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THE EFFECTS OF GLUTAMATE AGONISTS ON VOLTAGE-CLAMPED MOTONEURONS OF THE LOBSTER CARDIAC GANGLION

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Summary

The effects of L-glutamate and its analogues were studied in voltage-clamped motoneurons of the lobster cardiac ganglion. These excitatory amino acids caused a dose-dependent increase in membrane conductance and an inward current at the resting membrane potential. The EC_{50} for L-glutamate was $150\,\mu mol\,l^{-1}$. The rank order of potencies of the various agonists was quisqualate>L-glutamate= L-aspartate>kainate>cysteine. Kainate, unlike the other agonists, showed no desensitization. Of various antagonists studied, only the quinoxalinediones inhibited the response to glutamate. These antagonists also reduced the amplitude and duration of the pacemaker-driven burst potential, suggesting that glutamate may be released by some of the endogenous synapses within the ganglion. The reversal potential of the glutamate-induced current was −15 mV. When Na^+ was replaced with K^+, the glutamate-induced current still reversed between 0 and −20 mV. When Na^+ was replaced with the impermeant ion *N*-methyl-D-glucamine, the current was inhibited. The amplitude of responses evoked by glutamate and its analogues was reduced in salines containing either high or low concentrations of Ca^{2+}. These results of pharmacological and of reversal potential and ion substitution experiments indicate that glutamate acts on receptors of the non-NMDA (*N*-methyl-D-aspartate), quisqualate/kainate type to open a channel permeable to both Na^+ and K^+.

Introduction

Excitatory synapses onto motoneurons in the lobster cardiac ganglion originate from neurons within the ganglion and from the two pairs of cardioaccelerator fibers that enter the ganglion from the central nervous system (Cooke, 1988). The neurotransmitters released at these excitatory synapses are not identified. Acetylcholine (ACh) acting at muscarinic receptors on motoneurons causes a slow depolarization (Freschi and Livengood, 1989) incompatible with the fast time

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course of the excitatory postsynaptic potentials (EPSPs) in this system. Furthermore, neither muscarinic nor nicotinic antagonists block spontaneous EPSPs (Freschi and Livengood, 1989). Because glutamate is a widely distributed excitatory neurotransmitter in both vertebrates and invertebrates (Freeman, 1976), it is a good candidate for the neurotransmitter released by some of the excitatory synapses in the cardiac ganglion. The aims of this investigation were to determine what effect glutamate has on the motoneurons and to compare the physiology and pharmacology of the glutamate response with those in other crustacean nerve and muscle preparations. A preliminary account of these results has appeared (Hashemzadeh-Gargari and Freschi, 1991*a*).

Materials and methods

Detailed descriptions of our methods have been previously published (Freschi, 1991; Freschi and Livengood, 1989; Livengood, 1983). In this study, we used two microelectrodes, with tip resistances of 5–15 MΩ when filled with 3 mol l^{-1} KCl, to voltage-clamp motoneurons of lobster cardiac ganglion. The cardiac ganglion contains only five motoneurons, which are identifiable between preparations. Responses to glutamate analogues were identical in each of these five motoneurons. Lobsters (*Homarus americanus* Milne-Edwards) were obtained from local food stores and used the same day. Normal lobster saline (NLS) contained the following chemicals (in mmol l^{-1}): 440 NaCl, 15 KCl, 25 $CaCl_2$, 4 $MgCl_2$, 4 $MgSO_4$, 10 Hepes buffer. Two types of Na^+-free solutions were used in which either *N*-methyl-D-glucamine, a large organic ion unlikely to permeate the glutamate channel, or K^+ was substituted for Na^+. In the high-K^+ saline, acetate replaced Cl^- to keep the $[K^+]_o[Cl^-]_o$ product constant. Desired concentrations (1 μmol l^{-1}–10 mmol l^{-1}) of glutamate agonists were bath-applied by diluting concentrated stocks in NLS. For higher concentrations of these drugs, the final pH of the saline was adjusted by titration with NaOH. All chemicals were obtained

