Comparative cytochemical and pharmacological studies on the cholinergic innervation of the branchial heart of the cephalopod *Sepia officinalis* (L.)

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Summary. The dense motor innervation of the obliquely striated muscle cells in the branchial heart of Coleoida is composed of activating and inhibiting parts. The inhibiting cholinergic system investigated in this study is characterized histo- and cytochemically by a high acetylcholinesterase activity (EC 3.1.1.7) in the glycocalices of the nerve fibers with transparent synaptic vesicles, the muscle cells and, to a lesser extent, the ovoid interstitial cells, the functions of which are endocytosis and storage of catabolic substances. The pharmacological results from the isolated organ indicate a more nicotinic type of Ach-receptor, which can be reversibly blocked by D-tubocurarine and α-bungarotoxin, but not with the same intensity by tetraethylammonium or atropine.
Key words. Branchial heart; cephalopods; cholinergic innervation.

Introduction

The particular efficiency of the cephalopod circulatory system is due mainly to the fact that, apart from the systemic heart, additional auxiliary hearts exist, in the form of contractile veins[9] and in Dibranchiata also branchial hearts, and these promote a quick blood circulation. The branchial hearts of the dibranchiate cephalopods primarily have the task of overcoming the peripheral resistance of the blood vessels, capillaries and lacunae of the branchia; furthermore, they also have the function of making possible pressure filtration in the branchial heart appendages, i.e. the production of primary urine[21]. Their automatism is, like that of veins and the systemic heart, myogenic; the coordination with the activity of the remaining circulation is probably regulated by promoting and inhibiting neurons from the lobus visceralis or ganglion cardiacum[1,17,24]. Several findings with respect to the neuropharmacology of the systemic heart of the cephalopods have been reviewed, and also others concerning the effects of acetylcholine and other mimetics, which generally have a negative inotropic and chronotropic effect on the heart[2,6,7,12-14]. But apart from few in vivo observations[23], findings on the isolated organ concerning the receptors in obliquely striated muscle cells are missing[18]. First of all, an attempt has been made to describe the presumed cholinergic receptor system by means of pharmacological examinations and cytochemical findings.

Materials and methods

22 juvenile and adult, ♀ and ♂ *Sepia officinalis* (L.) – mantle length: 9–23 cm – from the Bassin d'Arcachon (Atlantic Ocean) were used in this study.

Electron microscopical methods. Fixation in 4% glutaraldehyde in phosphate buffer, 1000 mOsm; pH 7.3; 2 h at 4 °C increasing to 18 °C; followed by a postfixation in 1.5% OsO_4 in phosphate buffer (1½ h); embedding in araldite (Durcupan®-Fluka).

Histo- and cytochemical methods. Demonstration of the total nerve supply with the Bodian silver impregnation method[3]; demonstration of acetylcholine acetylhydrolase (AchE, EC 3.1.1.7) and acylcholine-acylhydrolase (ChE, EC 3.1.1.8) in cryomat sections of the branchial heart, ganglion cardiacum and nervus cor branchialis with histochemical and cytochemical methods[8,9]. The substrates acetylthiocholine iodide and butyrylthiocholine iodide were used in a solution brought to isotonia by 0.6 M saccharose, pH 5.5.

Both enzymes were blocked in control reactions by 10^{-6} M diisopropylfluorophosphate (DFP) in the preincubation solutions for 30 min; only the ChE was inhibited by addition of 10^{-4} M iso-OMPA (Tetraisopropylpyrophosphoramide) in the preincubation media for 60 min and 10^{-3} M iso-OMPA in the incubation media (60–80 min).

Pharmacological methods. Juvenile and semi-adult animals (ML:8–15 cm) were put under 1–2% ethyl alcohol anesthesia whereupon the branchial hearts were taken out and bound into a Straub-cannula by means of the a. branchialis (=afferent branchial vessel). Following ligature of the afferent vein truncus the organ, without ganglion cardiacum, was brought into a protective, water vapor saturated chamber. The vein truncus ligature was connected to a pressure transducer (Statham UC 2®) and then, in a quasi-isotonic suspension and with a constant hydrostatic pressure (3 cm H_2O), the mechanogram was registered (amplifier: Sachs HSE 300®; micrograph: Kipp and Zonen®). The equipment was calibrated with Newton weights.

In addition a perfusion method was applied; for that, a double barrelled glass cannula (∅ 1,8 mm) was implanted via the afferent branchial vessel, which permitted a permanent perfusion of the branchial heart lumen with the test solutions. In this case the changing hydrostic pressure was measured with a tightly connected Statham transducer Pb23BB® and a Burster® DMS-amplifier[21]. Both methods have demonstrated equivalent results.

Filtered isotonic seawater/glucose solution (pH 7.3) at 20–22 °C (1.7 g glucose/l seawater) continously air-bubbled served as artificial blood fluid and carrier-solution for the drugs to be tested; the osmolality was regularly controlled with a Knauer Semimicro-osmometer. After each test of an agonist it was rinsed for 10–30 min or longer until the original rhythm was attained again. The calculation of each mean and SD is based on 15–20 experiments. The experiments included the following drugs: acetylocholine chloride (Ach, Sigma), muscarine (Mus, Serva), nicotine (Nic, Merck), pilocarpine (Pil, Dr Thilo Co), carbachol (Car, Doryl®-Merck), D-tubocu-

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rarine chloride (Cur, Serva), atropine (Atr, Merck), tetraethylammonium-chloride (TEA, Merck), α-bungarotoxin (α-Btx, Sigma).

