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FURTHER OBSERVATIONS ON THE INTERACTION BETWEEN GLUTAMATE AND ASPARTATE ON LOBSTER MUSCLE

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1 The ability of bath-applied L-glutamate to enhance subsequent depolarizations produced by bath-applied L-aspartate on lobster muscle was further investigated by means of intracellular recording techniques.

2 Increasing the conditioning glutamate concentration or exposure time produced a greater enhancement of aspartate responses. Enhancement was also dependent on the time interval between glutamate and aspartate doses and was not prevented by overnight storage of preparations *in vitro*.

3 The dose-depolarization curve for *enhanced* aspartate responses (measured at a fixed time following a given dose of glutamate) was displaced to the left along the abscissa scale relative to control, with no detectable change in limiting log-log slope.

4 Conditioning doses of kainate or domoate (but not quisqualate, aspartate, or KCl) also enhanced aspartate responses; however, their conditioning effect was little affected by increasing the concentration, exposure time, or time interval before applying aspartate. The rate of onset and decline of the enhanced aspartate response always resembled that of the previous conditioning agonist.

5 D and L-Aspartate were approximately equieffective depolarizing agents whereas D-glutamate was approximately 1/40 as potent as L-glutamate. After a conditioning dose of D or L-glutamate, responses to D or L-aspartate were enhanced.

6 In a Na^+-free (Li^+) medium, both the glutamate depolarization and the conditioning effect towards aspartate were largely abolished. With kainate however, Na^+ was not apparently important either for evoking the kainate response or for producing the conditioning effect.

7 Bath-applied glutamate greatly enhanced and prolonged the time course of the iontophoretic aspartate potential with only a small effect on the glutamate potential; however, these effects were not maintained after washout of glutamate. In contrast, bath-application of aspartate depressed the aspartate potential while enhancing the glutamate potential. Some sites that were insensitive to iontophoretically-applied aspartate became clearly responsive to this agent during a bath-application of glutamate.

8 It is proposed that during conditioning with bath-applied glutamate, kainate or domoate, some agonist is trapped by extrajunctional sites and is subsequently displaced by bath-applied aspartate to produce the long-term enhancement effect.

Introduction

There is much evidence that favours an excitatory neurotransmitter role for L-glutamate at crustacean neuromuscular junctions (Takeuchi & Takeuchi, 1964; Kravitz, Slater, Takahashi, Bownds & Grossfeld, 1970; Gerschenfeld, 1973; Freeman, 1976). Recently, a co-transmitter function for L-aspartate at these synapses has also been suggested (Shank & Freeman, 1975; Shank, Freeman, McBride & Aprison, 1975); however, before such a role can be fully established, a clear understanding of the mutual interaction between these two amino acids on the postsynaptic membrane is necessary. Kravitz *et al.*, (1970) originally found that a bath-applied dose of aspartate having no effect on lobster muscle membrane potential or resistance, apparently enhanced the depolariz-

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ing effect of bath-applied glutamate when these two agents were applied simultaneously. Subsequently, this phenomenon has been shown to occur in some other invertebrate preparations (Crawford & McBurney, 1977a, b; Dudel, 1977; McCreery & Carpenter, 1978) and several interpretations have been suggested.

In a recent publication (Constanti & Nistri, 1978) we described a new glutamate/aspartate interaction on lobster muscle whereby the effect of bath-applied aspartate was markedly enhanced by a previous conditioning application of glutamate. In the present paper, we examine some concentration- and time-dependent features of this interaction and show that other conditioning agonists besides glutamate are capable of enhancing subsequent responses to aspartate. We also describe the contrasting effects produced by various bath-applied depolarizing agonists on the amplitude and time course of iontophoretic glutamate and aspartate potentials evoked at the same site on the muscle fibre membrane.

Methods

The experiments were carried out at room temperature (15 to 20°C) on claw opener muscles of the walking legs of the lobster *Homarus vulgaris* by means of conventional intracellular recording techniques. Full details of the dissection and methods for studying depolarizations evoked by bath-applied or iontophoretically-applied glutamate and aspartate are given elsewhere (Constanti & Nistri, 1976a; 1978). Briefly, superficial fibres were impaled at the centre with two microelectrodes, one for voltage recording (filled with 1.5 M potassium citrate) and the other for passing hyperpolarizing current pulses (800 ms; 0.25 Hz) through the membrane. Electrotonic potentials were displayed on an oscilloscope and recorded on a chart recorder. In some experiments, a second voltage-recording electrode was introduced at the tendon-end of the same fibre in order to calculate the membrane conductance (expressed as g_mL, where g_m is the conductance per unit length and L the fibre half-length) from 'short' cable theory (see Constanti, 1977). Muscles were continuously superfused *in situ* with the following solution (mM): NaCl 522, KCl 12, $CaCl_2$ 21, $MgCl_2.6H_2O$ 5 and Tris maleate 10, adjusted to pH 7.6 with 0.1 N NaOH. Drugs were dissolved in this solution, adjusted to pH 7.6 and applied via the bathing medium. Iontophoretic glutamate and aspartate potentials were obtained by the previously described method (Takeuchi & Takeuchi, 1964; Constanti & Nistri, 1976c; 1978) using standard double barrelled microelectrodes. One barrel was filled with 1 M L-Na glutamate (pH 8) and the second with 0.5 to 1 M L-Na aspartate (pH 8) (retaining currents were +5 to +30 nA). The voltage-recording electrode was placed as close as possible to a sensitive site in order to minimize potential rise-times. Potentials were recorded on a Racal Store-4 FM tape recorder (Scotch 220 tape) and later replayed for analysis onto a storage oscilloscope or a Medelec u.v. recorder.

Drugs

L-Glutamate (sodium salt), D-glutamic acid, D-aspartic acid and kainic acid were purchased from Sigma Ltd; L-aspartic acid was obtained from Hopkin & Williams and L-aspartate (sodium salt, used for iontophoresis) from K & K Labs. Domoic and quisqualic acids were gifts from Professor T. Takemoto (Tohoku University, Sendai, Japan). All other compounds were of reagent grade.

Results

Bath-application experiments

Glutamate/aspartate interaction. Bath-application of L-aspartate (1 to 8 mM) onto the lobster muscle at the beginning of an experiment (before testing other agonists) produced a large (up to 20 mV) depolarization of the membrane, with a slow rate of onset and offset and with sometimes a rapid 'fading' during a 2 min exposure. The amplitude of successive aspartate responses (dose interval 8 to 10 min) then decreased progressively to a plateau level (Figure 1a to c) and was maintained at this level even after washing for 30 min in drug-free solution (Constanti & Nistri, 1978). However, following a bath-application of glutamate, the subsequent aspartate response was markedly enhanced (Constanti & Nistri, 1978) and then declined progressively to a plateau as before. When plotted on a logarithmic scale against time, the initial decline of aspartate responses to a plateau could usually be fitted by a straight line indicating a simple exponential process ($T_{\frac{1}{2}} = 16.6 \pm 5.2$ min; mean $\pm$ s.d., $n = 6$). However, in some fibres, the decline was biphasic, with an initial fast component followed by a slower component ($T_{\frac{1}{2}} = 3.0 \pm 0.7$ min and 33.2 ± 16.7 min respectively; $n = 6$). In each fibre, the rate of decline of aspartate responses following a 2 min application of 100 μM glutamate paralleled that seen for the initial aspartate responses suggesting a similar underlying mechanism.