Fig. 1. Response of lobster cardiac ganglion motoneurons to glutamate and its analogues. (A) Some representative examples of responses from one neuron to the same concentration (0.5 mmol l^{-1}) of various analogues. The membrane holding potential (V_h was −45 mV). The thick black baseline in this and subsequent figures is the result of burst currents invading the soma from unclamped regions of the axon at the slow chart speed. The current steps recurring every 60 s in this and subsequent figures were evoked by 10 mV step commands to assess membrane conductance. The dashed line indicates the steady baseline current at the V_h. In this and subsequent figures, the glutamate- or analogue-evoked current was obtained by superfusion of the drug. The onset of drug superfusion is indicated by the downward arrow and the onset of drug washout is shown by the upward arrow. (B) Concentration–effect curves for glutamate and its analogues. For glutamate, the curve shows the mean (±S.E.M.) of four different experiments. Where the error bars are not present, they were smaller than the symbol. For the other analogues, the curves are from single experiments representative of at least five other experiments in which the analogue was applied over a more limited concentration range. The responses were normalized to the maximum response.

from Sigma (St Louis, MO) and from Research Biochemical Incorporated (Natic, MA).

Results

Concentration–effect relationships of L-glutamate and its analogues

We found that bath-application of L-glutamate caused depolarization of the motoneurons associated with an increase in the frequency of spontaneous burst potentials (data not shown). Under voltage-clamp, L-glutamate and its analogues caused a dose-dependent current (Fig. 1A,B) associated with an increase in chord conductance. We determined concentration–effect relationships by applying various concentrations of L-glutamate and its analogues with washout between applications (Fig. 1B). For L-glutamate, the EC_{50} was 150 μmol l^{-1}, and we found

