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Myocardial Depolarizing Response to Glutamate in the Myogenic Heart of the Branchiopod Crustacean *Triops longicaudatus*

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ABSTRACT—Fine structure of the heart and the effects on the heartbeat of some transmitter candidates in crustacean cardioregulatory system were examined in the myogenic heart of the branchiopod crustacean *Triops longicaudatus*. Electron microscopy revealed that, in each myocardial cell, myofibrils are confined in the part facing the epicardium and intercalated disks are present between the myofibrillar regions of adjacent myocardial cells. No neural elements were found in the heart, suggesting lack of extrinsic cardioregulatory nerves from the central nervous system. Gamma aminobutyric acid and acetylcholine produced no detectable changes in the myogenic activity of the heart at concentrations up to 10^{-3} M, respectively. Glutamate induced a depolarizing membrane response in the cardiac muscle with a threshold concentration of approximately 1×10^{-5} M. The amplitude of the depolarizing response was concetration-dependent and saturated at approximately 1×10^{-4} M. The myogenic activity of the heart increased in frequency with glutamate of less than approximately 3×10^{-5} M. With higher dose of glutamate, action potential adaptation occurred in the cardiac muscle and the heart exhibited a systolic arrest.

INTRODUCTION

The neurogenic heart of many crustaceans is regulated by the central nervous system via bilateral cardioregulatory nerves each of which contains inhibitory and acceleratory nerve fibers (reviewed by Maynard, 1960, 1961; Hagiwara, 1961). These nerve fibers make synaptic contacts on the cardiac ganglion neurons or both the cardiac ganglion neurons and the myocardium, regulating the frequency, force and tonus of the neurogenic heartbeat (reviewed by Tanaka *et al*., 1996). Mainly on the basis of pharmacological experiments, gamma-aminobutyric acid (GABA) is suggested commonly in several crustaceans to be a transmitter of the cardioinhibitory nerve and some other substances, acetylcholine (ACh), glutamate and dopamine, are designated as the transmitter candidates of the cardioacceleratory nerve (reviewed by Cooke, 1988; Yazawa and Kuwasawa, 1992). However, phylogenetic diversity of the cardioregulatory system in crustaceans have not been investigated thoroughly (e.g. Hill and Kuwasawa, 1992).

On the basis of morphological observations that found no neurons in the heart, the heart of branchiopods, one of the lower orders within the Crustacea, has been suggested to be myogenic (reviewed by Krijgsman, 1952; Maynard, 1960; Prossor, 1973). Recently, Yamagishi *et al*. (1997) showed electrophysiologically that the heart of *Triops longicaudatus* is myogenic and the heartbeat occurs according to endogenous activity of the myocardium. Based on the observations that ACh applied externally to the body produces inhibitory effects on the heartbeat, cholinergic nervous regulation has been suggested in the heart of *Daphnia* (Baylor, 1942; Bekker and Krijgsman, 1951). However, no direct evidence for extrinsic nervous regulation has been reported in the myogenic heart of branchiopods.

We therefore examined the possibility of extrinsic nervous regulation in the myogenic heart of *Triops longicaudatus* using electron microscopic and electrophysiological methods. We also examined the effects on the myogenic activity of the heart of some substances (GABA, ACh, glutamate) designated as neurotransmitter candidates in crustacean cardiac and cardioregulatory systems. The results show that the *Triops* heart may not be innervated by extrinsic cardioregulatory nerves from the central nervous system; in addition the myogenic activity of the heart is affected by glutamate but not by GABA and ACh.

MATERIALS AND METHODS

Animals

Over 80 adult specimens of the fresh water tadpole shrimp *Triops longicaudatus* (Branchiopod, Crustacea), 15 to 20 mm in body length, were used. Collection and maintenance of the animals were described previously (Yamagishi, *et al*., 1997).