Results

1) Ultrastructural aspects of the branchial heart

Differing from the ultrastructure of the central heart[18a], the branchial heart shows a more heterogenic wall construction[18b]. Numerous ovoid-polygonal interstitial cells (fig. 1) with high vesicle content, dense bodies and dictyosomes, the whole volume of which is 2–3 times greater than that of the muscle cells, are bedded in the branched network of obliquely striated muscle cells and single collagenous fibers. Their role in the organ's hemodynamics, however, is only indirect (figs. 1 and 2).

The obliquely striated muscle cells have a very markedly high content of sarcosomes and glycogenosomes. At the level of the obliquely arranged Z-patches, tubular sarcolemma invagination and interdigitations with intercalated disk-like sarcolemmal contacts are regularly observed (fig. 3). In the case of larger fibers – containing up to 6–8 myofibrils – the latter continues cell-inward as a T-system, which builds only indistinctly visible diads and triads with longitudinal sarcotubules. The polyaxonal nerve fibers mentioned earlier are found to branch intramurally (fig. 1); their runners show a close parallel approach to the muscle fibers. Near the neuromuscular synapsis, mostly groups of axons are onesidedly (towards the sarcolemma) without any myelin sheath, and make contact with crypt-like invaginations on the muscle-cell surface, resulting in a minor intersynaptical gap smaller than 200 Å. The terminally widened axons contain few mitochondria and plentiful transparent synaptical vesicles (∅ 60–100 nm) (fig. 4) or dense cored granula.

2) Histochemical and cytochemical localization of the AchE and ChE

The histochemical experiments show a strong reaction for AchE within the ganglion cardiacum, the nervus cor branchialis, the intramural nerve fibers and the muscle cells but distinctly weaker activity in the ovoid interstitial

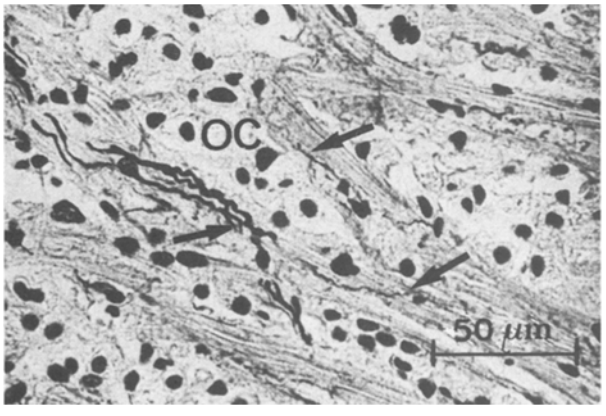

Figure 1. Part of the branchial heart wall of *Sepia* colored by the Bodian-method; terminal nerve fibers (arrows) in contact with the branched muscle cells; ovoid interstitial cells (OC).

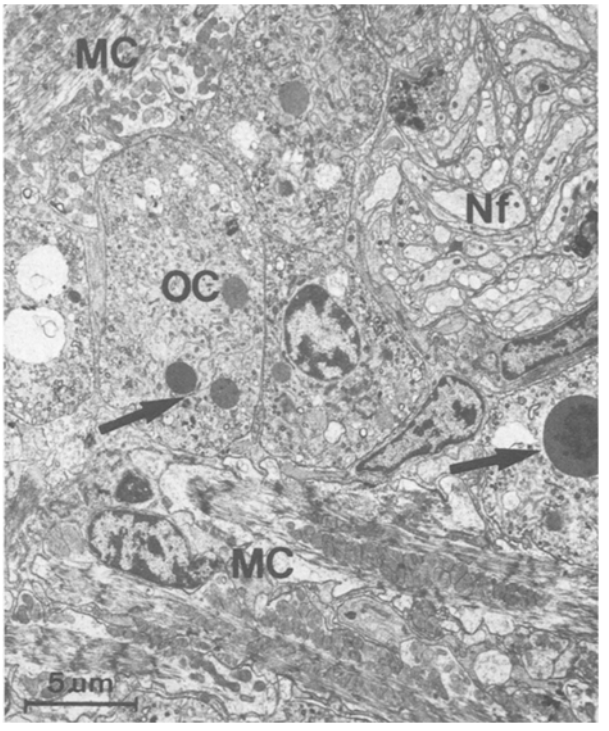

Figure 2. Section of the peripheral wall of branchial heart; between the obliquely striated muscle cells (MC) with a high content of sarcosomes a polyaxonal nerve fiber (NF) and ovoid interstitial cells (OC) with dense bodies (arrow), endocytosis vesicles and endoplasmic reticulum.

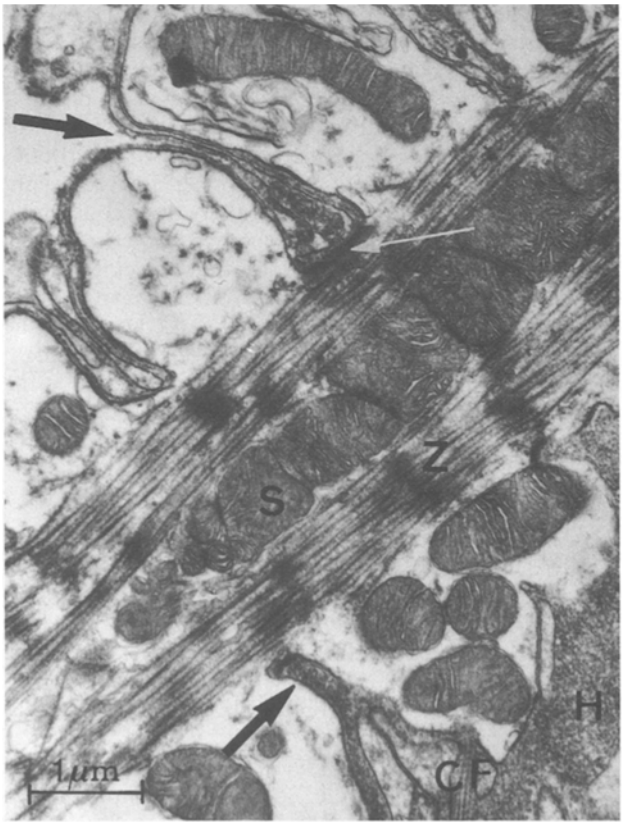

Figure 3. Part of an obliquely striated muscle cell; T-invagination and lateral interdigitations (black arrows) on the level of Z-patches (Z) with intercalated disk-like contacts of sarcolemma (white arrow); sarcosomes (S); hemocyanin (H); collagenous fibers (CF).