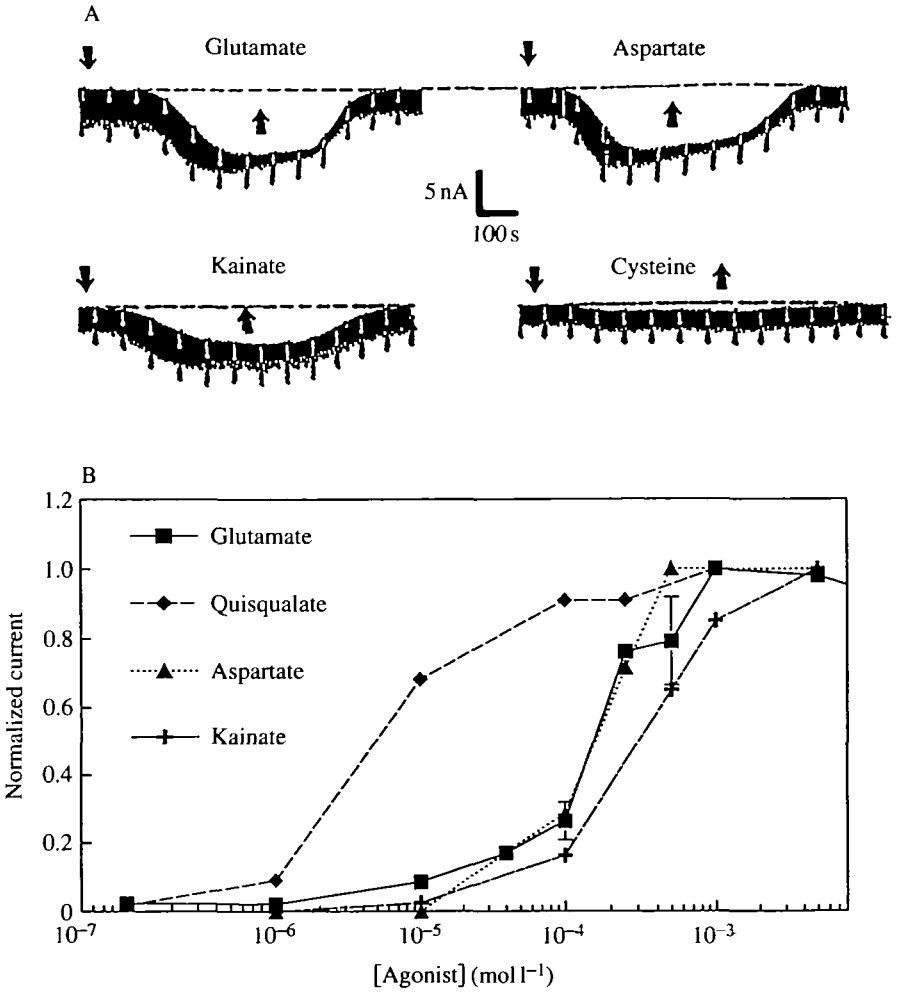

Fig. 1

similar potencies for kainate and L-aspartate (Fig. 1B). Quisqualate was about 50 times more potent than L-glutamate. The rank order of potency of the various agonists was: quisqualate>L-glutamate=L-aspartate>kainate>cysteine. Ibotenic acid, *N*-methyl-D-aspartate (NMDA), L-glutamic acid-γ-methyl ester and glutamate diethyl ester were ineffective agonists. D-Glutamate did evoke a current, but the threshold concentration was 0.5 mmol l^{-1} and the EC_{50} was at least 20 times greater than that of L-glutamate. At high concentrations, glutamate, aspartate and quisqualate caused desensitization. The desensitization to quisqualate was particularly strong and rapid (Fig. 2B). In contrast, there was no desensitization to kainate at concentrations up to 5 mmol l^{-1} (Fig. 2C). Addition of spermine and glycine did not modify the response to L-glutamate.