Preparations and electrophysiology

The heart is a long tubular organ situated at the dorsal side of the thorax. Gross anatomy of the heart was detailed in a previous

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paper (Yamagishi *et al.*, 1997). The heart was isolated together with the dorsal carapace. The preparation was fixed, ventral side up, in the experimental chamber by pinning the dorsal carapace keeping the heart intact. In some cases, the heart isolated completely from the body was used. The preparation was continuously perfused with aerated physiological saline solution throughout the experiments. Based on the ionic composition of the hemolymph of *Triops longicaudatus* (Horne, 1966), the saline had the following composition (mM): NaCl 75, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, Tris-HCl (pH7.4) 5 (Yamagishi, *et al.*, 1997). The experiments were performed at a temperature of 20 to 24°C.

Membrane potential of the cardiac muscle cells was recorded using a conventional glass capillary microelectrode filled with 3 M KCl (10–30 MΩ). The data were stored in an FM magnetic tape recorder and displayed on a chart recorder. A heart rate counter (Nihon Koden AT 601G) was used to determine the instantaneous frequency of the muscle action potential.

All the chemicals used in this study, gamma aminobutyric acid (GABA), acetylcholine (ACh) and glutamate were obtained from Wako Pure Chemicals. Each of the chemicals was dissolved in the saline solution just prior to experimentation and applied to the preparation by changing the perfusing saline from normal saline to chemical-containing saline.

Electron microscopy

The heart was isolated together with the dorsal body wall and prefixed for 1 h at 4°C with 2.5% glutaraldehyde in physiological saline solution. After washing, the specimen was postfixed for 1.5 hr at 4°C with 2% osmium tetroxide in distilled water and dehydrated through a graded ethanol series (50 to 90%). After en bloc staining with saturated uranyl acetate in 95% ethanol for 1.5 hr, the specimen was dehydrated with absolute ethanol, transferred to n-butyl glycidyl