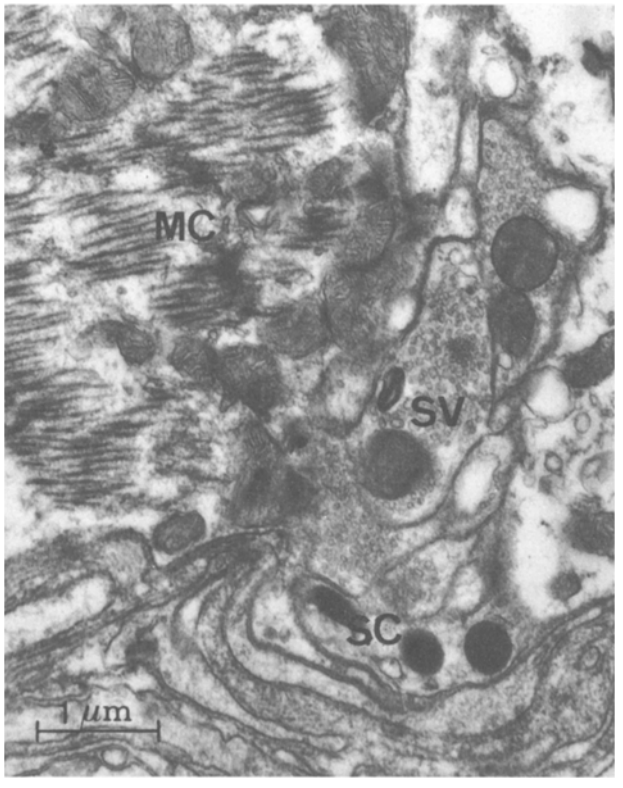

Figure 4. Neuromuscular synapse in the branchial heart wall; the terminal axons contain many transparent synaptic vesicles (SV); Schwann cell (SC).

cells and the outer epithelium, if the substrate acetylthiocholineiodide and the inhibitor iso-OMPA are used (fig. 5). All activity is inhibited after a preincubation with DFP or a preincubation with 10^{-3} M iso-OMPA combined with the substrate butyrylthiocholineiodide and 10^{-4} M iso-OMPA in the incubation medium, but weakly inhibited after an iso-OMPA preincubation and an incubation with butyrylthiocholineiodide without any inhibitor. Cytochemically the AchE activity is located above all in the sarcolemma area of the obliquely striated muscle, but also, to a certain extent, in the glycocalices and endocytosis vesicles of the ovoid cells (fig. 6). Moreover, outer axolemma and lemnoblast membranes of single polyaxonal nerve fibers lacking granulated vesicles react positively (fig. 7).