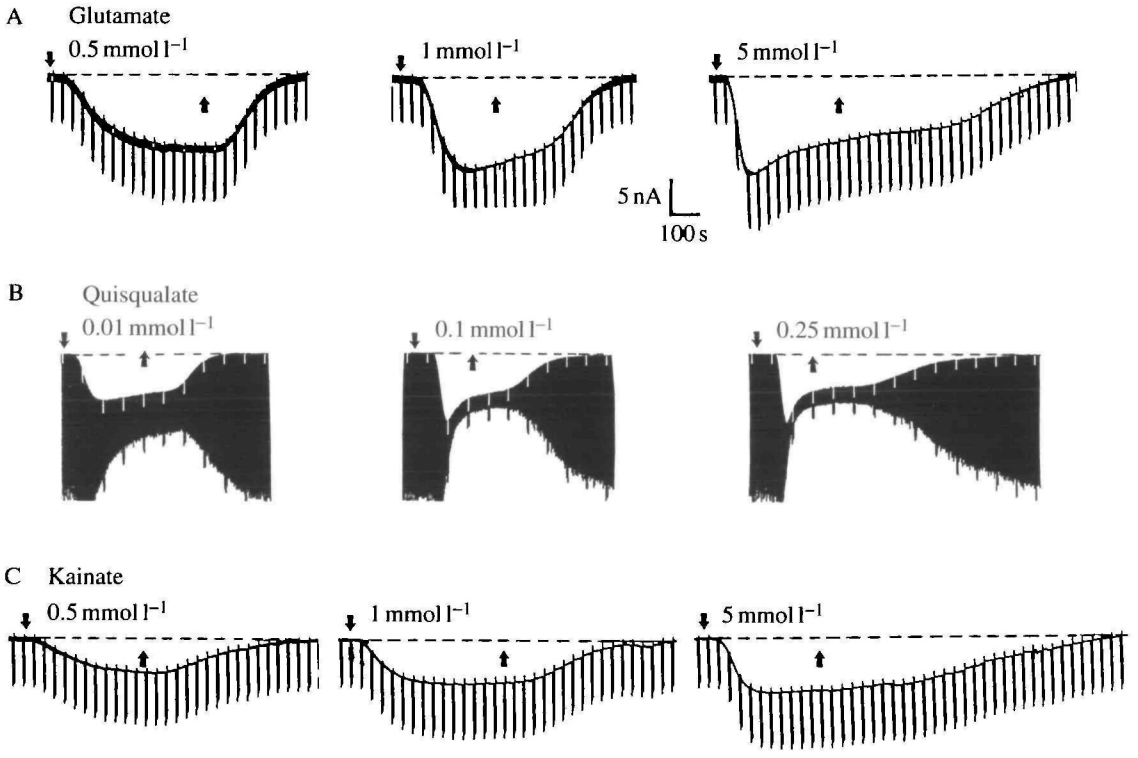

Fig. 2. Currents evoked by glutamate and quisqualate but not kainate undergo dose-dependent desensitization. (A) The current induced by 0.5 mmol l^{-1} glutamate (first trace) did not desensitize. Desensitization was seen during the application of 1 mmol l^{-1} glutamate (middle trace). Greater desensitization is seen during superfusion of 5 mmol l^{-1} glutamate (last trace). (B) Desensitization is seen with the lowest concentration (0.01 mmol l^{-1}) of quisqualate (first trace). The desensitization occurred rapidly at the higher concentrations shown in the middle and right-hand traces. (C) For kainate, there was no desensitization by the time washout (upward arrows) began for concentrations of 0.5 and 1 mmol l^{-1} (left-hand and middle traces, respectively). There is minimal desensitization by the time washout of 5 mmol l^{-1} kainate was begun (last trace).

