prepared opening fronto-ventrally of the left eye. In the control group of experiments (sham) this was saline; in a second group it was saline with octopamine (0.1 M); and in the third group it was saline with epinastine (0.02, 0.1, or 0.25 M; Fig. 6).

2.4. Chemicals

Epinastine (3-amino-9, 13*b*-dihydro-1*H*-dibenz(*c*,*f*)imidazo(1,5-*a*)azepine hydro-chloride) was a generous gift of Boehringer Ingelheim. [^{3}H]tyramine (30 Ci/mmol), and [^{3}H]LSD (40 Ci/mmol) were purchased from NEN Dupont (Bad Homburg, Germany). [^{3}H]mianserin was purchased from Amersham-Buchler (Braunschweig, Germany). [^{3}H]NC-5*Z* (2[2,6-diethyl-4-azidophenylimino]imidazolidine; 40 Ci/mmol) was a generous gift of Dr. J.A. Nathanson (Mass. Gen. Hosp., Boston, USA). All other chemicals were of highest quality available.

3. Results

3.1. Pharmacology

The tetracyclic substance epinastine (Fig. 1) is a histamine H_1 receptor antagonist of the newest generation.

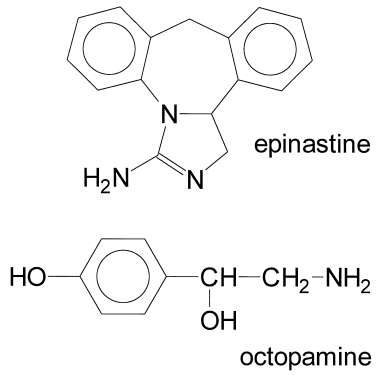

Fig. 1. Chemical structures of high affinity antagonist epinastine, and the natural ligand octopamine.

First clinical studies revealed its usefulness as an antihistaminic agent (Adamus et al., 1987). We used this substance for our study because it shows structural features comparable to substances we identified as high affinity antagonists for the locust neuronal octopamine receptor, e.g., mianserin, and maroxepine (Fig. 1; Roeder, 1990). In the locust nervous tissue, epinastine is able to displace specific [^{3}H]NC-5*Z* binding (a high affinity octopamine receptor agonist; Roeder and Nathanson, 1993) at low concentrations (Fig. 2). The estimated K_d of epinastine for [^{3}H]NC-5*Z* binding is 2.0 nM ($\pm$0.4 nM) which is amongst the highest affinities for octopamine receptor antagonists studied so far. This value is in the same order of magnitude as the affinities of, e.g., mianserin and maroxepine.

To study whether this high affinity of epinastine is common for insect neuronal octopamine receptors, or if it is restricted to locusts, we estimated its affinity also for the honey bees neuronal octopamine receptor. In honey bees, its affinity (1.1 nM $\pm$ 0.7 nM; Fig. 2) is slightly higher.

The major drawback of all other high-affinity octopamine receptor antagonists is their pharmacological side activity. Antagonists such as mianserin have affinities in the same order of magnitude for other receptors for biogenic amines in the insect central nervous system, e.g., a histamine H_1 receptor (Roeder, 1994). One major application of antagonists relies on their ability to specifically inhibit the respective neurotransmission. The use of antagonists with high affinities for other targets in the system makes physiological experiments in the insect nervous system, where, e.g., mianserin should block the effects of octopamine, hard to interpret, because the respective effects could also result from interactions with other receptors such as the insect histamine H_1 receptor.

To test whether epinastine is better suited for this purpose we evaluated its affinity for the other known receptors for biogenic amines in the central nervous tissue of the locust. These receptors are the neuronal 5-HT receptor, the neuronal dopamine receptor, the neuronal histamine H_1 receptor, and the neuronal tyramine receptor. Interestingly, the affinity of epinastine for each of these receptor types is relatively low (Fig. 3). Epinastine, even at concentrations above 10–100 μM, is not able to displace specific 5-HT- or dopamine-sensitive [^{3}H]LSD-binding, [^{3}H]mianserin binding, or [^{3}H]tyramine binding to locust nervous tissue membranes. The estimated K_i values for these receptors are, 41 μM for the dopamine receptor, 112 μM for the 5-HT receptor, 225 μM for the tyramine receptor, and 830 μM for the histamine H_1-receptor (Fig. 3). The affinity for the locust neuronal dopamine receptor is the highest in this group followed by the 5-HT, the tyramine, and the histamine H_1 receptor. 41 μM, the highest affinity in this group, is about 50 000 times as high as epinastine's affinity for the octopamine receptor. This means, that these receptor systems are not at all disturbed at epinastine concentrations that leads to a total blockade of octopaminergic neurotransmission.