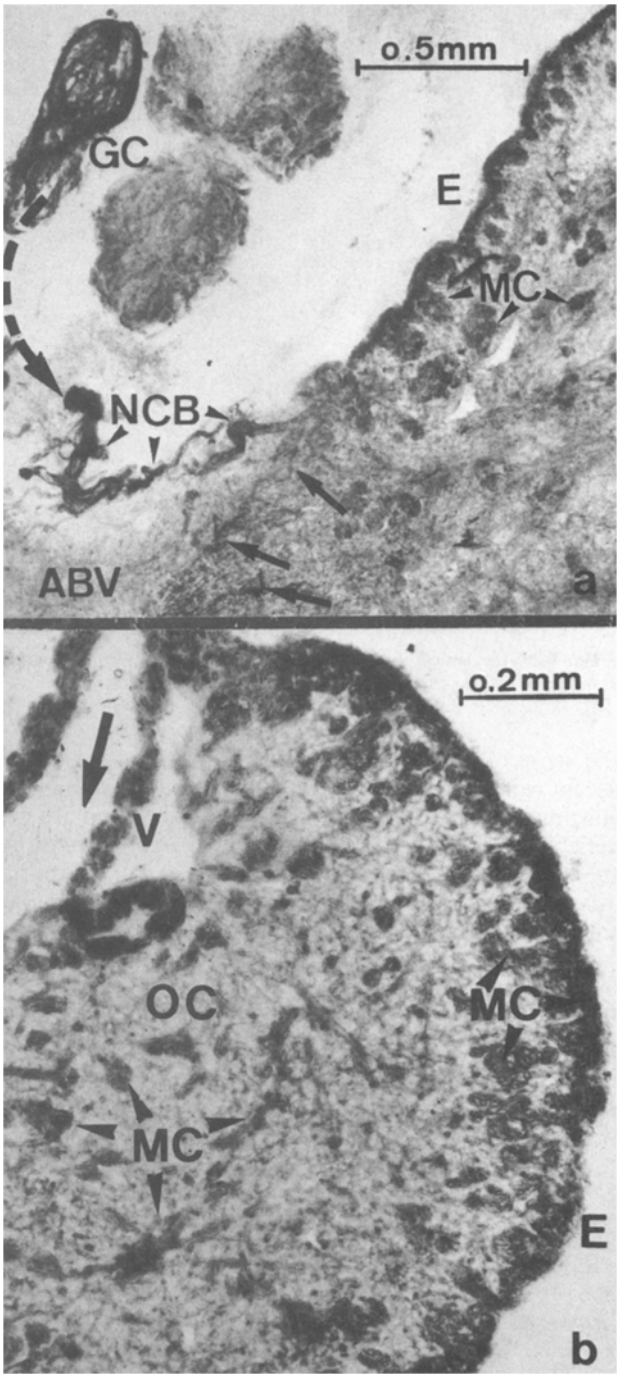

Figure 5. Positive AchE reaction (E.C. 3.1.1.7) – after pre- and incubation with iso-OMPA – within: the ganglion cardiacum (GC), its branch to the branchial heart = n. cor branchialis (NCB) – entering into the branchial heart near the insertion area of the afferent branchial vessel (ABV, interrupted arrow) –, the intramural nerves (small arrows in a) and the muscle cells (MC); the epithelium (E) and the mass of ovoid cells (OC) show no, or only weak reactions; valvulae (V) at the entrance of V. cava (= renal pole, big arrow in b).

3) Pharmacological classification of the cholinergic receptor

a) The effect of acetylcholine and some mimetic drugs According to the morphological peculiarities, the isolated, pharmacologically uninfluenced branchial heart of *Sepia officinalis* does not always present, compared to the systemic heart and notwithstanding constant experimental conditions, a very regular actogram. Periodic fluctuations of the muscle tonus are frequent, not seldom with changing frequencies and amplitudes. In addition, the organs display differences, individual and due to age, in their normal activity which must be considered when evaluating the pharmacological reaction.

Acetylcholine generally induces a negative inotropic, with rising doses also an increasing negative chronotropic effect. A concentration as low as 5.5×10^{-10} M already leads to a slight amplitude reduction, whereas after 2–3 more strongly inhibited contractions, in many cases, in

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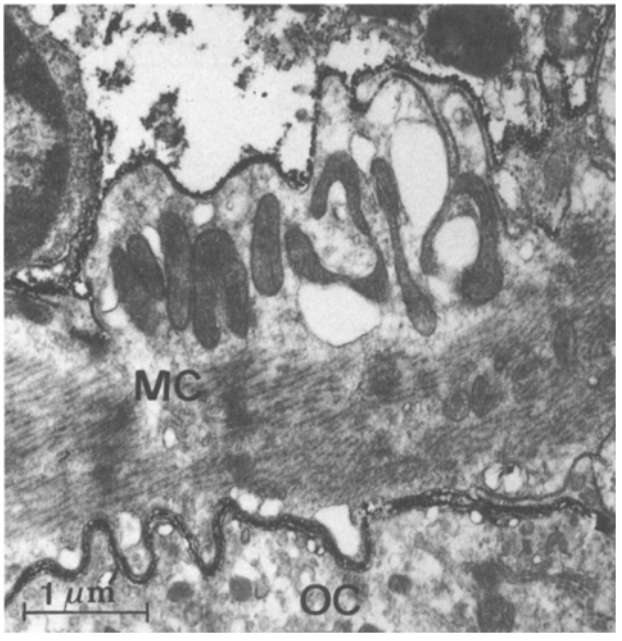

Figure 6. Cytochemical reaction of the AchE in the glycocalices of the muscle cell (MC) and ovoid interstitial cell (OC) (unstained section).

the sense of a post-inhibitory overshoot, an amplitude ascent can again follow, temporarily even exceeding the starting amplitude. Inhibiting effects of this kind are reversible up to ED 50 ($\sim 10^{-5}$M±); the sigmoid path of the concentration-response curve (figs. 8 and 9), however, ascends steeply within this range, consequently even the smallest further rise of dosage leads to extreme inhibiting

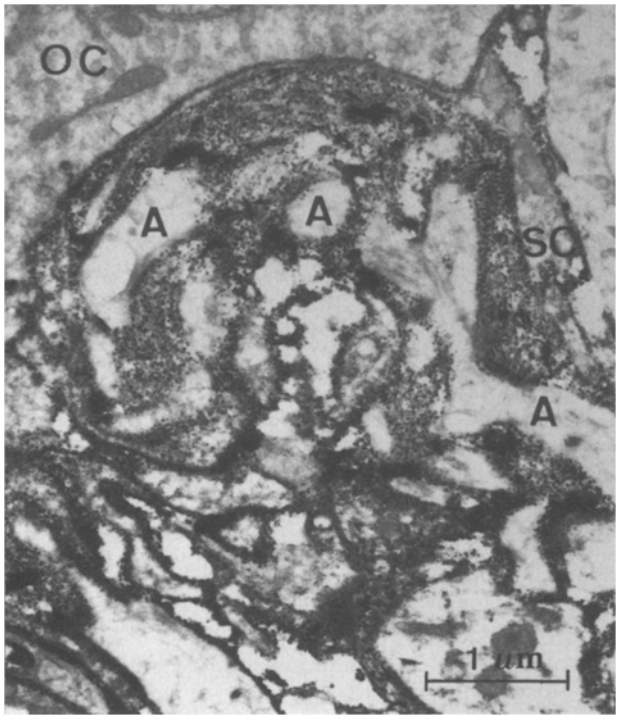

Figure 7. Reaction of the AchE in a terminal polyaxonal nerve fiber; axons (A); Schwann cell (SC); ovoid interstitial cell (OC) (unstained section).