The conductance increase produced by 1 mM aspartate at plateau was relatively small (mean value $\pm$ s.d. for 7 fibres was 1.2 ± 0.5 μS; mean resting conductance in these fibres was 14.3 μS). Even for initial aspartate depolarizations exceeding 10 mV, where the conductance increase was probably slightly overestimated due to delayed rectification of the membrane, aspartate-evoked conductance increases rarely

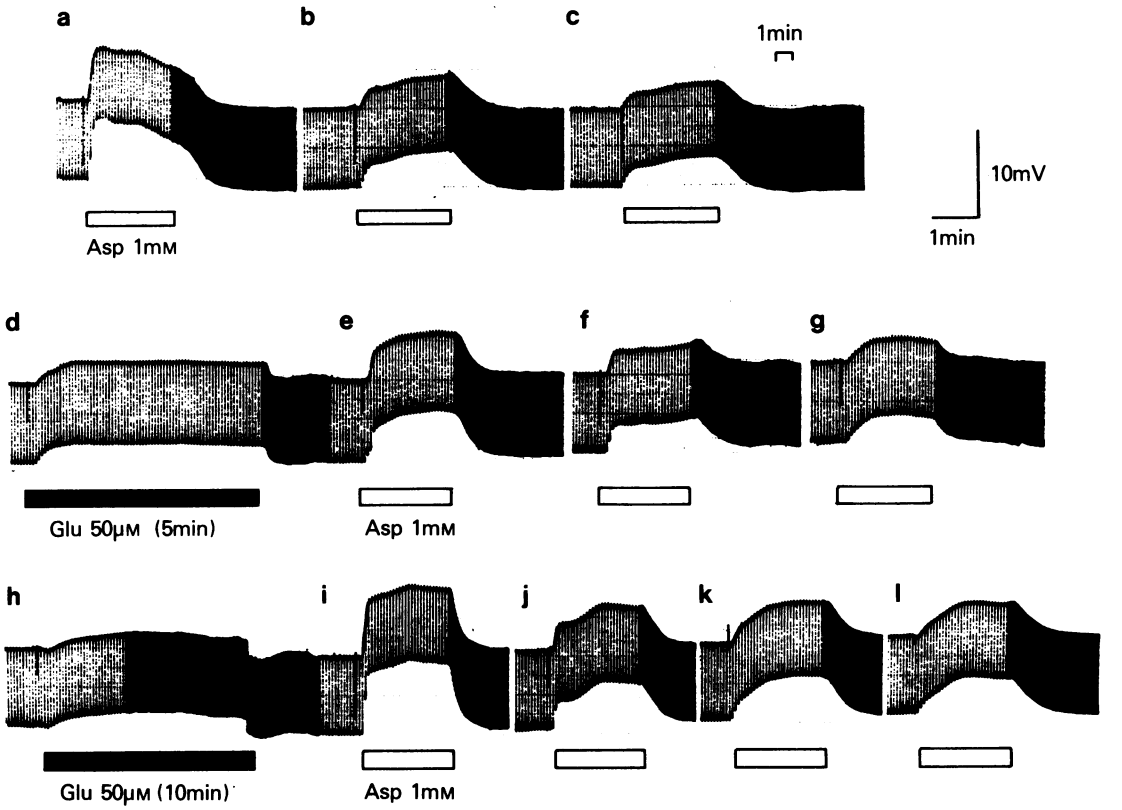

Figure 1 Depolarizations produced by L-aspartate (Asp 1 mM, open bars) and L-glutamate (Glu 50 μM, filled bars) recorded at the centre of a lobster muscle fibre. Downward deflections are hyperpolarizing electrotonic potentials (intracellular current = 240 nA). (a to l). Continuous record reading left to right shows the effect of two different glutamate exposure times on the amplitude of subsequent aspartate responses; (a to c) repeated aspartate responses (dose interval = 10 min) show progressive reduction in amplitude at the beginning of an experiment (see text); (e) enhanced aspartate response, tested 5 min after the end of a 50 μM glutamate application (5 min exposure) (d); (f to g) progressive reduction in aspartate response; (i) further enhancement of aspartate response, 5 min after a 10 min exposure to 50 μM glutamate (h); (j to l) successive aspartate responses are now slower to decline to plateau level. Resting potential = −80 mV; chart speed was slower during decline of responses.

exceeded 5 μS; these values were comparable to those produced by a low concentration (10 μM) of γ-aminobutyric acid (GABA) on this preparation (see Constanti, 1977). From semilogarithmic plots, the initial rate of decline of 1 mM aspartate-evoked conductance responses was always biphasic and generally faster than the corresponding decline of the voltage responses. For small depolarizations, there was usually an excellent agreement between the amplitude of responses recorded at the centre and at the tendon-end of a fibre, indicating a uniform effect of the amino acids on the membrane.

Figure 1 also compares the effects of two different exposure times to glutamate (5 and 10 min) on the amplitude of subsequent aspartate depolarizations; the longer the conditioning exposure to glutamate, the greater was the enhancement of the first subsequent aspartate response (usually tested 5 min after the end of the glutamate application) and the slower the decline to the plateau response level (compare Figure 1 (e) to (g) and (i) to (l)).

The degree of enhancement by glutamate was also dependent on the time interval between the end of the glutamate dose and the application of the aspartate dose. When this interval was increased, the enhancement decreased in a progressive manner

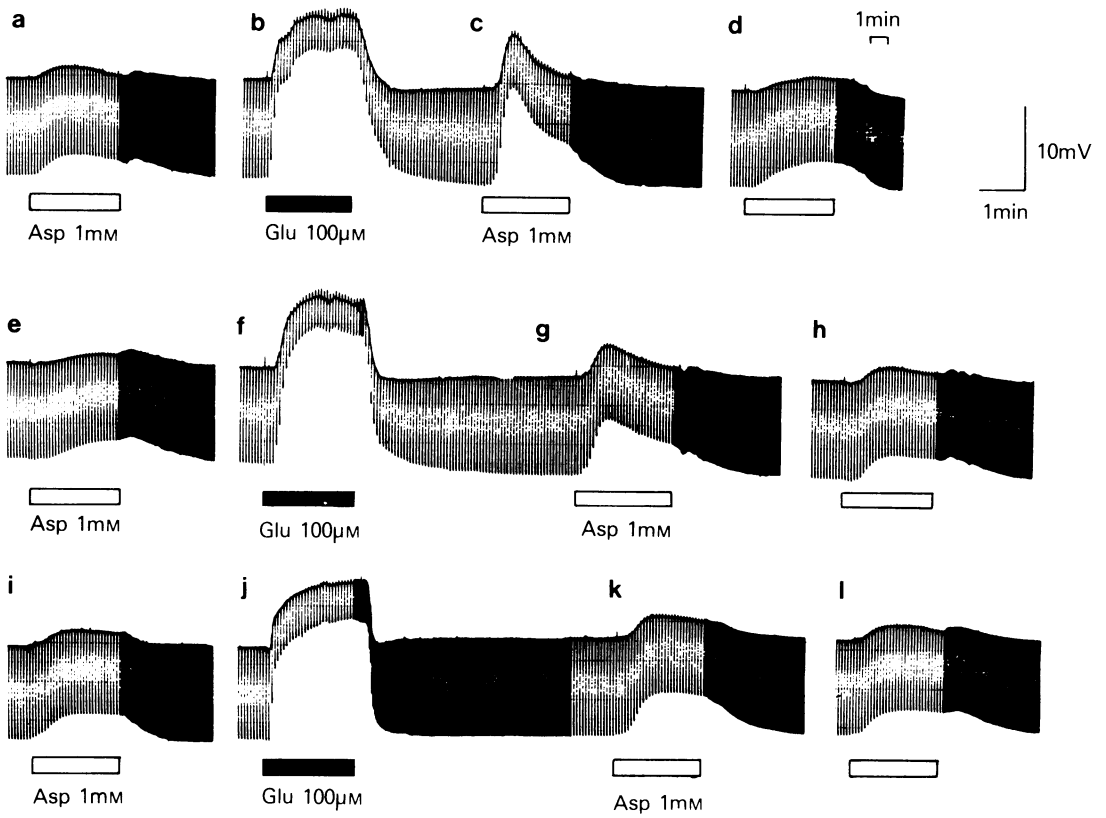

Figure 2 Depolarizations evoked by aspartate (Asp 1 mM, open bars) and glutamate (Glu 100 μM, filled bars) in a single muscle fibre (resting potential = −80 mV; current = 300 nA). (a to l) Continuous record shows that the degree of aspartate enhancement depends on the time interval following the conditioning dose of glutamate; (a, e and i) control aspartate responses measured at plateau; (c and d) aspartate responses obtained 3 and 10 min respectively after the end of a glutamate application (b); (g and h) aspartate responses 5 and 15 min after glutamate (f); (k and l) responses 15 and 25 min after glutamate (j). Note the progressively smaller enhancement of the aspartate response with increasing time interval between glutamate and aspartate doses; (a to d) represents a typical test sequence as referred to in the text.