To show that epinastine is not only a high affinity competitor for the octopaminergic receptor but really an antagonist, its ability to inhibit octopamine induced cAMP formation was evaluated. Coincubation of octopamine (10

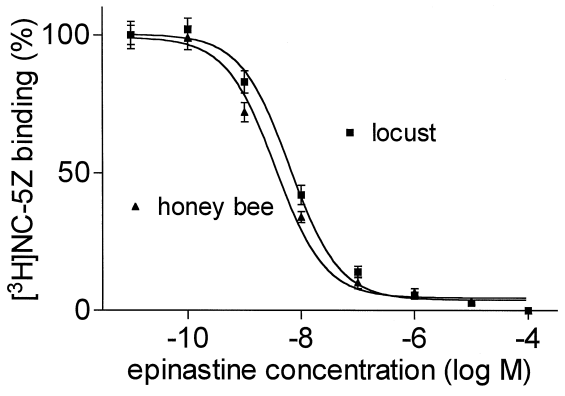

Fig. 2. Increasing concentrations of epinastine compete with specific [^{3}H]NC-5*Z* for the locust and the honey bee neuronal octopamine receptor. Each value (% of control) is the mean of at least 3–4 different experiments performed in triplicate. S.D. is indicated as vertical bars.

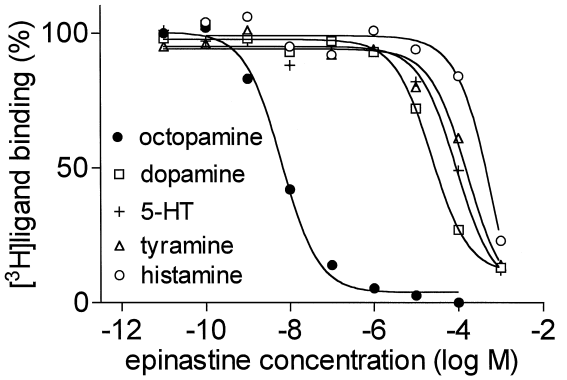

Fig. 3. Displacement of various radioligands by increasing concentrations of epinastine. For octopamine receptors, [^{3}H]NC-5*Z* (1 nM), for the dopamine receptor [^{3}H]LSD (2 nM) in the presence of 10 μM serotonin, for serotonin receptors [^{3}H]LSD (2 nM) in the presence of 10 μM dopamine, for tyramine receptors [^{3}H]tyramine (10 nM), and for histamine receptors, [^{3}H]mianserin (1 nM) were used as radioligands.

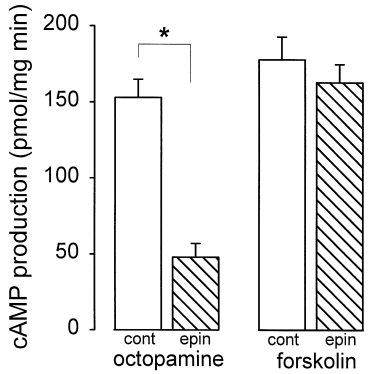

Fig. 4. Effect of epinastine (epin) on octopamine- and forskolin-induced adenylyl cyclase from the locust nervous tissue. For controls (cont) 10 μM octopamine or foskolin were used. The effect of epinastine was measured at equimolar concentrations. The mean of eight independent experiments is given together with the corresponding S.D. Only for octopamine, a significant difference is to be seen ($*$; $P < 0.05$).

μM) together with an equimolar concentration of epinastine abolished the octopamine-induced cAMP production totally. The measured cAMP-formation was at the control level (Fig. 4). As the octopamine concentration used in this experiment is about 1000 times higher than its K_D-value, the epinastine concentration required to totally abolish octopamine-induced cAMP production is as low as expected. Forskolin-induced cAMP production is not reduced in the presence of epinastine, which shows that epinastine really antagonises octopamine-induced cAMP formation in the insect brain. This makes physiological studies dissecting the octopaminergic neurotransmission within the insect nervous system possible. Therefore, epinastine is an important supplement to the pharmacological tools currently available.