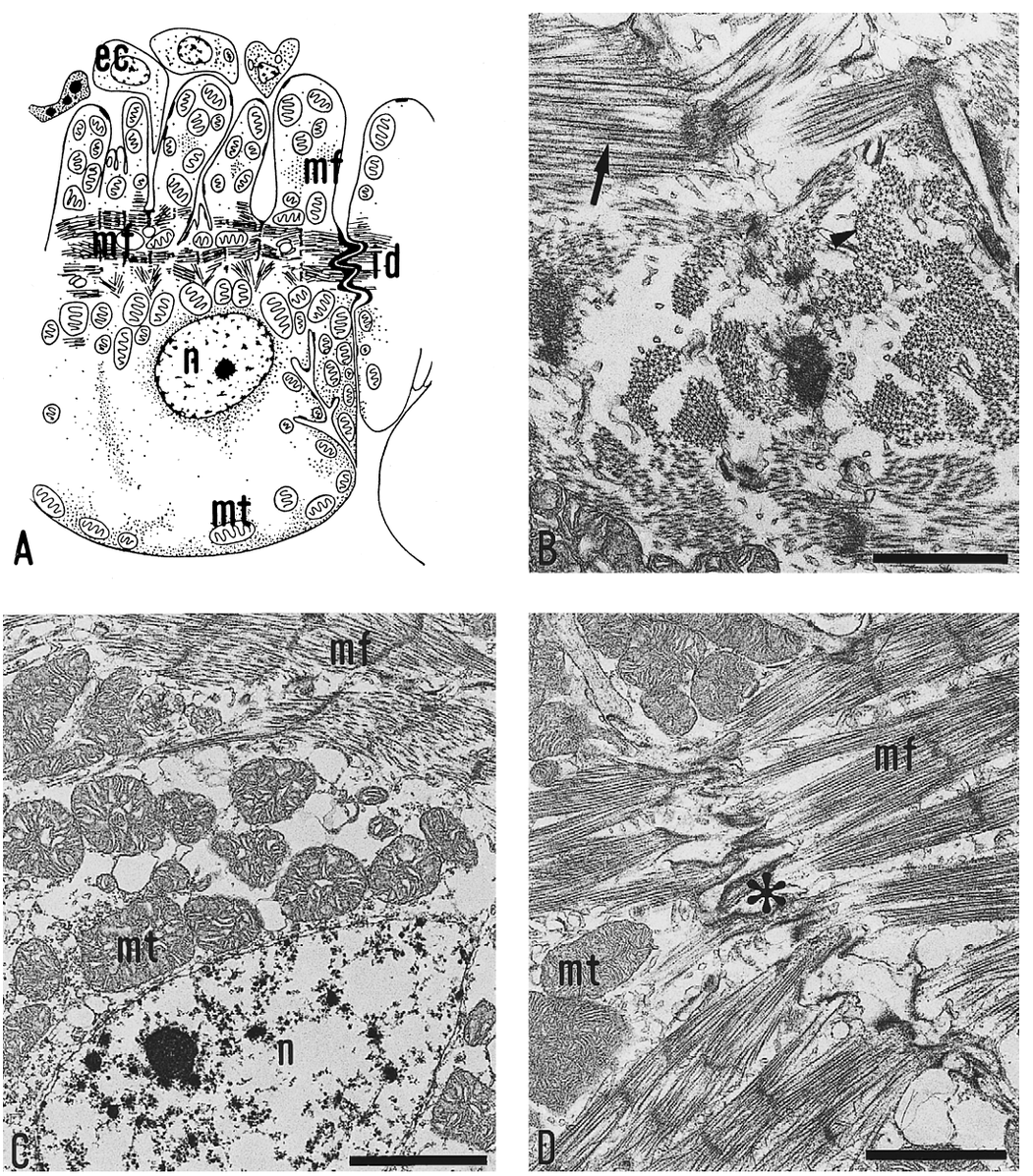

Fig. 1. Schematic drawing (A) and electron micrographs (B–D) of transverse section of the myocardial cells. (B) Myofibrils cross each other (arrow and arrowhead) in the myocardial cell. (C) Nucleus of a myocardial cell. (D) Intercalated disc (asterisk) between adjacent myocardial cells. Abbreviations: ec, epicardial cell; id, intercalated disc; mf, myofibrils; mt, mitochondorion; n, nucleus. Scale bars, 1 μm (B) and 2 μm (C, D).

ether (QY-1), and then embedded in epoxy resin (Agar 100). Ultrathin sections were cut with a diamond knife. The sections were stained in a saturated uranyl acetate solution and examined with a JEOL JEM-1010 transmission electron microscope at 80 kV.

RESULTS

Fine structure of the heart

The heart of *Triops longicaudatus* consists of a single layer of myocardial cells (Yamagishi, *et al.*, 1997). Electron microscopically revealed fine structure of the myocardial cells are summarized in Figure 1A. The epicardium is composed of a rough layer of epicardial cells (A). In each myocardial cell, myofibrils are long striated, and localized at the epicardial side of the cell and cross each other forming a contractile network (B). Many large mitochondria are present around myofibrils. A nucleus is located at the luminal side of the myocardial cell (C). Intercalated discs characterized by a high electron dense and interdigitating structure are present between the myofibrillar regions of adjacent myocardial cells (D). We could detect no neural elements in the heart.

Effects of GABA, ACh and glutamate

The heartbeat of *Triops longicaudatus* is myogenic and each beat occurs in association with a slow depolarizing potential (action potential) of the cardiac muscle (Yamagishi, *et al.*, 1997). Under perfusion with normal saline in the preparations used, the action potential was in the range from 150 to 230 min^{-1} in frequency and from 13 to 25 mV in amplitude.

Both GABA and ACh produced no detectable changes in the myogenic activity at concentrations up to 10^{-3} M, respectively (n=12) (data not shown).

We next examined the effects of glutamate on the myogenic activity of the heart (Fig. 2). Glutamate of a concentration 1×10^{-5} M produced no definite changes in the myogenic activity of the cardiac muscle (A). Glutamate of higher concentrations (B, 2×10^{-5} M; C, 3×10^{-5} M) induced depolarizing membrane response in the cardiac muscle; maximum membrane potential in the myogenic activity decreased (B, 1 mV; C, 4 mV) and the frequency of the muscle action potential increased (B, 19% , from 160 to 187 min^{-1}; C, 32%, from 161 to 213 min^{-1}). With increasing the concentration of glutamate (D, 4×10^{-5} M; E, 5×10^{-5} M; F, 1×10^{-4} M), the amplitude of the depolarizing response was larger (D, 9 mV; E, 13 mV; F, 23 mV). In association with the membrane depolarization, the action potential of the cardiac muscle was successively smaller in amplitude and then vanished completely. During the depolarizing response, the heart exhibited a systolic arrest (visual observations). After washout of glutamate, the membrane potential of the cardiac muscle recovered gradually and the myogenic activity was restored. The depolarizing response of the cardiac muscle to glutamate was also obtained in the heart preparations isolated completely (n=3), which showed direct