(Figure 2); after an interval of 15 min, very little enhancement was usually detected following a 2 min exposure to 100 μM glutamate.

The effect of varying the conditioning concentration of glutamate (keeping glutamate exposure time, aspartate concentration and test-interval after glutamate constant) was also investigated (Figure 3a). In each experiment, aspartate was applied successively at the outset and also after each glutamate response until a stable response level was obtained. For each sequence, 1 mM aspartate was tested 3 min after the end of a glutamate application; thereafter, before the next dose of glutamate, 1 mM aspartate was applied repeatedly until the plateau response level was regained. The enhancement of the aspartate response was clearly dependent on the previous glutamate concentration, and the dose-effect curve had an approximate ED_{50} of 100 μM; similar results were obtained when higher doses of aspartate were used. In the converse approach, glutamate dose, exposure time and test interval were fixed while aspartate concentration varied; this allowed the dose-response curve for the enhanced aspartate response to be compared with the control curve obtained for plateau responses (Figure 3b). The aspartate curve was consistently shifted to the left along the abscissa scale by glutamate, with sometimes an additional slight upwards displacement; however, the log-log transfor-

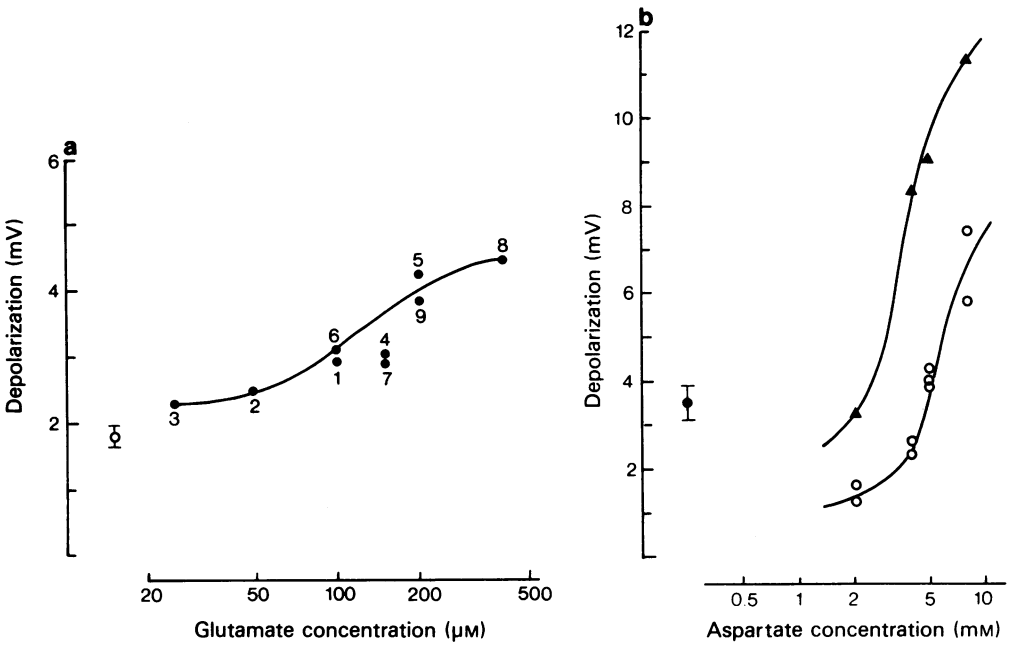

Figure 3 (a) Effect of different conditioning concentrations of glutamate (2 min applications) on the amplitude of a 1 mM aspartate depolarization (●); (○) represents the mean ± s.e. mean of 10 plateau responses to 1 mM aspartate measured throughout the experiment on a single fibre. Ordinate scale: membrane depolarization (mV); abscissa scale: conditioning concentration of glutamate (μM). Numbers near symbols indicate the order of glutamate dose applications. (b) Effect of a fixed conditioning dose of glutamate (150 μM) on the shape of the aspartate dose-depolarization curve obtained on a single fibre (different from a). (○) Control aspartate responses measured at plateau level; (▲) amplitude of the first enhanced aspartate response obtained 3 min after the end of a glutamate application; note the lateral shift and upward displacement of the curve; (●) represents the mean ± s.e.mean of 4 control responses to 150 μM glutamate. Ordinate scale: depolarization (mV); abscissa scale: concentration (mM) of test aspartate dose. Each test sequence was conducted as described in the text.

mation of the enhanced and control curves gave similar limiting slope values (in Figure 3b, values of 1.1 and 1.2 respectively for control and enhanced curves were obtained) suggesting that the kinetics of the underlying aspartate/receptor interaction were not markedly affected during the enhancement (Werman, 1969).

A problem in these latter experiments was the slight depression of the glutamate response shortly after a large dose of aspartate; this suggested that some mutual inhibition was occurring between these two agents when applied in this order (see also Figure 3 of Constanti & Nistri, 1978); some similar findings were obtained in other preparations (Clements & May, 1974; Dudel, 1977; McCreery & Carpenter, 1978). The depression was avoided by separating each test sequence by at least 15 min washing in normal solution.

Effects of different conditioning agonists

We have shown previously (Constanti & Nistri, 1978), that the depolarizing agonists glutamate, quisqualate, kainate and a related analogue domoate did not mimic the action of aspartate on lobster muscle, in that successive responses to these agents were reproducible and uninfluenced by a conditioning dose of glutamate. In the present study, we found that conditioning exposures to kainate, domoate but not quisqualate were also capable of enhancing subsequent responses to aspartate (Figure 4). Four features were immediately apparent in such experiments:—(i) equieffective doses of domoate and kainate produced a similar degree of enhancement of the aspartate response, and subsequent aspartate responses declined to a plateau level with a similarly slow rate. Unlike the experiments with glutamate (Figures 1 and 3),