It is possible that the responses to glutamate and its analogues were secondary to their evoking the release of another transmitter. Against this hypothesis was our finding of responses in salines containing tetrodotoxin, Mn^{2+} or elevated Mg^{2+} concentrations (data not shown) that were similar to those obtained in NLS as described above.

Effect of glutamate antagonists

We applied various glutamate antagonists known to block glutamate responses in both vertebrates and invertebrates. Both kynurenic acid and L-glutamic acid diethyl ester failed to block glutamate responses. 6-Cyano-7-nitroquinoxaline-2,3-

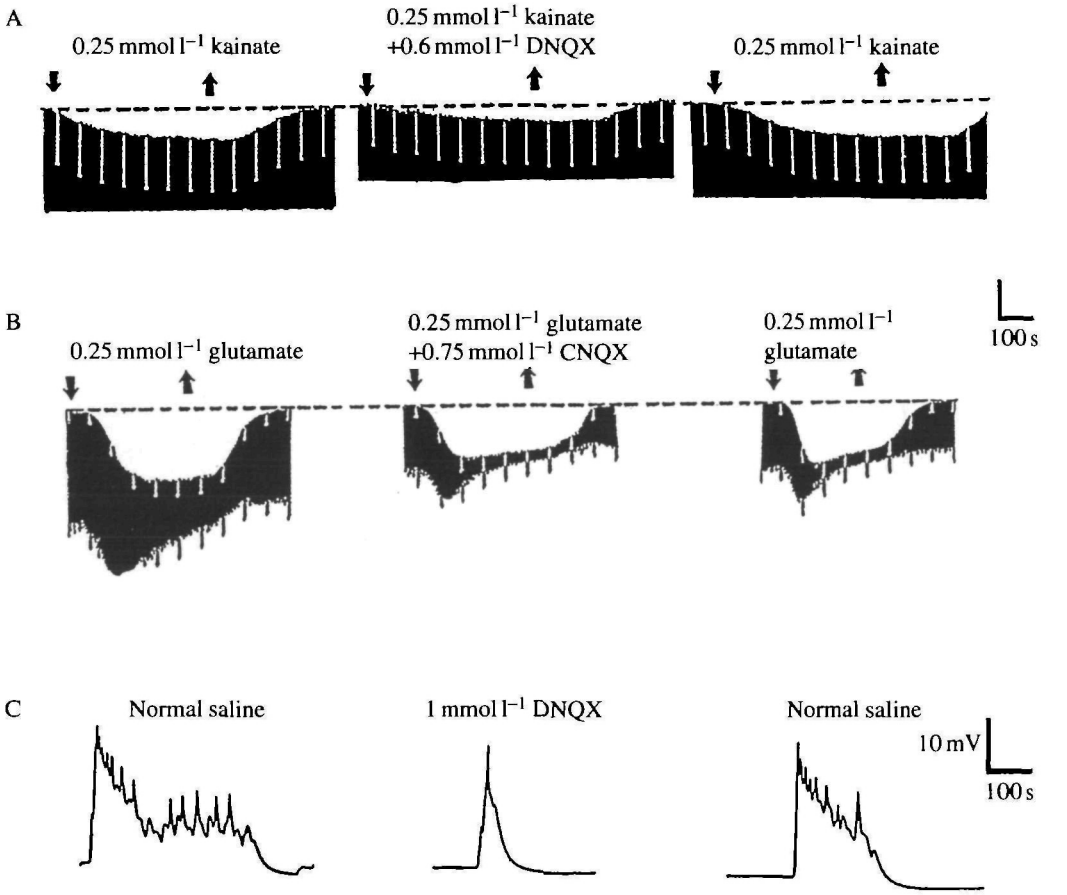

Fig. 3. (A,B) Inhibition of the kainate (A) and glutamate (B) currents by quinoxalinediones. The first traces are the control responses to kainate (A) and glutamate (B). The middle traces are the responses after incubation in the antagonist for 10 min. (The antagonists had no effect on holding current.) The last traces are the responses to kainate (A) and glutamate (B) after washout of the antagonist. V_h −49 mV (A) and −54 mV (B). (C) Bursting configuration in normal saline (left-hand trace), during superfusion with 1 mmol l^{-1} DNQX (middle trace) and after washout of the DNQX (right-hand trace). Calibration bars: vertical, 1 nA in A and 2.5 nA in B.

dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX), however, partially blocked quisqualate, kainate and glutamate responses (20–40 % inhibition, Fig. 3A,B).

Possible role of glutamate in intrinsic ganglionic synapses

The pacemaker burst recorded in the soma of the motoneuron is a complex potential consisting of EPSPs, regenerative Ca^{2+} and K^+ potentials and back-propagated Na^+ spikes (Cooke, 1988). Although the specific EPSP components of the burst cannot be identified with certainty, we tested the hypothesis that glutamate may be released by some of the endogenous synapses by looking at the effect of DNQX on the burst potential waveforms. Bath-application of 1 mmol l^{-1} DNQX inhibited the amplitude and duration of the burst potential (Fig. 3C), and washout of DNQX largely restored the burst pattern.

Ionic mechanism of the glutamate-induced current

To determine the ionic basis of the glutamate response, we measured the amplitude of the current at various membrane holding potentials (V_h). The reversal potential for the L-glutamate-evoked response in NLS was between 0 and −20 mV (mean=−15 mV; N=5) as shown in Fig. 4A,D. Similar reversal potentials were obtained for L-aspartate and kainate (Fig. 4D). When Na^+ was replaced by K^+, the membrane depolarized to appoximately 0 mV. When the neuron was voltage-clamped at this new membrane potential, glutamate evoked an outward current (Fig. 4B). Shifting V_h was difficult because the high-K^+ saline caused a large decrease in the cell input resistance. In three different experiments, however, we noted that when V_h was shifted to −20 mV, the glutamate-evoked current was inward (Fig. 4B). Thus, the reversal potential in the high-K^+ saline was between 0 and −20 mV, similar to that found in NLS. The responses to

Fig. 4. Relationship between evoked current and membrane potential. (A) Representative current traces from a motoneuron in normal lobster saline (NLS) voltage-clamped at the V_h (in mV) indicated to the left of each trace. The dashed line represents the steady baseline current at that V_h before the drug was applied. Note that the current jumps evoked by command voltage steps have been cropped in some of the traces. (B) Effect of replacing Na^+ with K^+ on the glutamate-evoked response. A neuron was voltage-clamped at 0 mV (first trace) and at −20 mV (second trace) in saline in which NaCl had been replaced by potassium acetate. (C) Effect of replacing Na^+ with N-methyl-D-glucamine. After the control response (first trace) had been obtained in NLS, the cell was superfused with saline in which N-methyl-D-glucamine was substituted for Na^+, and the response to glutamate was obtained in this solution (middle trace). After the Na^+-free saline had been replaced by NLS, the response to glutamate was again obtained (last trace). The currents from the voltage-step commands in the first trace were not applied during the subsequent traces. (D) Normalized evoked current plotted against V_h. The data for glutamate are the averages from five different experiments. The data for aspartate and kainate are from single representative experiments. The currents were normalized to the inward current obtained at −60 to −80 mV. The curves were derived from least-squares fits of the data to a polynomial function.