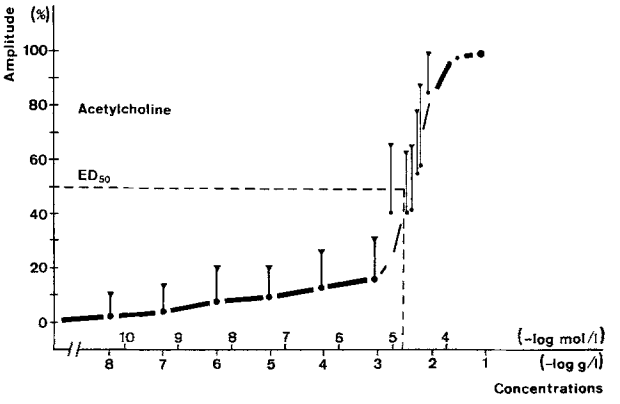

Figure 8. The effect of Ach on the frequency of the branchial heart.

effects – at 5.5×10^{-5} M a partial standstill – after which the organ, despite repeated washing out, does not return to its original activity. Significant for the individual reaction of the different organs are the relatively high standard deviations, especially in this curve area.

Nicotine induces a similar reaction, so that accordingly the concentration-response curves resemble each other in their course (figs. 10 and 11).

The affinity towards the receptor is, however, 10^1 times higher than that of Ach. Concentrations of 6×10^{-11} M already lead to a weak negative inotropic effect, doses of 6×10^{-6} M, on the other hand, to diastolic standstill, and even higher concentrations (6×10^{-5} M) to standstill with irreversible organ damage.

Muscarine, shows 10^{-3} to 10^{-4} times less intrinsic activity and affinity towards the receptor by showing a weak negative inotropic action (fig. 12) and hardly a chronotropic one at first, at concentrations between 5×10^{-6} and 10^{-5} M. Even unphysiologically high doses above 5×10^{-4} M ($=10^{-1}$ g/l) do not result in a complete standstill of the organ (figs. 12 and 13).

Carbachol was only tested in some single experiments at concentrations of 4×10^{-7} to 10^{-5} M; it also acts as a cholinomimetic, whereby – as with Mus – the negative inotropic reaction is stronger than the chronotropic effect. Concentrations higher than 4×10^{-4} M lead to diastolic standstill and irreversible organ damage.

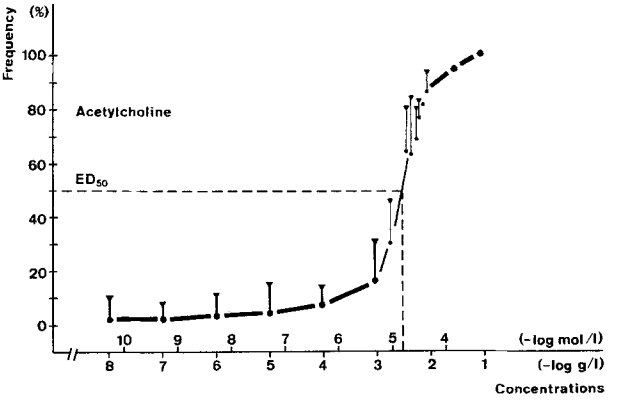

Figure 9. The effect of Ach on the amplitude of the branchial heart.

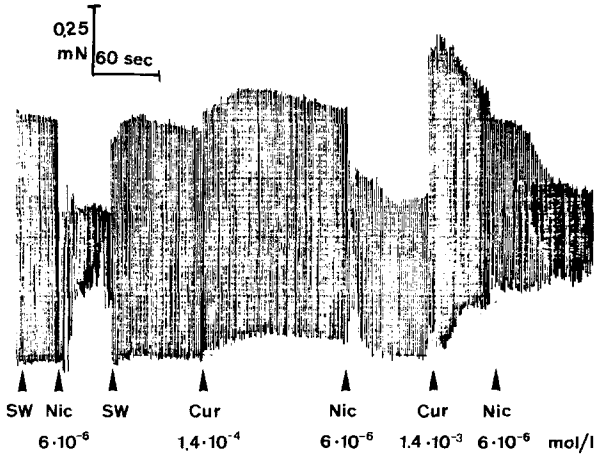

Figure 10. The effect of Nic on the branchial heart and its blocking by Cur; seawater-glucose solution (SW).

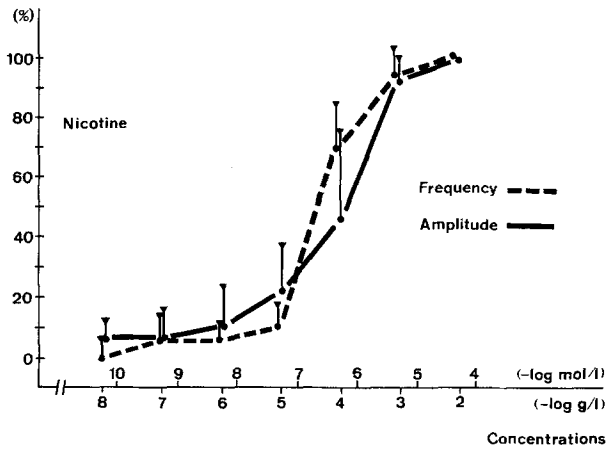

Figure 11. Concentration-response curves for Nic.

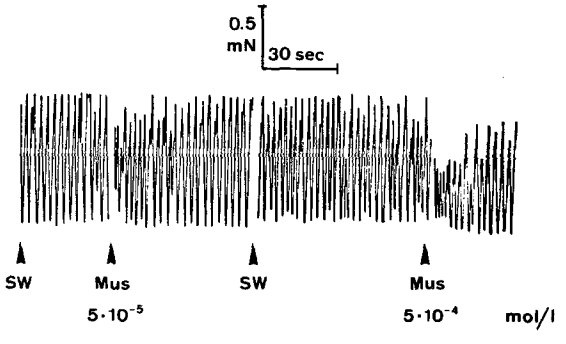

Figure 12. Mus shows, only in very high doses, a weak inotropic but no chronotropic effects; seawater-glucose solution (SW).