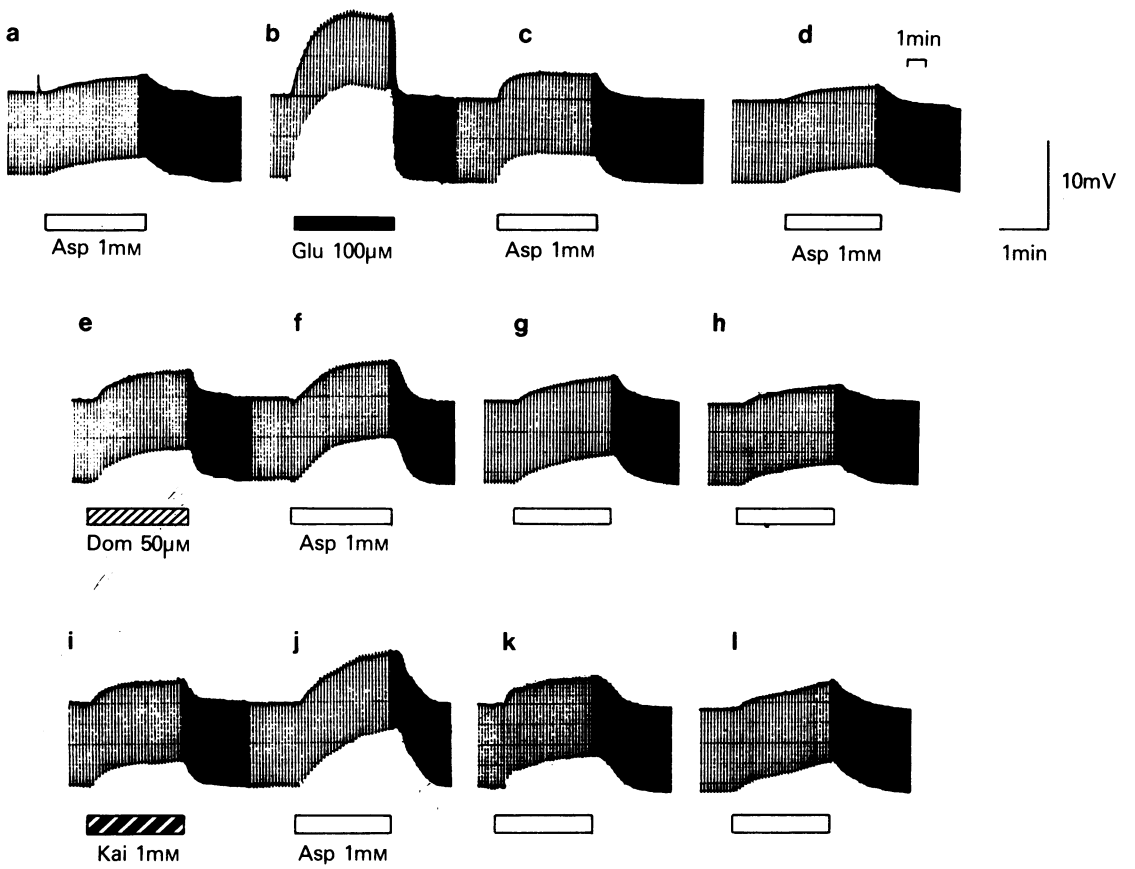

Figure 4 Effects of different conditioning agonists (2 min exposures) on the amplitude of subsequent aspartate depolarizations (Asp 1 mM, open bars). (a to l) Continuous record from a single fibre (resting potential = −76 mV; current = 160 nA). (a to d) Control sequence shows that the response to aspartate is enhanced 5 min after a glutamate response (b) (Glu 100 μM, filled bar). (e to h) and (i to l) Similar sequences show that the aspartate response is also enhanced by a previous 2 min application of domoate (e) (Dom 50 μM, hatched bar) or kainate (i) (Kai 1 mM, thick hatched bar) respectively. Aspartate dose interval was 10 min. Comparable results were obtained in 5 other preparations.

prolonged exposures or greater conditioning concentrations of kainate or domoate produced only small additional increases in the aspartate response. Moreover, the degree of enhancement of aspartate after kainate or domoate did not decrease with increasing test-interval (up to 30 min) as seen with glutamate (Figure 2); (ii) a dose of glutamate giving a depolarization similar to or greater than that of kainate or domoate, always produced a relatively smaller aspartate enhancement and a faster decline to a plateau; (iii) the first enhanced aspartate response after a given conditioning agonist had a rate of onset and offset resembling that of the previous agonist (this is shown clearly by responses (c), (f) and (j) of Figure 4). Thus, following glutamate, aspartate responses were faster in onset and offset than after domoate or kainate; (iv) a previous dose of aspartate did not enhance responses to kainate or domoate and no mutual interaction between kainate and domoate was found. There was also no interaction between quisqualate and either glutamate, domoate or kainate.

An important finding was that the ability of agonists to enhance subsequent aspartate responses could be disrupted by the presence of aspartate during the conditioning period. Thus for example, application of a mixture of 50 μM glutamate plus 1 mM

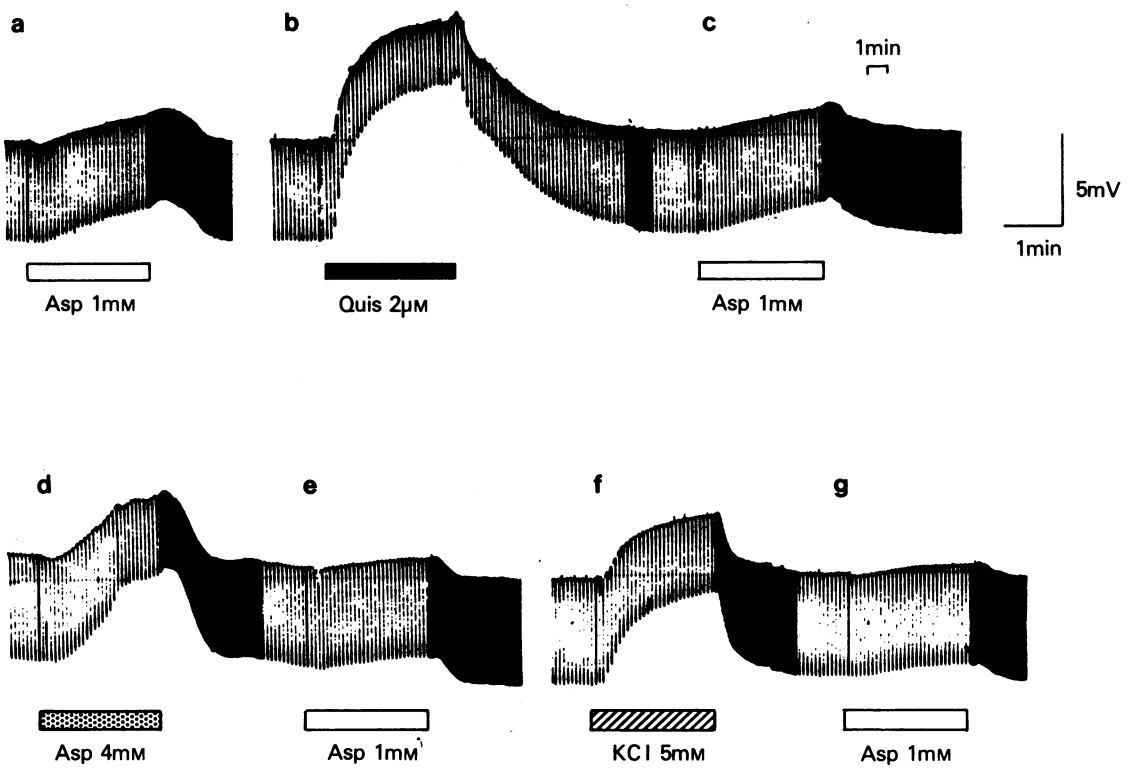

Figure 5 (a to g) Continuous record from a single fibre (resting potential = −88 mV; current = 300 nA) showing that responses to aspartate (Asp 1 mM, open bars) are not enhanced by previous applications of quisqualate (Quis 2 μM, filled bar), L-aspartate itself (Asp 4 mM, stippled bar) or potassium chloride (KCl 5 mM, hatched bar).

aspartate produced a large depolarization but the next response to 1 mM aspartate was not enhanced. This could perhaps, explain why previous workers studying the combined effects of glutamate and aspartate (Shank & Freeman, 1975; Shank *et al.*, 1975; Crawford & McBurney, 1977a, b) failed to observe the aspartate enhancement phenomenon we describe.

Rather surprisingly, aspartate responses were unaffected or even slightly depressed by a previous application of quisqualate (Figure 5 a to c), by a larger dose of aspartate itself or by an application of 5 mM potassium chloride (Figure 5 d to g). Furthermore, a sustained fibre depolarization produced by steady current through the intracellular electrode, did not affect aspartate responses. These results emphasized that membrane depolarization *per se* was not responsible for the aspartate-enhancing effects on lobster muscle.

Effects of D-*aspartate and* D-*glutamate.* Bath-applications of D-aspartate (1 to 8 mM) produced responses comparable to those of L-aspartate obtained on the same fibre, and repeated doses of D-aspartate applied at the beginning of an experiment, produced responses that decreased progressively to a plateau as seen with the L-isomer. In contrast, D-glutamate evoked reproducible depolarizations but was about 40 times less potent than L-glutamate on a molar basis. A conditioning dose of D-aspartate did not influence responses to L-aspartate or *vice versa*; however, a previous application of D or L-glutamate clearly enhanced subsequent responses to D or L-aspartate (Figure 6i). Thus, the membrane sites mediating the depolarizations were more selective towards the L-form of glutamate but were almost equally sensitive to both D and L-forms of aspartate (Figure 6ii). On the other hand, the mechanism(s) operating in the enhancement of aspartate responses by conditioning glutamate did not appear to discriminate between these D and L enantiomers.