3.2. Electrophysiology

In the electrophysiological experiments the large action potentials of one descending giant neuron (DCMD) were recorded from the pro-meso-thoracic connective (Fig. 5, top). Each experiment consits of 6–17 tests with 10 min interval at a time. Each test starts after a dark period during which the DCMD shows a low spontaneous activity. When the screen of the stimulating personal computer was brightened (66 cd/m^2) the neuron answered, with a burst of action potentials (on sensitivity). When a black dot was moved down and up (for 2.3 s in one stimulus unit; Fig. 5, middle) six times every motion stimulus excited the DCMD. However, the answer to a stimulus unit decreased successively. The same result was generated by subsequent forward or backward movements of the black dot. A last burst (off effect) was caused by the final darkening of the screen (to 3 cd/m^2). After this the activity was low again. In Fig. 5 (below), histograms demonstrate the decrease of the impulse frequency during the two successive stimulus periods, i.e., the habituation of the neuron to repeated stimuli (Rowell, 1971).

The histograms shown in Fig. 5 are the starting-point for judging the pharmacological effects of octopamine and epinastine on the visual pathway of *Schistocerca*. The number of action potentials of the first histogram of a test (total frequency during vertical dot movement) was added to that of the second histogram (total frequency during horizontal dot movement). The resulting summed activity

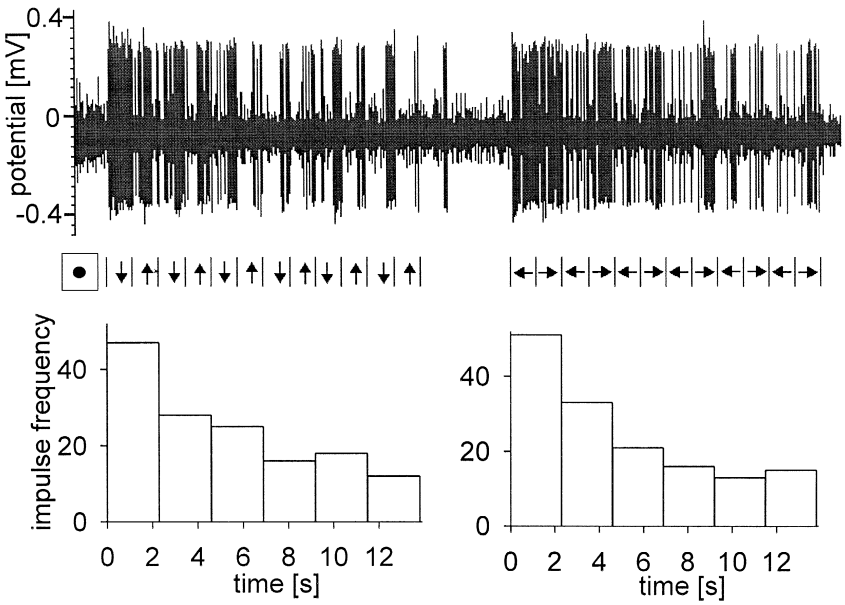

Fig. 5. This example of an electrophysiological test shows extracellular recording of action potentials (impulses) of the DCMD (top), generated by vertical (left) or horizontal (right) movements of a black disc (middle). The respective histograms (below) show the decrease of the impulse frequency, i.e., the habituation of the DCMD. The number of all impulses of these two histograms together give the value of the activity of this test (impulses/test). This procedure was put through all tests, giving the data base for the evaluations shown in Figs. 6 and 7.

(impulses/test) is further evaluated, and shown in Fig. 6. In the control group (sham) of seven animals unsupplemented saline was injected after the third test into the head capsule at the fronto-ventral margin of the stimulated eye. In these animals a continuous habituation of the DCMD-activity is seen from the first test ($t = 0$ min) to the end ($t = 160$ min) of the experiment (Fig. 6A). However, in no case it decreases to zero.

The second group of experiments was performed to prove the effect of octopamine on the DCMD-activity. Therefore, after the third test an octopamine–saline solution (0.1 M) was injected into the head capsule. In four animals with a high initial activity (> 200 impulses/test) octopamine did not cause any significant effect (Fig. 6B, upper curve). Only in four animals with low initial activity (< 100 impulses/test), the DCMD-activity increased after octopamine application (Fig. 6B, lower curve), i.e., octopamine dishabituated the neuron (Bacon et al., 1995)

The critical group of experiments for the present study was the third one in which the effect of epinastine on the activity of the DCMD, and on its habituation was investigated. In all these experiments, its activity decreased after epinastine application more or less fast to zero, depending on the concentration of the epinastine–saline solution (Fig. 6C). High concentrations of epinastine (0.1 or 0.25 M) suppressed in four animals the DCMD-activity totally within few minutes. With lower concentration (0.02 M) the activity decreased more slowly, i.e., after about 20 min. In some experiments, the action potentials of other descending neurons with smaller amplitude (Fig. 5), whose frequency was not influenced by the moving dot stimulation, also vanished.