Pilocarpine was also only tested in few single experiments; similar to Car it primarily reduces the amplitude at concentrations from 5.5×10^{-9} to 10^{-6} M, while the frequency rises slightly and the phase of diastolic repolarization is shortened with rising concentrations.

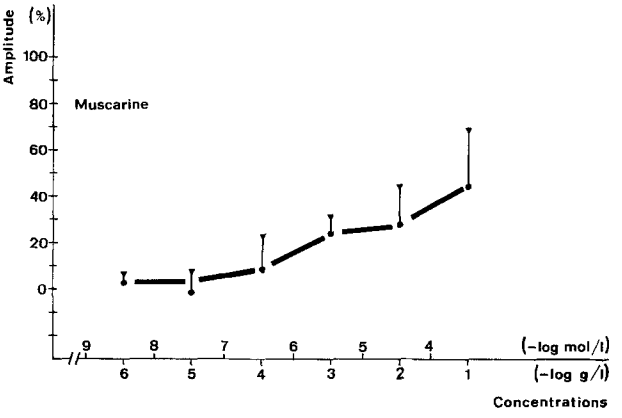

Figure 13. Concentration-response curve for Mus, plotted as a percentage of the maximal response to the reference agonist (Ach).

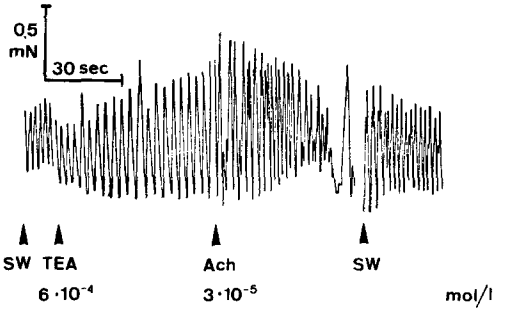

Figure 14. Blocking trial of Ach by TEA; seawater-glucose solution (SW).

b) The effect of acetylcholine-blocking drugs

Atropine seems to have a certain intrinsic activity on the isolated branchial heart of *Sepia;* concentrations between 3.5×10^{-7} and 3.5×10^{-6} M change the organ's basic tonus, whereby due to a strengthened diastole the amplitude rises as a whole, the frequency however sinks. Inhibition of the Ach effect (5.5×10^{-5} M) with 3.5×10^{-5} M can only be obtained to a small degree.

Curare (D-tubocurarinechloride) possesses only a slight positive inotropic effect. A stable receptor block for a period of several minutes can be attained at a concentration of 1.4×10^{-5} M in comparison to Ach (5.5×10^{-6} M) and Nic (6×10^{-6} M) (fig. 10).

Tetraethylammonium, on the other hand, obviously shows a much smaller affinity towards the receptor. Although the drug's effect also appears merely as a weak positive inotropic reaction, it is not possible to bring about any other similar type of stable receptor block. Concentrations of 6×10^{-4} M block the effect of Ach (3×10^{-5} M) and Nic (6×10^{-6} M) weakly and for a short time only (fig. 14 and 15).

α-Bungarotoxin, applied in concentrations of 10^{-6} M for 10–13 min, has not an own intrinsic activity on the organ. Added in a concentration of 10^{-6} M it completely blocks the effect of normally toxic concentrations of 5.5×10^{-5} or 2.8×10^{-4} M Ach (fig. 16).

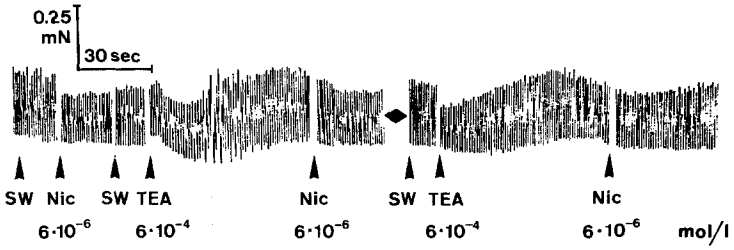

Figure 15. The blocking of the Nic effect by TEA; seawater-glucose solution (SW).

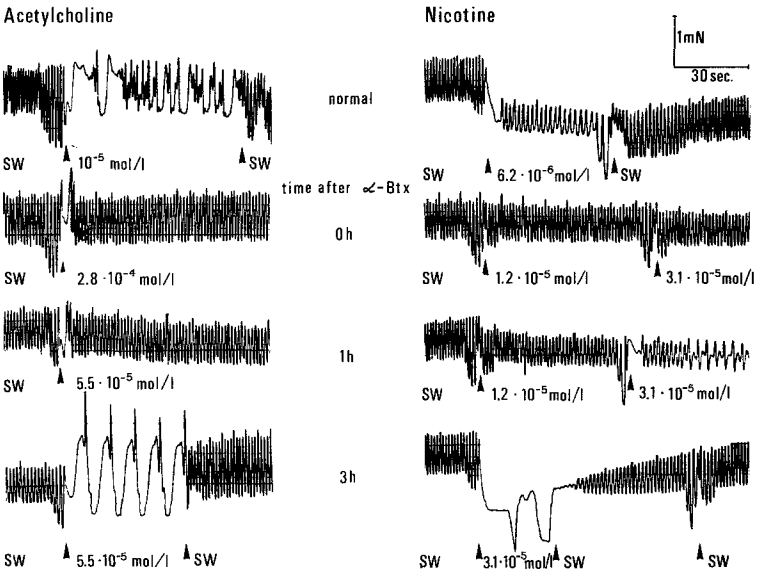

Figure 16. Effects of Ach and Nic on the activity of a branchial heart before and 0 h, 1 h and 3 h after application of 10^{-6} M α-Btx.