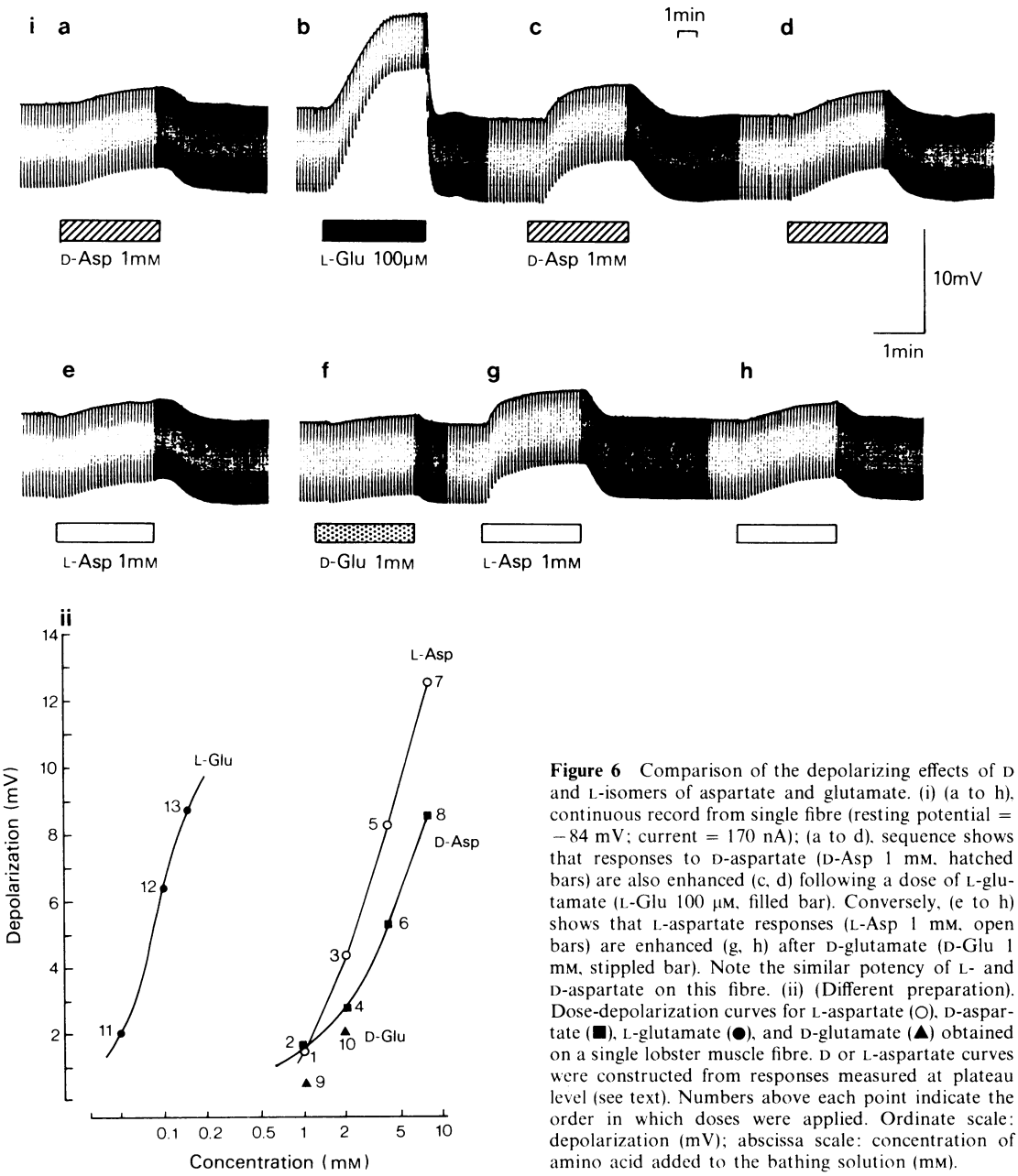

Figure 6 Comparison of the depolarizing effects of D and L-isomers of aspartate and glutamate. (i) (a to h), continuous record from single fibre (resting potential = −84 mV; current = 170 nA); (a to d), sequence shows that responses to D-aspartate (D-Asp 1 mM, hatched bars) are also enhanced (c, d) following a dose of L-glutamate (L-Glu 100 μM, filled bar). Conversely, (e to h) shows that L-aspartate responses (L-Asp 1 mM, open bars) are enhanced (g, h) after D-glutamate (D-Glu 1 mM, stippled bar). Note the similar potency of L- and D-aspartate on this fibre. (ii) (Different preparation). Dose-depolarization curves for L-aspartate (○), D-aspartate (■), L-glutamate (●), and D-glutamate (▲) obtained on a single lobster muscle fibre. D or L-aspartate curves were constructed from responses measured at plateau level (see text). Numbers above each point indicate the order in which doses were applied. Ordinate scale: depolarization (mV); abscissa scale: concentration of amino acid added to the bathing solution (mM).

Effect of Na^+-free solution. The depolarizing action of glutamate on crustacean muscle is known to be largely Na^+-dependent (Onodera & Takeuchi, 1975; Constanti & Nistri, 1976b). Thus, 10 min after the Na^+ content of the lobster saline was replaced by equimolar Li^+ (solution adjusted to pH 7.6 with LiOH) both glutamate (100 μM) and aspartate (1 to 2 mM) depolarizations were almost abolished (a rapidly fading component of the glutamate response in Na^+-free solution has previously been noted; Constanti & Nistri, 1976b) but recovered fully after a 30 min wash in normal solution. Similar findings were