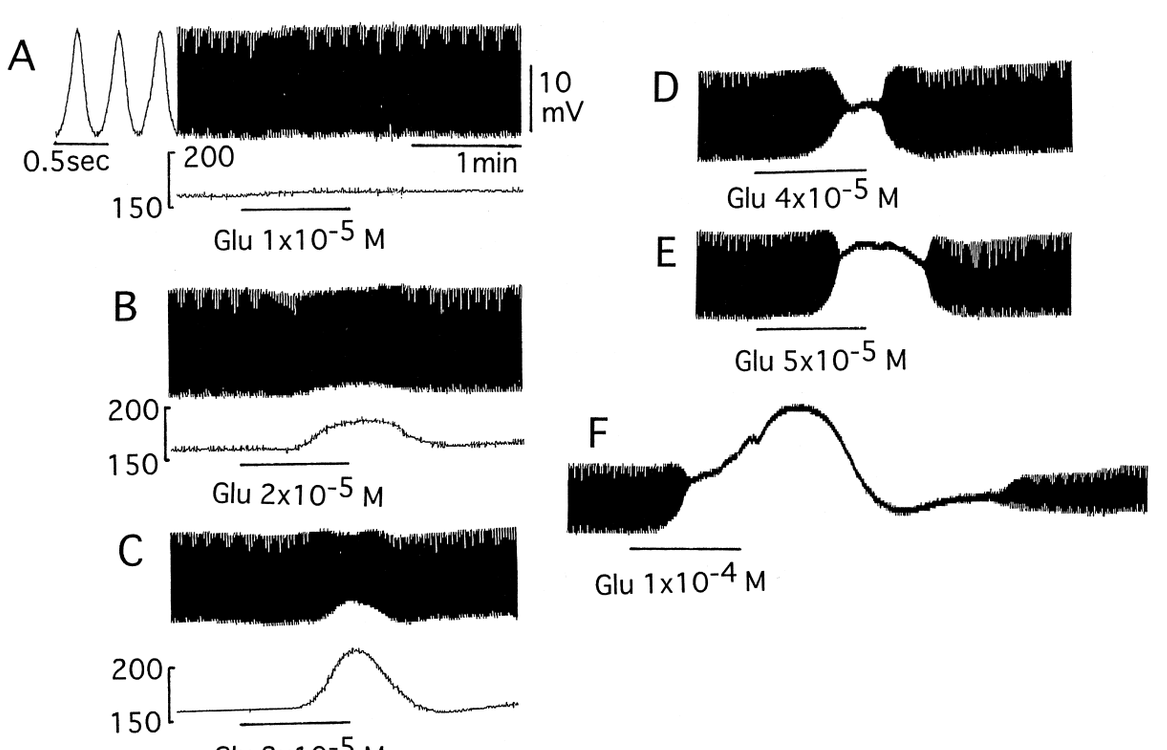

Fig. 2. Effects of glutamate on myogenic activity of the heart. In A, B and C, intracellular activity of the cardiac muscle (upper trace) and frequency of the cardiac muscle activity (lower trace) are shown. In D, E and F, only intracelluar activity of the cardiac muscle is shown. Glutamate was applied during the period (1min) indicated by the horizontal bar under each record. Glutamate concentration; (A) 3×10^{-5} M, (B) 4×10^{-5} M, (C) 5×10^{-5} M, (D)1×10^{-4} M. Note the different time scale in the left portion of the trace in A.

effects of glutamate on the heart.