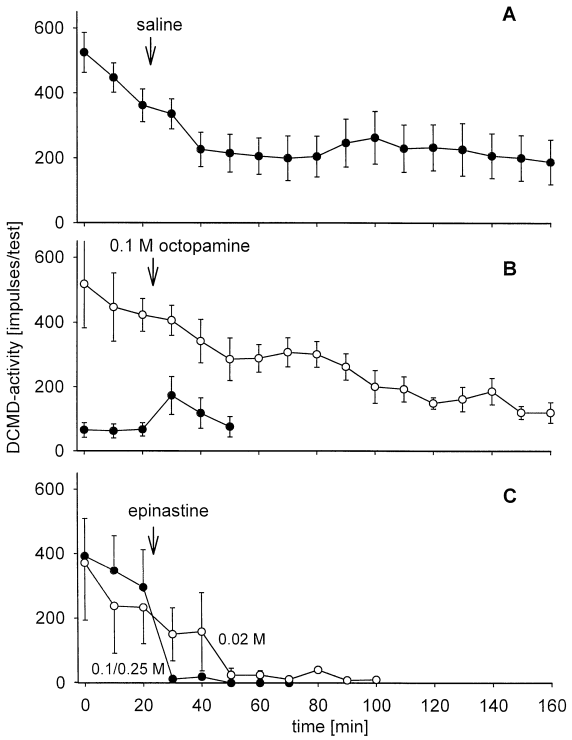

Fig. 6. Dependence of DCMD-activity on successively repeated tests, and injection of different compounds (10 μl) into the head capsule (arrow). (A) In sham experiments ($n = 7$), pure saline was injected after the 3rd test, by which the habituation process was not influenced. (B) After injection of saline with octopamine (0.1 M), the same was true in aroused locusts ($n = 4$) with high initial DCMD-activity (above), whereas in locusts ($n = 4$) with low initial activity (below), octopamine application caused dishabituation (arousal) of the DCMD. (C) Injection of saline with epinastine in low concentration (0.02 M; $n = 4$) decreased its activity after about 30 min, and in high concentration (0.1 or 0.25 M; $n = 4$) immediately after a few minutes almost to zero. Bars indicate standard error of the mean (S.E.).

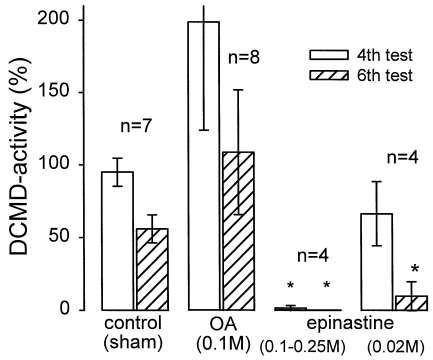

Fig. 7. Statistical evaluation of the experiments shown in Fig. 6. The DCMD-activity (%) of the 4th, and 6th tests of each experiment is normalised to the respective 3rd test (= 100%), before injection of saline into the head capsule. Statistically significant (*; $P < 0.05$) is only the decrease of DCMD-activity after application of 0.1–0.25 M epinastine, and the decrease of its activity 20 min after application of 0.02 M epinastine. Bars indicate S.E.

To obtain statistically relevant results, corresponding experiments were taken together and displayed. Because the starting DCMD-activity varied strongly from experiment to experiment, we decided to normalise the data. The third test (the test before application of the drug) was set to 100% and only the 4th and 6th test displayed. After octopamine application, the visually evoked mean activity increased about twofold, which is nevertheless statistically not significant ($P > 0.05$; Fig. 7). In contrast, addition of high concentrations of epinastine (0.1–0.25 M) abolished the DCMD-activity measured directly after application (4th test) as well as 20 min later (6th test). Both values are statistically significant lower compared with the control experiments. Lower concentrations of epinastine had a smaller, delayed effect, which was 20 min after application (6th test) statistically significant different from the control ($P < 0.05$, Fig. 7).