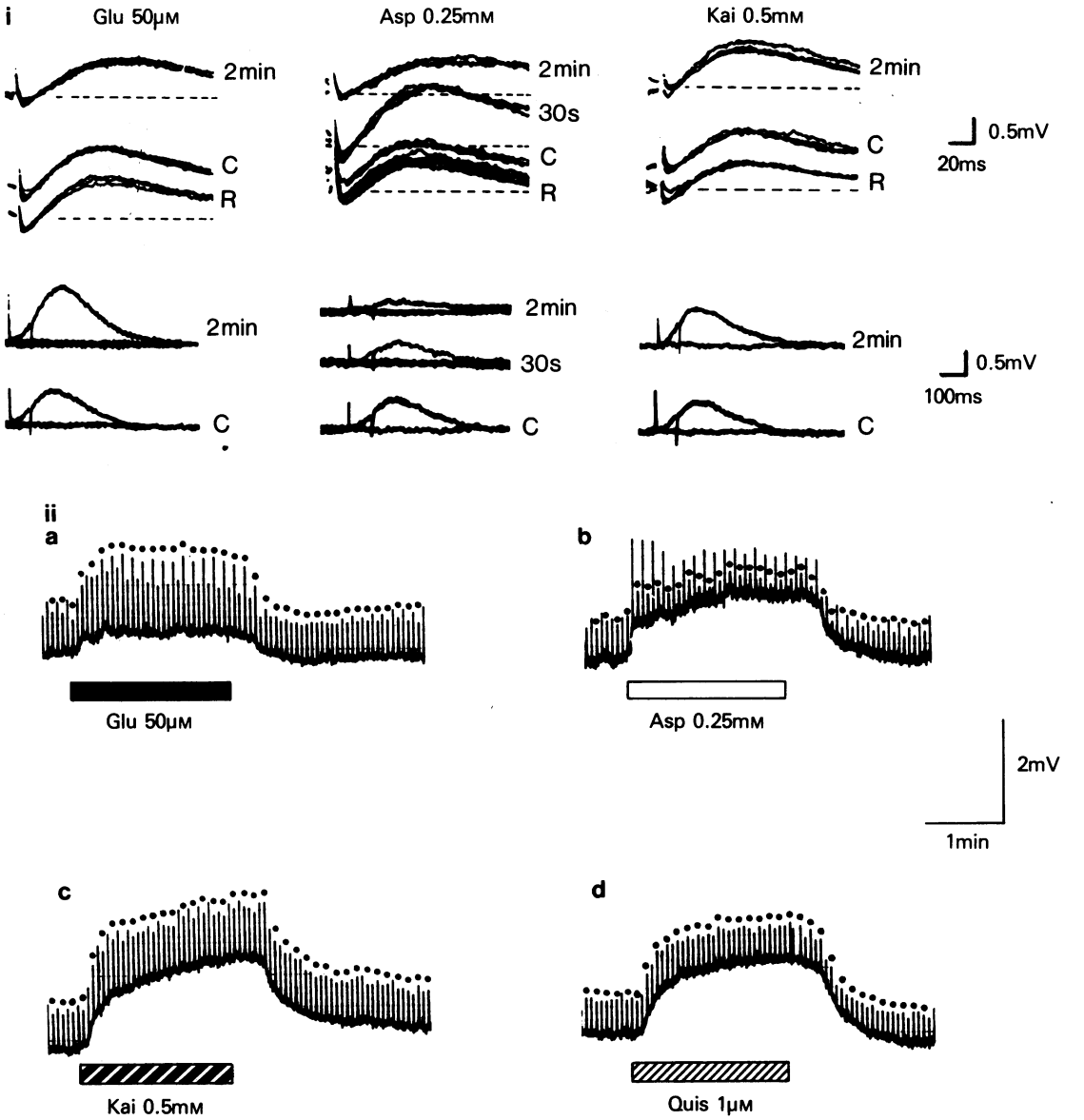

Figure 7 Effect of bath-applied depolarizing agonists on the amplitude and time course of alternate iontophoretic glutamate and aspartate potentials (evoked 1 every 4 s) recorded at the same site on the fibre membrane. (i) Upper set: glutamate potentials on fast time sweep; lower set: aspartate potentials (ejection parameters for glutamate and aspartate were 450 nA, 20 ms, $i_{ret} = +30$ nA and 130 nA, 90 ms, $i_{ret} = +5$ nA respectively). Several sweeps were superimposed. Each column shows the effect of applying a different agonist (glutamate, aspartate or kainate) where (C) is the control potential; (30 s, 2 min) are the potentials recorded after 30 s or 2 min exposure to agonist and (R) is the recovery potential after 3 min wash. Aspartate recovery potentials were exactly superimposed on control. (ii) Corresponding chart record of experiment shown in (i), displaying successive aspartate (dots) and glutamate potentials (1 every 4 s). Bars below traces indicate periods when agonists were bath-applied: (a) glutamate; (b) aspartate; (c) kainate or (d) quisqualate (not shown in (i)). Note the clearly contrasting effects of glutamate, aspartate and kainate on the iontophoretic potentials but the absence of any effect of quisqualate.

obtained when choline was substituted for Na^+, although choline solutions produced a consistent and irreversible increase in membrane resistance (30%).

It was of interest to test whether the ability of glutamate to enhance aspartate would be affected in a Na^+-free medium. We established initially that a 5 min application of 100 μM glutamate enhanced the aspartate response even after a wash interval of 30 min (the latter period was required for full recovery of the aspartate response after return to normal solution from Na^+-free). After a 5 min application of 100 μM glutamate in Na^+-free solution and immediate return to normal solution, the aspartate response tested after 30 min did not show any enhancement; the presence of Na^+ was therefore necessary for both the glutamate depolarization and for conditioning the muscle fibre towards aspartate. When glutamate was subsequently reapplied in normal solution, the usual aspartate enhancement could be demonstrated. We previously noted that small doses of glutamate (25 to 50 μM) but not aspartate were more effective in a Na^+-free medium (Constanti & Nistri, 1976b); however, in this solution, a conditioning dose of 25 μM glutamate did not enhance the effect of aspartate.

In similar experiments, the effect of Na^+-removal on the aspartate-enhancing action of kainate was investigated. In contrast to glutamate, responses to kainate (1 or 2 mM for 5 min) in Na^+-free solution were hardly affected (cf. Constanti & Nistri, 1976b) and 30 min after return to normal solution, the aspartate response was *still* markedly enhanced. With kainate therefore, the presence of Na^+ was not apparently important for either evoking the response or for producing the conditioning effect.

Preparations stored overnight. On 4 occasions, the long-term viability of the enhancement phenomenon was examined by storing a dissected preparation overnight in lobster saline solution at 5°C, to be used for an experiment the following day. Intact muscle fibres in such preparations had resting potentials and membrane resistances in the control range (about −76 mV; 50 kΩ respectively) and showed the usual sensitivity to depolarizing agonists (cf. Frank, 1974). However, the initially large response given by aspartate at the beginning of experiments was now absent, and a plateau response level was attained after fewer repeated applications of aspartate; the enhancement of aspartate responses after conditioning with agonists was not noticeably affected.

Iontophoresis experiments

Effect of bath-applied agonists on glutamate and aspartate potentials. Glutamate and aspartate were applied iontophoretically with a twin-barrelled pipette to single sensitive sites on the membrane. At these sites, brief depolarizations (glutamate potentials) of about 0.5 to 6 mV amplitude and with time to peak (t_{max}) ⩽ 100 ms were obtained. Aspartate potentials however, were consistently small in amplitude (about 0.5 to 1.5 mV), had a relatively long time course (t_{max} > 200 ms), and were enhanced after an iontophoretic application of glutamate only when the two agents were pulsed within about 200 ms of each other; the long-term interaction between glutamate and aspartate as seen with bath-applications was not therefore detectable in iontophoresis experiments (Constanti & Nistri, 1978). Occasionally, no aspartate potential was visible at glutamate-sensitive spots even when very high ejecting currents (up to 2 μA) were used.

We attempted to answer three specific questions; first, whether a bath-application of glutamate would enhance the aspartate potential and if so, would such an enhancement be maintained for a long time after the washout of glutamate (cf. Figure 2); second, whether bath applications of kainate or domoate would enhance the aspartate potential and third, would bath-applied agonists induce sites that were insensitive to iontophoretic aspartate to become responsive to this amino acid?

Figure 7i shows glutamate and aspartate potentials obtained either in control solution (C) or after 30 s to 2 min exposure to bath-applied agonist; the corresponding chart records are shown in Figure 7ii (aspartate potentials are marked by dots above the trace). In 50 μM glutamate, the glutamate potential was slightly enhanced initially, but then depressed with a prolongation in time course; the aspartate potential on the other hand, was clearly enhanced and the t_{max} increased. The aspartate potential attained a steady amplitude while glutamate was present (Figure 7iia) and was not further enhanced by a longer exposure to glutamate (not shown). On washout of glutamate, the iontophoretic potentials returned to control level within 3 min. In the presence of 0.25 mM aspartate, the reverse result was obtained whereupon the glutamate potential was enhanced initially but then depressed and prolonged (cf. Constanti & Nistri, 1978) whereas the aspartate potential was almost abolished (Figure 7iib). Again, recovery was rapid after washout of agonist, although after aspartate, the recovery was normally slower than after glutamate. It is important to mention that following repeated doses of bath-applied aspartate, the resulting membrane depolarizations declined to a plateau as described earlier. However, the effects on the glutamate and aspartate potentials were unchanged. Kainate (0.5 mM) or domoate (50 μM, not shown) produced only a small enhancement of the aspartate and glutamate potentials (Figure 7iic) whereas quisqualate (1 μM) had no effect on either (Figure 7iid); these enhancement effects could not therefore have been a