Figure 3 shows a dose-response relationship between glutamate and the depolarizing response. Each amplitude (mean±sem, n=6–18) of the depolarizing response of the cardiac muscle was plotted against each concentration of glutamate applied. The threshold concentration of glutamate to induce the response ranged from 1 to 2×10^{-5} M. The response was larger in amplitude with increasing the concentration of glutamate and almost saturated at approximately 1×10^{-4} M.

The membrane potential response of the cardiac muscle to glutamate was further examined in the heart preparations that had become quiescent after perfusion for a long time (several hours) (n=5) (Fig. 4). Glutamate induced a depolarizing membrane response in the cardiac muscle with a threshold concentration of 1 to 2×10^{-5} M (A and B). With increasing the concentration of glutamate (B, 2×10^{-5} M; C, 5×10^{-5} M), the amplitude of the depolarizing response was larger (B, 5 mV: C, 15 mV). Ordinarily, no myogenic activity appeared during the depolarizing response.

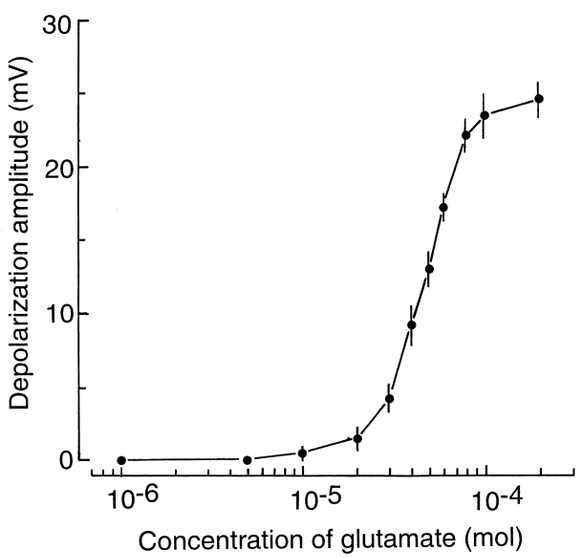

Fig. 3. Dose-response relationship between glutamate and the muscle response. Values are means±SEM (n=6–18).

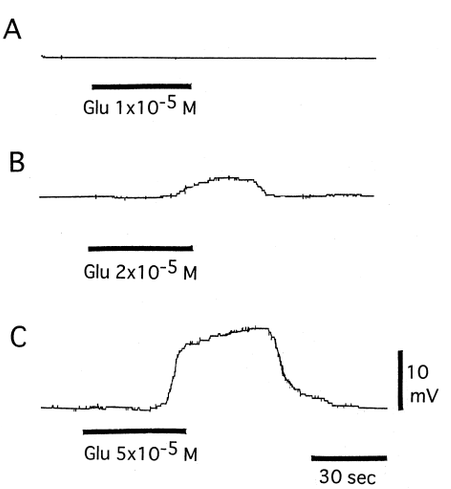

Fig. 4. Effects of glutamate on the myocardium in a quiescent heart preparation. Membrane potential recorded intracellulary from the cardiac muscle is shown in each record. Resting potential of the cardiac muscle was –48 mV. Glutamate (Glu) was applied during the period (40 sec) indicated by the horizontal bar under each record. Glutamate concentration; (A) 1×10^{-5} M, (B) 2×10^{-5} M, (C) 5×10^{-5} M.

DISCUSSION

Electron microscopy confirmed a previous light microscopic observation (Yamagishi *et al.*, 1997) that the heart of *Triops longicaudatus* consists of a single layer of polarized myocardial cells. Moreover it revealed that intercalated disks are present between the myofibrillar parts of adjacent myocardial cells (Fig. 1). These morphological features of the *Triops* heart are similar to those reported in another tad pole shrimp *Lepidurus arcticus* (Tjønneland *et al.*, 1980).