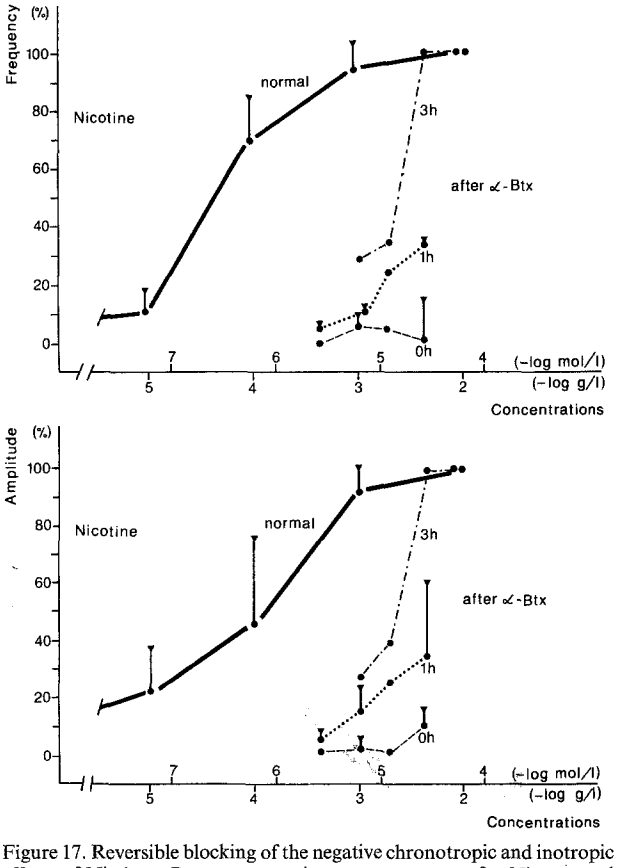

Figure 17. Reversible blocking of the negative chronotropic and inotropic effects of Nic by α-Btx; concentration-response-curves for Nic: normal and 0–3 h after incubation with 10^{-6} M α-Btx.

The blockade of the effect of Ach and Nic, however, only persists for 3 h. After 1 h the inhibitory effect of 1.2×10^{-5} M Nic on amplitude and frequency is only blocked to 80%, that of 3.1×10^{-5} M to 62 resp. 51% (fig. 17a and b).

Discussion

The branchial heart of the dibranchiate cephalopods has a double function. Its hemodynamic task[7,19,21,24] is to maintain the blood circulation in branchia and branchial heart appendages. Further, it is active in catabolic processes and in the storage of excreta and reserve substances, the latter being coupled to the above (e.g. $Cu^{++}$and Fe^{+++})[20,22].

In the course of physiological-pharmacological examinations about the motoricity of the isolated branchial heart, it must be taken into account that – in contrast to corresponding experiments on the central heart[12] – we do not have a pure nerve-muscle preparation but, as proved by our electron-microscopic findings, a hollow muscle which is to a great extent mixed with ovoid interstitial cells[18b,21] equipped with a very efficient enzyme inventory[19]. Although the enzymes primarily carry out the catabolic functions mentioned, it must be taken into account that, during the pharmacological experiments presented, the enzymes also acted as an interfering factor whose effects could not always be calculated. The obliquely striated branchial heart muscle, however, does not show any significant differences in its ultrastructure

compared to central heart muscle[18a,b]. The high sarcosome content and the development of a T-system are remarkable facts. A specific pacemaker tissue has not been differentiated[18b].

Because of the numerous intramural nerve fibers it can be supposed that every muscle cell is reached via the motor neurons from the lobus visceralis or the ganglion cardiacum. The neuromuscular synapse is reminiscent in many cases of the motor endplate in vertebrates, groups of naked axons being apparently firmly anchored in crypt-like infoldings in the sarcolemma; therefore, during the various contraction phases, there is always a tight synaptical contact. A double innervation as in vertebrates is more than likely; evidence comes from the presence of granulated and nongranulated vesicles in axons and different terminal nerve fibers, and is also provided by our histo- and cytochemical findings. The cholinergic examined in the course of this study is demonstrated by the histo- and cytochemical localization of the structure-bound acetylcholin-acetylhydrolase (AchE, EC 3.1.1.7), whereas the ChE (EC 3.1.1.8) is found to a distinctly lesser extent. The AchE was found to react strongly not only in single nerve fibers but also and above all in the glycocalyx and in coated endocytosis vesicles of muscle cells but weaker in the ovoid interstitial cells or round cells[22].

It has to be assumed that during the pharmacological experiments, even on the longer-isolated organ, the natural terminal axon neurotransmitters and their catabolic enzymes, especially the high content of AchE (EC 3.1.1.7), act at the same time. This provides an explanation for the frequently observed variability of intensity and length of the branchial heart reaction following the addition of a certain drug.