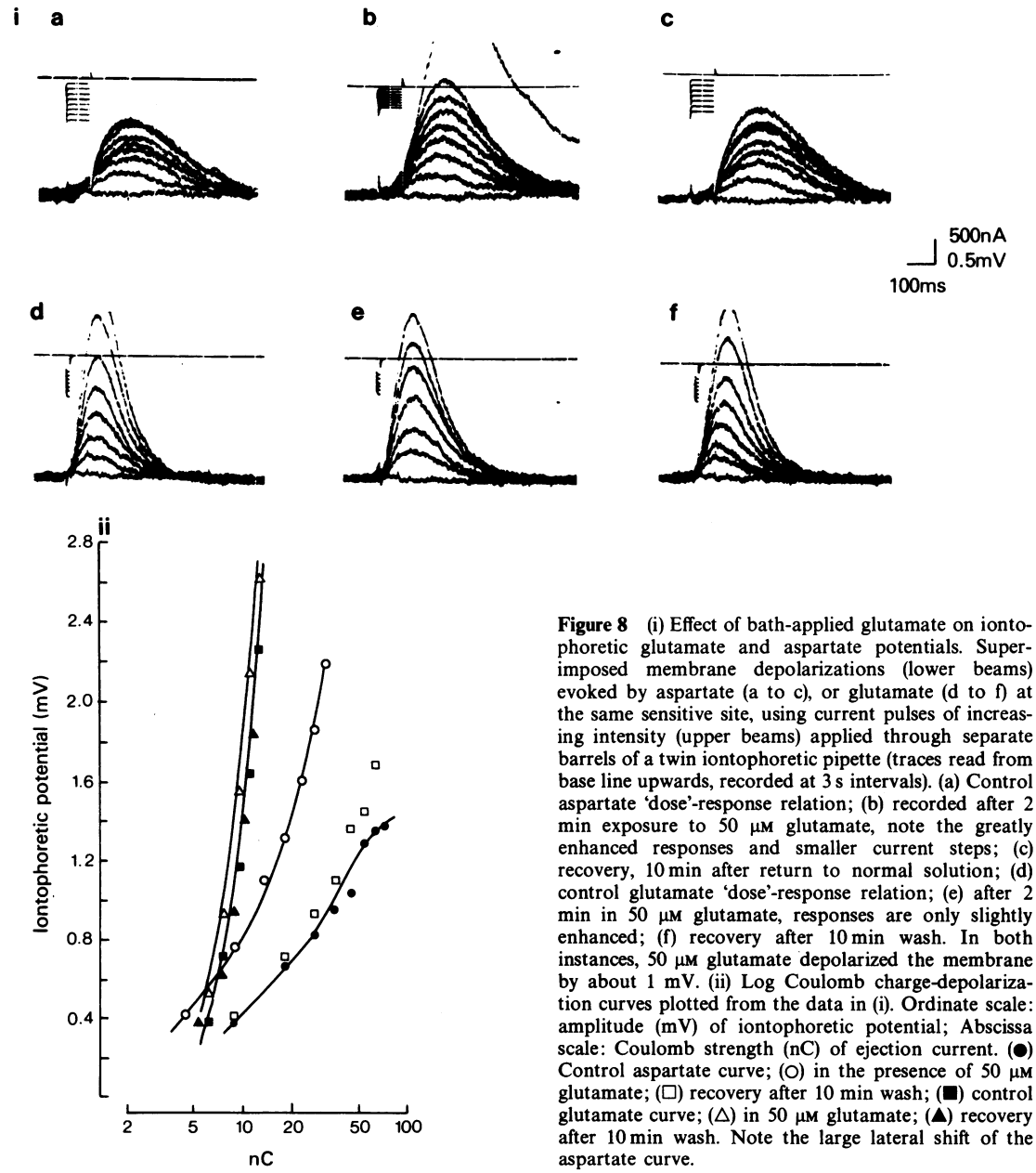

Figure 8 (i) Effect of bath-applied glutamate on iontophoretic glutamate and aspartate potentials. Superimposed membrane depolarizations (lower beams) evoked by aspartate (a to c), or glutamate (d to f) at the same sensitive site, using current pulses of increasing intensity (upper beams) applied through separate barrels of a twin iontophoretic pipette (traces read from base line upwards, recorded at 3 s intervals). (a) Control aspartate 'dose'-response relation; (b) recorded after 2 min exposure to 50 μM glutamate, note the greatly enhanced responses and smaller current steps; (c) recovery, 10 min after return to normal solution; (d) control glutamate 'dose'-response relation; (e) after 2 min in 50 μM glutamate, responses are only slightly enhanced; (f) recovery after 10 min wash. In both instances, 50 μM glutamate depolarized the membrane by about 1 mV. (ii) Log Coulomb charge-depolarization curves plotted from the data in (i). Ordinate scale: amplitude (mV) of iontophoretic potential; Abscissa scale: Coulomb strength (nC) of ejection current. (●) Control aspartate curve; (○) in the presence of 50 μM glutamate; (□) recovery after 10 min wash; (■) control glutamate curve; (△) in 50 μM glutamate; (▲) recovery after 10 min wash. Note the large lateral shift of the aspartate curve.

mere consequence of the membrane depolarization evoked by bath-applied agonists.

The enhancement of the aspartate potential by glutamate was further investigated by comparing iontophoretic Coulomb dose vs. response relationships for glutamate and aspartate at single sites (Figure 8i). Figure 8ii shows that the dose-depolarization curve for aspartate was much less steep than that for glutamate (limiting log-log slopes were 0.8 and 3.1 respectively; cf. the values of 0.8 and 2.1 obtained for bath-applied aspartate and glutamate in Constanti & Nistri, 1978) and that in the presence of 50 μM glutamate, the aspartate curve was clearly shifted to the left along the abscissa scale with no indication of a maxi-

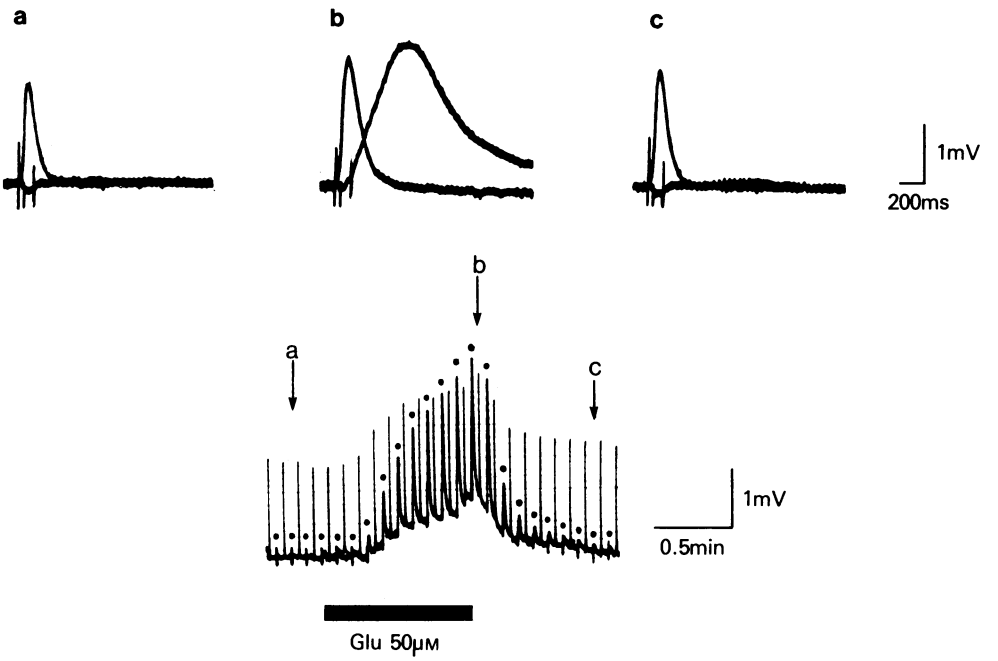

Figure 9 Effect of bath-applied glutamate on iontophoretic glutamate and aspartate potentials. At this site, no aspartate potential was visible at the beginning of the experiment. Ejection currents (recorded but not shown) were 365 nA, 20 ms, $i_{ret} = +10$ nA for glutamate and 600 nA, 80 ms, $i_{ret} = +5$ nA for aspartate. (a) Control glutamate and aspartate 'potentials' superimposed (1 pulse every 3 s); (b) after 1 min in 50 μM glutamate, the aspartate potential became visible and its amplitude exceeded that of the glutamate potential; (c) recovery potentials, 80 s after washing out glutamate. The corresponding chart record is shown below (dots indicate aspartate potential; arrows show points where frames were taken).

mum (in the range studied). In contrast, the glutamate curve was only slightly displaced to the left. Interestingly, the log-log slope of the aspartate curve in glutamate solution was hardly affected, suggesting that the kinetics of the aspartate/receptor interaction were unaltered.

Finally, at 3 out of 5 sites where no aspartate potential could be evoked, application of 50 or 100 μM glutamate depolarized the membrane and caused the gradual appearance of an aspartate potential; the amplitude of this potential increased dramatically during the depolarization and eventually exceeded the glutamate potential, which itself, was only slightly enhanced (Figure 9). After washout of glutamate, the aspartate potential rapidly declined in amplitude until eventually it was no longer visible. Bath-applications of kainate, domoate, aspartate or quisqualate depolarized the membrane but failed to reveal an aspartate potential. In the other instances, glutamate (or other agonists) produced the expected effects on the glutamate potential (cf. Figure 7) but never caused the aspartate potential to become visible; perhaps at these sites, the fibres were truly insensitive to aspartate.