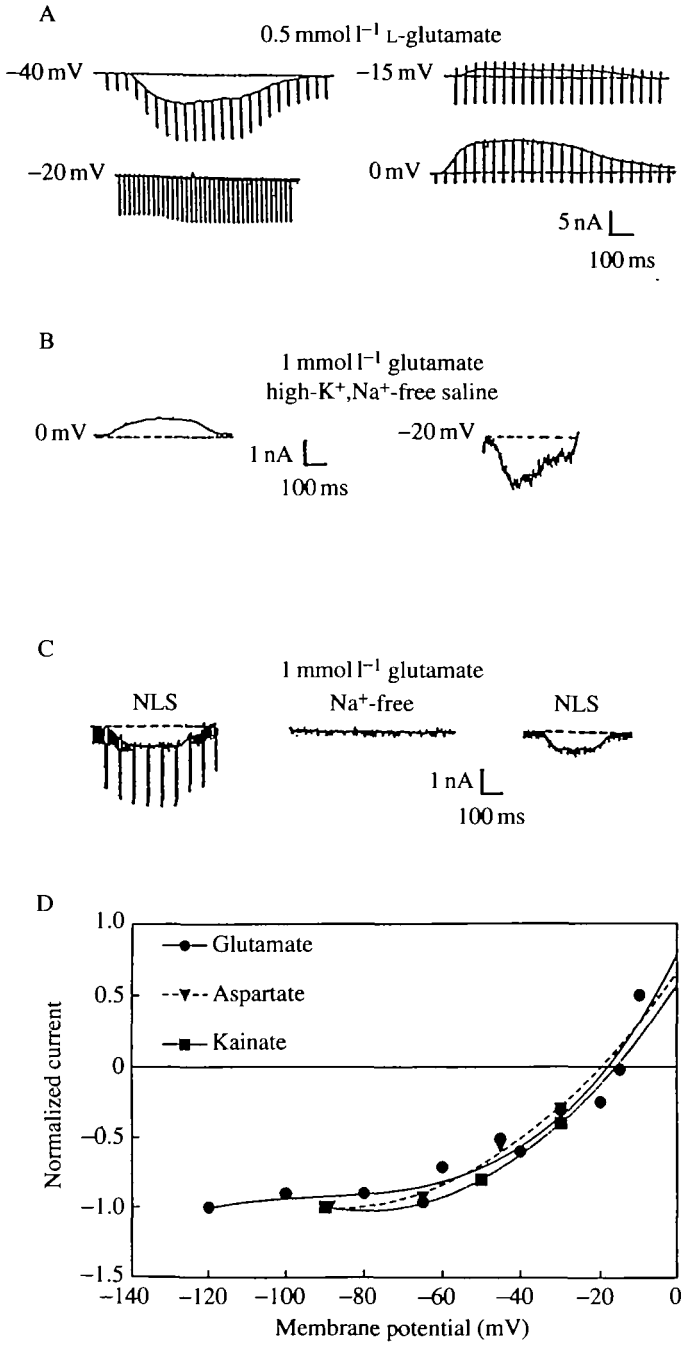
A
0.5 mmol l⁻¹ L-glutamate
−40 mV
−15 mV
−20 mV
0 mV
5 nA
100 ms
B
1 mmol l⁻¹ glutamate
high-K⁺,Na⁺-free saline
0 mV
−20 mV
1 nA
100 ms
C
1 mmol l⁻¹ glutamate
NLS
Na⁺-free
NLS
1 nA
100 ms
D
Glutamate
Aspartate
Kainate
Normalized current
1.0
0.5
0
−0.5
−1.0
−1.5
−140
−120
−100
−80
−60
−40
−20
0
Membrane potential (mV)

Fig. 4

L-glutamate (Fig. 4C) and kainate were blocked in solutions where Na^+ was replaced by *N*-methyl-D-glucamine. The glutamate-evoked current remained unchanged in both high-Mg^{2+} and Mg^{2+}-free saline, and it was not modified in saline containing 10 mmol l^{-1} Mn^{2+}. The glutamate response was decreased in the presence of high extracellular Ca^{2+} concentrations (100 mmol l^{-1}) (Fig. 5A) and in saline containing low Ca^{2+} concentrations (5 mmol l^{-1} Ca^{2+}, 20 mmol l^{-1} Co^{2+}). The amplitude and reversal potential of the glutamate-evoked current were not affected by replacing NaCl with sodium isethionate in the superfusate (data not shown). After incubation for 2.5 h in Concanavalin-A (0.2 mg ml^{-1}), there was no significant change in the amplitude of the glutamate-induced currents and no change in the glutamate reversal potential (Fig. 6).