The pharmacological findings on the isolated organ prove the existence of a cholinergic receptor system in the obliquely striated muscle of the branchial heart and are in agreement with earlier in vivo experiments concerning this organ[24]. The receptor excitation due to Ach is like that seen with the central heart[12,13]; eventually, at concentrations higher than 10^{-9} M, it leads to negative chronotropic and inotropic effects, i.e. by means of a comparatively strong K^+-permeability leading to a dose-dependent inhibition of the branchial heart. Nic, as already demonstrated on the central heart (ED 50 5×10^{-7} M) presents the strongest effect among the applied agonists and proves itself, to be more than ten times more effective than Ach (ED: $> 10^{-5}$ M) with respect to affinity towards the receptor as well as its intrinsic activity. Mus possesses a distinctly weaker effect than Nic. An inhibition equivalent to ED 50 of Ach can only be attained near unphysiologically high concentrations of 10^{-3} M and more. As far as the systemic heart is concerned, however, even such concentrations have no inhibiting effect[12].

The mimetics, Car and Pil, with which supplementary tests were done in a few single experiments, act on the branchial heart receptors in the same way. Car, however, as proved on the systemic heart, appears to have a higher intrinsic activity and receptor affinity than Pil, the latter producing weak negative inotropic effects but hardly chronotropic effects.

The blocking experiments carried out for the purpose of more exact determination of the cholinergic receptor prove that Atr, which possesses a weak positive inotropic effect, is not able to counterbalance the effect of Ach at a concentration of 3.5×10^{-5} M, whereas Cur (1.4×10^{-5} to 10^{-4} M), with little positive inotropic or tonotropic effect, stably blocks not only the inhibiting effect of Ach (5.5×10^{-6} M) but also that of Nic (6×10^{-4} M) for several minutes. Comparable concentrations of TEA (6×10^{-4} M), however, block only weakly the inhibiting effects of Ach (3×10^{-5} M), and Nic (6×10^{-6} M). In vertebrates α-Btx reduces the binding capacity of the motor endplate and ganglionic receptors for Ach and Nic[4,5,10] in a similar way to Cur. In the branchial heart of *Sepia* we can also see that this α-neurotoxin, at a concentration of 5×10^{-6} M, completely blocks the effects of normally-toxic concentrations of Ach (2.8×10^{-4} M) and Nic (3.1×10^{-5} M). However, in contrast to its effect on the above-mentioned vertebrate tissues and in agreement with results on nicotinic receptors in *Aplysia* neurones[11,14], the binding seems to be reversible. It can be concluded that the cholinergic receptor in the obliquely-striated branchial heart muscle resembles more the nicotinic receptor type in vertebrates; the fact that the receptor can primarily be blocked by Cur and α-Btx but only to a smaller degree by TEA, points out structural similarities to the receptor of the motor endplate. But, considering that Atr has a weak blocking effect also, and Mus likewise has a weak agonistic effect, opposite to that seen on the central heart[12], the conformity between the two receptor types is only relative. Above all, it is probable that although the cephalopods are highly organized, receptor differentiation does not exist to the same extent as in vertebrates.

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Short Communications

The gross morphology of the stinging and non-stinging states of the ant *Tetramorium caespitum* L. (Hymenoptera, Formicidae, Myrmicinae)

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Summary. The change in sting morphology of *Tetramorium caespitum* is described in detail. The sting-tip may protrude/retract from the sting-ending during its stinging/non-stinging process respectively. A possible trail laying function of the stings terminal membranous sheath is proposed.
Key words. Sting; *Tetramorium caespitum;* ant; trail laying.

Tetramorium caespitum is the only endemic species of the genus *Tetramorium* in Britain. It is a small and aggressive ant, the workers being individually tough and persistent. The main method of fighting involves three stages, the attachment of the workers mandibles to the appendages of the enemy; the ventral arching of the gaster and the insertion of the sting into the joints[1]. Once sting penetration is achieved the venom, predominantly proteinacious in nature[2], is injected. Detailed investigations of the sting structure and mechanism of several formicine genera have been reported[3,4]. We present here a study of the changes occurring in the sting during the act of stinging based upon scanning electron and light microscopic studies.

Materials and methods. Workers of *T. caespitum* were obtained from heathland surrounding Furzebrook Research Station, Wareham, Dorset. To obtain the stinging position workers were irritated by aggressive handling whilst the non-stinging position was achieved by anesthetizing live specimens with CO_2. In both cases the gaster was removed and examined. For light microscopical studies the gasters were fixed for 24 h in alcoholic Bouin's fixative, dehydrated in a graded series of ethanol solutions, and mounted on slides in Damar. Stains were not employed on the specimens. Using this procedure the Dufour's gland was not observed in the whole mount preparation (fig. 1). Scanning electron microscopy studies were performed on stings individually mounted on specimen stubs and coated with gold. Samples were observed using a Cambridge stereoscan electron microscope (Model S600). In some cases the sting lancets were removed from the stings, in their stinging position, to ensure that the sting-tip (ST) was the distal end of the protruding sting lancet. Observations were chiefly made on the distal sting-ending.

Results and discussion. As is typical of formicine ants the gaster of *T. caespitum* is elongate, 1.20–1.35 mm long and 0.56–0.59 mm in diameter (fig. 1). The venom gland (VG) which is approximately spherical with a diameter of 0.28 mm, extends 0.32 mm anteriorly from the sting bulb via the main duct. The Dufour's gland is smaller than the venom gland being 0.16 mm long and 0.04 mm in diameter. This bulbous gland lies anterior to the sting bulb and ventral to the main duct. The sting (S), hidden by the 7th abdominal segment, may extend from its fully retracted position up to 0.3 mm from the posterior tip of the gaster, at its thickest its diameter is 30 µm. The sting curves slightly downwards at its anterior end and sclerotization is extensive. The poison canal (PC) from the sting bulb to the sting-ending decreases in diameter and at its termination it is 1.5 µm. A triangular membranous sheath (TS) is attached to the distal sting-ending (fig. 2), this extends dorsally to 70 µm perpendicular to the sting. The sting-ending viewed dorsoposteriorly is super-