It is generally believed that the synergism seen when glutamate and aspartate are bath-applied in combination onto crustacean muscle is due to an enhancement, by aspartate, of the effects of glutamate (Shank & Freeman, 1975; Shank *et al.*, 1975; Crawford & McBurney, 1977a; Dudel, 1977). Our finding above that glutamate can enhance aspartate effects clearly indicates that this phenomenon must also be taken into consideration in any future analysis of glutamate/aspartate synergism.

Discussion

Bath-application experiments

The sustained enhancement of the effect of one synaptically active agent by a previous conditioning appli-

cation of another is not a new concept. For example, in the superior cervical ganglion, bath-applied dopamine can potentiate responses to cholinoceptor agonists over a period of hours (Libet, Kobayashi & Tanaka, 1975). In guinea-pig liver slices, some α-adrenoceptor agonists enhance subsequent hyperpolarizing responses to β-adrenoceptor agonists (Jenkinson & Koller, 1977). However, at crustacean synapses, such a 'modulatory' role of glutamate towards the effects of aspartate has not been reported previously.

We have shown that bath-application of glutamate onto lobster muscle can potentiate aspartate-evoked responses long after glutamate has been washed away and at a time when the membrane potential and conductance have returned to their control level (Constanti & Nistri, 1978). We also observed that the aspartate enhancement was dependent upon the conditioning concentration and length of exposure to glutamate, the time interval between glutamate and aspartate applications and the presence of Na^+ in the bathing medium; this enhancement was not prevented by the overnight storage of preparations *in vitro*. Since we could not detect a change in the limiting log-log slope of the aspartate dose-depolarization curve during the enhancement, then glutamate was probably not affecting the order of the aspartate/receptor interaction.

We previously proposed (Constanti & Nistri, 1978) that bath-applied aspartate was exerting a weak-postsynaptic agonist effect on the fibre membrane as well as an indirect effect through release of glutamate from an unidentified store. When the muscle was conditioned with glutamate, some of this amino acid was presumably trapped by the store then released slowly during washout unless accelerated by bath-application of aspartate. The initial aspartate responses at the beginning of an experiment might have been large because of release of trapped glutamate originating from the haemolymph (Shank *et al.*, 1975) and/or tissues damaged during the dissection; the results of the overnight storage experiments would indeed support this latter proposal (the existence of a store that can trap bath-applied agonists has also been suggested by Adams (1975) to explain the current 'humps' seen following washout of cholinoceptor agonists from frog muscle). On this basis, our present data would indicate that the glutamate store has a finite capacity although it shows no stereospecificity since both D or L-glutamate enhanced D or L-aspartate responses. The finding that kainate and domoate, two heterocyclic glutamate analogues (Shinozaki & Shibuya, 1974b; 1976; Takeuchi & Onodera, 1975) also enhanced aspartate responses implies that some other agonists besides glutamate can be trapped, although the store may show different capacities and rates of spontaneous release for these different agents. There were three particular points of interest: (i), aspartate itself was not apparently trapped since large doses of aspartate did not enhance subsequent aspartate effects (Figure 5); (ii) in the presence of aspartate, other agonists were not trapped since their ability to enhance aspartate responses was abolished by including aspartate during conditioning and (iii), the shape of enhanced aspartate responses always resembled that of the previous conditioning agonist (Figure 4). All these findings suggest that release of glutamate (or the other agonists) from a store plays some role in the long-term enhancement of aspartate effects.

Up to now, we have conveniently explained our observations in terms of a simple binding and displacement model; however, the fact that the conditioning effect of glutamate could be prevented by Na^+-removal suggests the involvement of a glutamate transport system in the enhancement effect particularly since such systems in both crustacean (Iversen & Kravitz, 1968; Baker & Potashner, 1971) and mammalian (Bennett, Logan & Snyder, 1973) neural tissues are strongly Na^+-dependent. Glutamate sequestered during conditioning would thus be liberated following competition by aspartate for the carrier. However, in order to explain all our results, we have to imply that kainate and domoate (but not quisqualate) were also transported by the glutamate (or some other) carrier in a Na^+-independent manner. This possibility has yet to be tested experimentally although it is relevant that the uptake of glutamate into crustacean muscle is unaffected by the presence of kainate (Shinozaki & Shibuya, 1974b).

Iontophoresis experiments

Further insight into the possible mechanism of aspartate enhancement by agonists was provided by the results of iontophoresis experiments. When glutamate and aspartate were iontophoretically-applied onto single sites, no mutual interaction was seen other than that expected for the summation of two agonists in the receptor region (Constanti & Nistri, 1978). On the other hand, when glutamate and aspartate (and to a slight extent kainate and domoate but not quisqualate) were bath-applied, the effects on the iontophoretic glutamate and aspartate potentials were quite different and were not maintained after washout; these effects could not therefore be responsible for the long-term enhancement of aspartate action seen in bath-application experiments.

On crayfish muscle, kainate and domoate are considered to act mainly on the extrajunctional glutamate receptors that surround the junctional glutamate-sensitive sites (Shinozaki & Shibuya, 1974b; Takeuchi & Onodera, 1975) while quisqualate is thought to act predominantly on the junctional receptors (Shinozaki & Shibuya, 1974a). Since bath-

application of agonists is likely to influence extrajunctional membrane areas more than iontophoretic applications, we tentatively suggest that during conditioning with bath-applied glutamate, kainate or domoate, some agonist remains bound to extrajunctional sites and is subsequently displaced by bath-applied aspartate to produce an enhanced response. Presumably, aspartate has a high binding and displacing ability but is only weakly retained, whereas the other agonists have a slow dissociation rate from these sites. In keeping with this proposal is the suggestion that the glutamate analogue ibotenic acid might bind to extrajunctional glutamate receptors on feline spinal interneurones for up to 1 h (MacDonald & Nistri, 1978).

The contrasting effects produced by various bath-applied agonists on iontophoretic glutamate and aspartate potentials require a different explanation. On crayfish muscle, aspartate produces no depolarization; however, in low doses it enhances glutamate effects, possibly by diminishing glutamate desensitization. At higher doses, aspartate antagonizes glutamate by a competitive mechanism (Dudel, 1977). On lobster muscle, where aspartate does have a depolarizing action, the initial increase and subsequent depression of glutamate potentials in the presence of aspartate might also be explained partly by the block of desensitization and partly by agonist summation followed by mutual antagonism.

Since aspartate potentials were either enhanced or 'unmasked' by glutamate, it is possible that rapid desensitization to iontophoretic aspartate developed and that glutamate somehow reduced this phenomenon. This interpretation is not inconceivable since (i), aspartate potentials were depressed by bath-applied aspartate and (ii), the iontophoretic aspartate dose-response curve was displaced to the left by glutamate with no change in limiting slope, indicating an increased effectiveness without change in order of reaction. However, whereas desensitization to glutamate can be readily demonstrated by repeated iontophoretic applications (Nistri & Constanti, 1978) such conventional desensitization to iontophoretic aspartate was never visible in our experiments. Nevertheless, if the rate of onset of aspartate desensitization was faster than the rate of generation of the iontophoretic potential, then the observed responses would always be partially desensitized. This 'occult' desensitization (Werman, 1976) has been shown not to alter estimates of drug/receptor kinetics even though the response amplitude may be decreased. It is important to stress that the long-term enhancement of bath-applied aspartate responses by glutamate could not be explained by blockade of either 'occult' or conventional desensitization (cf. Constanti & Nistri, 1978) particularly since the shape of the enhanced aspartate response differed according to the previous conditioning agonist and the simultaneous bath-application of aspartate and glutamate prevented rather than induced the enhancement of subsequent aspartate effects.

In conclusion, our present findings suggest that extrajunctional receptor sites may be important in determining the action of excitatory amino acids on lobster muscle. As the influence of these sites was apparently maintained for relatively long periods, it is tempting to suppose that long-term 'modulation' of postsynaptic responses to these amino acids might generally arise outside the synapse itself from areas considered to have little functional significance.

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