Discussion

An excitatory effect of glutamate on the isolated crustacean cardiac ganglion was noted by Cooke (1966), but the mechanism of action and role in synaptic

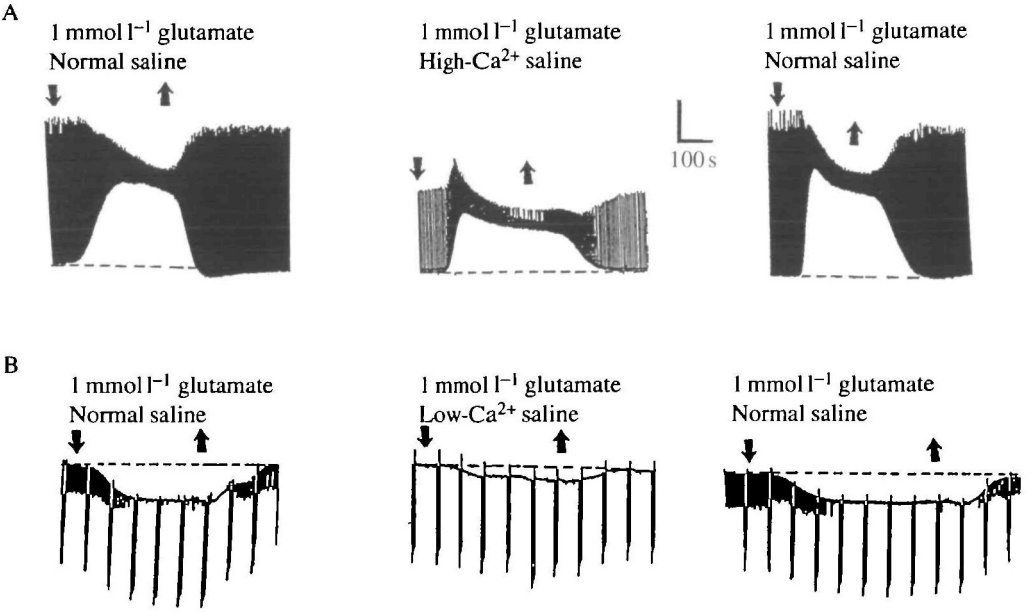

Fig. 5. Effect of changes in extracellular Ca^{2+} concentrations on the response evoked by glutamate. (A) Under current-clamp, the depolarization elicited by 1 mmol l^{-1} glutamate (first trace) was reduced in amplitude in the presence of 100 mmol l^{-1} Ca^{2+} (middle trace). The amplitude was restored after reintroduction of normal saline (third trace). (B) In a voltage-clamped motoneuron, the current evoked by 1 mmol l^{-1} glutamate in normal saline is shown in the left-hand trace. After superfusion with saline containing 5 mmol l^{-1} Ca^{2+} and 20 mmol l^{-1} Co^{2+}, the glutamate-induced current was inhibited (middle trace). The right-hand trace shows restoration of the control response after washout of the low-Ca^{2+} saline with normal saline. Calibration bar: vertical, 10 mV, A, 5 nA, B.

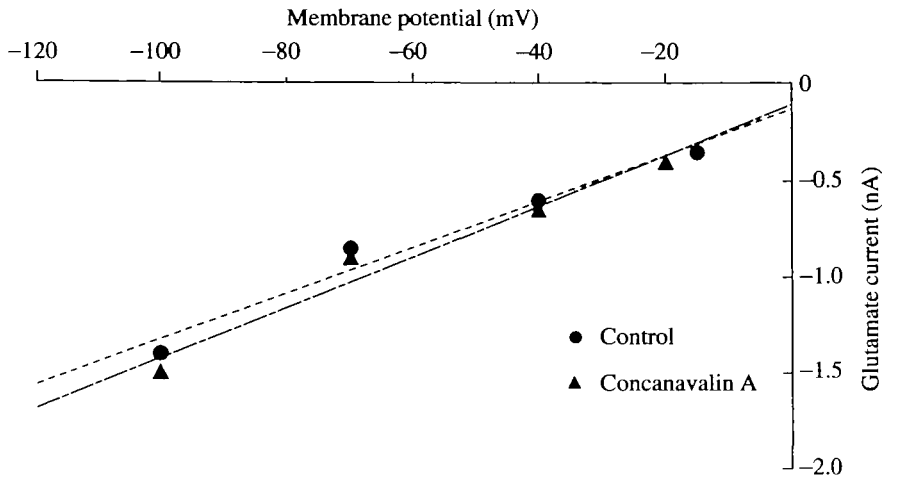

Fig. 6. Lack of effect of Concanavalin A on the current–voltage relationship of the glutamate-evoked current. Glutamate, 0.5 mmol l^{-1}, was applied at various voltage-clamped holding potentials before (control) and after 2.5 h incubation with Concanavalin A (0.2 mg ml^{-1}). The response amplitude at each membrane holding potential was similar under the two conditions. The slope (conductance) and extrapolated *x*-intercept (reversal potential) were unchanged in the presence of Concanavalin A.