The crustacean heart is generally innervated by extrinsic cardioregulatory nerves from the central nervous system (reviewed by Maynard, 1961). Despite using a vital staining method with methylene blue, we could not find any extrinsic nerves in the *Triops* heart (data not shown). We also failed to detect any neural elements in the heart with light (Yamagishi *et al.*, 1997) or electron microscopy (cf. Fig. 1). Electron microscopy of the heart has been performed in several branchiopods and no neural elements were found in the heart (in *Daphnia pulex*, Stein *et al.*, 1966; Steinsland, 1982; in *Lepidurus arcticus*, Tjønneland *et al.*, 1980; in *Branchinecta paludosa*, *Artemia salina*, *Banchipus schaefferi* and *Streptocephalus* sp., Økland *et al.*, 1982). The heart of branchiopods may not be innervated by cardioregulatory nerves from the central nervous system.

GABA had no effects on the myogenic activity of the *Triops* heart suggesting lack of the GABA receptor in the myocardium. In many crustaceans, GABA is suggested in common as a transmitter of the cardioinhibitory nerve (reviewed by Cooke, 1988; Yazawa and Kuwasawa, 1992; McMahon, 1995). Moreover, the cardioinhibitory nerve of some isopods innervates both the cardiac ganglion and myocardium and the cardiac muscle is suggested to have the GABA receptor (Delaleu and Holley, 1976; Tanaka *et al.*, 1992 ; Mori *et al.*, 1997).

ACh had no effects on the myogenic activity of the *Triops* heart. ACh has been known to accelerate the neurogenic heartbeat of crustaceans (reviewed by Krijgsman, 1951; Maynard, 1960). In several branchiopods, the effects of ACh on the heartbeat were examined by immersing whole animals in solution containing ACh. ACh causes a decrease in the frequency of the heartbeat of *Daphnia magna* and *Daphnia pulex* (Baylor, 1942; Bekker and Krijgsman, 1951), but no effects on the heartbeat of *Artemia salina* and *Eubranchipus serratus* (Prosser, 1942). From the effects of ACh, it has been thought that the heart of *Daphnia* is innervated by cholinergic cardioregulatory nerves but those of *Artemia* and *Eub-*

ranchipus are not (Prosser, 1942; reviewed by Krijgsman, 1952). However, in the results on the *Daphnia* heart, the possibility of humoral effects on the heartbeat induced by indirect application of ACh can not be excluded. Further investigation on nervous regulation of the *Daphnia* heart is required.

Glutamate induced a depolarizing membrane response in the *Triops* cardiac muscle in a concentration-dependent manner (Figs. 2 and 3). The action potential of the cardiac muscle increased in frequency with glutamate of low concentrations and vanished completely with higher dose (Fig. 2). This change in the myogenic activity is probably due to action potential adaptation of the cardiac muscle to the depolarizing response. The depolarizing membrane response of the cardiac muscle to glutamate was confirmed in the quiescent heart (Fig. 4).

In the neurogenic heart of several crustaceans, glutamate induced a depolarizing membrane response in the myocardium and is suggested to be a transmitter of the motoneurones in the cardiac ganglion (in decapods, Hallet, 1971; Benson, 1981; in isopods, Holley and Delaleu, 1972; Yazawa *et al*., 1998; Sakurai *et al*., 1998). However, there are no neurons in the *Triops* heart and the heartbeat is myogenic (Yamagishi *et al*., 1997). In the isopod *Ligia exotica*, the threshold concentration of glutamate to induce a depolarizing membrane response in the cardiac muscle is approximately 1×10^{-5} M and the response saturates at approximately 5×10^{-3} M (Sakurai *et al*., 1998). While in the *Triops* heart, the depolarizing response of the cardiac muscle to glutamate occurred with a threshold concentration of approximately 1×10^{-5} M and almost saturated at approximately 1×10^{-4} M (Fig. 3). Thus, the amplitude of the depolarizing response of the *Triops* myocardium increases largely to a slight increase in glutamate concentration.

It is unlikely that the *Triops* heart is regulated by extrinsic cardioregulatory nerves from the central nervous system. However, presence of the glutamate receptor in the myocardium and its response characteristics to glutamate may indicate some regulation mechanism of the heartbeat. Further investigation on the physiological role of the glutamate receptor in the *Triops* myocardium is needed.

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