4. Discussion

The physiological characterization of a neurotransmitter system depends on the availability of highly specific agonists and antagonists. High affinity and especially high specificity are required to ensure that relatively small concentrations of the corresponding compound could elicit

the physiological response without influencing other receptor systems. As mentioned earlier, numerous agonists from different classes of compounds fall in this group (Nathanson, 1985; Roeder, 1995). Although high affinity antagonists for the octopamine receptor are available (Roeder, 1990), none of them has a high degree of specificity. Compounds such as mianserin or maroxepine have also high affinities for other receptors such as a locust histamine or 5-HT receptor. These compounds has to be excluded from physiological studies, because an observed physiological response could not be attributed without any doubt to an interaction with the octopamine receptor. Our candidate compound that should serve as an antagonist even suited for physiological studies is epinastine that shares structural features with other high affinity antagonists of the octopamine receptor such as mianserin or maroxepine. Its high affinity is only the first criterion that has to be fulfilled. Even of greater importance is the high degree of specificity required. To exclude high affinity interactions with other, comparable receptors, we checked the affinity of epinastine for other receptors for biogenic amines in the insect nervous system. Fortunately, the receptor with highest affinity for epinastine in this group, the dopamine receptor has an affinity that is about 50 000 times lower compared with that of the octopamine receptor. This difference in affinity is sufficient to block octopamine receptors totally without influencing any of the related receptors for biogenic amines in the nervous system of the locust.

Therefore, epinastine supplements the tools for the physiological dissection of octopaminergic neurotransmission within the insect nervous system. The lack of highly specific antagonists was a major drawback for physiological studies. This holds true especially for those experiments were endogenous octopamine plays the major role. Differences in the octopamine content leading to divergent physiological responses could thus not be attributed to the octopaminergic system. Using epinastine, this is possible, because octopaminergic neurotransmission could easily be blocked specifically by relatively low concentrations of this antagonist acting at its target sites.

Epinastine high-affinity properties of insect neuronal octopamine receptors seems to be a general phenomenon. This conclusion is justified because the relationship between locusts and honey bees is rather low, as their evolutionary lines split about 300 million years ago. In addition, locusts and honey bees are representatives of the two lines of modern insects, pauro- and holometabolic insects, respectively.

To demonstrate that epinastine is indeed suited to act as an antagonist of octopaminergic neurotransmission, we choose the visual pathway of the locust. Within this pathway, a descending interneuron, the DCMD is known to be modulated by octopamine (Bacon et al., 1995). It is believed that octopamine has a dishabituating effect in this system. Injection of octopamine led to an increased activity in this neuron (statistically not significant, Fig. 7). The effects of epinastine are more pronounced. Injection of relatively high concentrations resulted in a dramatical reduction of the visually evoked DCMD activity. This effect, that occurs relatively quick after injection of the small test volume into the head capsule, is statistically significant. It has to be regarded that the concentration of epinastine acting at the target site within the central nervous system is several orders of magnitude lower than the injected concentration. The total volume of the head capsule exceeds 500 μl, which leads to an at least 1:50 dilution of the injected substance (in the case of 0.1 M epinastine, its concentration in the head capsule is a few micromoles). Unfortunately, the nervous system of insects is effectively separated from the hemolymph thus making in vivo pharmacological studies in insects very complicated. Although it could not be estimated how much epinastine enters the central nervous system, it is highly probable that only a small percentage of the substance injected into the headcapsule could act at ist target site within the optic lobe. Taken these effects together, epinastine should be present at ist target site within the optic lobe at nanomolar concentrations which could be expected form the in vitro studies. In classical pharmacological studies wash out of the substance of interest, showing its reversibility, are required. As mentioned above, this is usually not possible in the insects nervous system, because a constant flow of the ringer solution towards the target within the central nervous system is not possible. The only way to compensate this drawback is to perform numerous experiments to get statistically relevant results. Nevertheless, the quick, statistically relevant drop of the DCMD activity following epinastine application shows that it could easily reach its target sites within the insect nervous system, which is the third prerequisite for an antagonist to be suited for in vivo pharmacology. The picture obtained using lower epinastine concentrations fits into this scenario. The drop in activity occurs with a little delay, because the critical epinastine concentration reaches its target within the insect nervous system later.

Taken together epinastine combines all requirements of an antagonist suitable for the physiological dissection of octopaminergic neurotransmission. It has high affinity and high specificity for octopamine receptors, and it reaches easily its targets within the insect nervous system. Therefore, it should be a powerful tool for studies dealing with the diverse and interesting actions of octopamine within the insect nervous system.

Acknowledgements

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