transmission have not been determined. Our results show that glutamate depolarizes motoneurons of the lobster cardiac ganglion by activating a cation conductance. We found no evidence of K^+- or Cl^--mediated inhibitory responses such as those seen in some crustacean (Marder and Paupardin-Tritsch, 1978; Marder and Eisen, 1984; Sansom and Usherwood, 1990) and molluscan neurons (Kehoe, 1978). Because similar effects were seen with quisqualate, kainate and aspartate, but not NMDA, it is likely that the response is mediated by a non-NMDA glutamate receptor of the quisqualate/kainate type. The pharmacological profile is similar to that of the depolarizing glutamate receptor on locust and crayfish skeletal muscle (Sansom and Usherwood, 1990; Shinozaki, 1988) in that quisqualate was the most potent agonist. Unlike these other arthropod receptors, however, we found that kainate and aspartate had agonist activity on the receptors of lobster motoneurons. The rapid and prolonged desensitization by quisqualate and the lack of desensitization by kainate have also been noted in studies of glutamate receptors in mammalian brain (Barnard and Henley, 1990). Our studies show that glutamate increases membrane conductance to both Na^+ and K^+. The reversal potential of −15 mV is within the voltage range expected for excitatory postsynaptic cation channels, which usually show a K^+ to Na^+ permeability ratio between 1.1 and 1.5 (Takeuchi and Takeuchi, 1960; Edwards, 1982). This mechanism of increased cation conductance is similar to that seen in numerous other invertebrate tissues and in mammalian neurons through non-NMDA glutamate receptors and through nicotinic ACh channels (Barnard and Henley,

1990; Edwards, 1982; Freeman, 1976). This conductance is different from that activated by muscarinic agonists, proctolin and phosphodiesterase inhibitors in the cardiac ganglion (Freschi, 1989; Freschi and Livengood, 1989; Hashemzadeh-Gargari and Freschi, 1991*b*). These latter chemicals activate a voltage-dependent conductance and the resulting current reverses at a membrane potential of +24 mV (Freschi and Livengood, 1989; Freschi, 1989). The mechanism of depression of the glutamate response by increased and by decreased concentrations of extracellular Ca^{2+} is unclear, but Barker (1975) found that elevated Ca^{2+} concentrations and low concentrations of Co^{2+} depressed the glutamate response in crustacean muscle. He found that the depression by divalent cations was selective for Na^{+}-dependent glutamate responses. Furthermore, it has been shown that crayfish muscle glutamate receptors are strongly Ca^{2+}-dependent. When extracellular Ca^{2+} concentration is reduced, glutamate-induced reponses are markedly diminished (Sansom and Usherwood, 1990).

Although the quinoxalinediones are potent antagonists of non-NMDA receptors in the mammalian nervous system (Honore *et al.* 1988), we found that high concentrations of these antagonist caused only partial inhibition. Nevertheless, these were the only compounds of those we tested that had an inhibitory effect. We also used these antagonists to determine whether spontaneous EPSPs might be due to glutamatergic synapses. We found that the components of the burst potential were reduced by DNQX, consistent with the hypothesis that glutamate may contribute to part of the burst waveform. We cannot exclude the possibility, however, that the drugs were acting on other components of the burst potential. Although it is difficult to study isolated EPSPs from intrinsic ganglionic synapses, it should be possible to stimulate extrinsic cardioaccelerator nerves selectively and to record the resulting EPSPs before and during the application of DNQX.

In summary, in this paper we describe the pharmacology and ionic mechanism of the action of glutamate and a number of its agonists on motoneurons of the lobster cardiac ganglion. We present evidence to suggest that such glutamate receptors may contribute to excitatory transmission at the synapses onto these motoneurons.

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