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The dependence of glutamate uptake by crab nerve on external Na^+ and K^+

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SUMMARY

Glutamate uptake by crab nerve is activated by external Na^+ and inhibited by external K^+. Kinetic analysis indicates that two Na^+ or K^+ are required for activation or inhibition.

The cellular concentrations of glutamate and aspartate in the walking leg nerves of the spider crab, *Maia squinado,* are 37 and 225 mmoles/kg nerve, respectively. If these negatively charged amino acids are passively distributed across the nerve cell membrane with an internal potential of about −60 mV, they should each be 10-fold more concentrated in the external medium. Although the *in vivo* external concentrations are somewhat in doubt they are much less than 1 mM. It follows that the distribution of glutamate and aspartate is unlikely to be passive. The high cellular levels of these amino acids may be explained in a number of ways: (1) the activity of cellular glutamate and aspartate may be much less than the measured concentration; (2) they may be synthesized inside the cells faster than they can leak out, or (3) they may be concentrated by an active uptake process. The first suggestion is unlikely as glutamate and aspartate are two of the major anions in crab nerve[1]; but it seems probable that the other two mechanisms may contribute to maintaining the high intracellular concentration of these amino acids. The present report is concerned with the mechanism of glutamate uptake. This is of interest not only because glutamate can be accumulated against a steep electrochemical gradient but also because glutamate is thought to be a transmitter substance in crab nerve[2-4], and uptake into the nerve may be an important means of terminating its transmitter action.

Nerves were removed from the walking legs and claws and kept for up to 1 h at room temperature in artificial sea water containing 10 mM KCl, 460 mM NaCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$ and 2.5 mM $NaHCO_3$ (10 mM K^+ (Na^+) artificial sea water)[5]. The nerves were subsequently incubated for 5 or 10 min at 16° in various test solutions.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

When the concentration of Na^+ or K^+ in these media was varied, choline$^+$ was used to maintain isotonicity. Glutamate influx was followed with L-[U-^{14}C] glutamate. After incubation, nerves were removed from the test solutions and washed in ice-cold artificial sea water to remove extracellular radioactivity. They were then blotted on filter paper, their middle portions digested in Nuclear Chicago Solubilizer (NCS) and counted by liquid scintillation. The counts were corrected for quenching. Over 90% of the radioactivity in the nerves was extractable with 5% trichloroacetic acid and identified as glutamate. It should be stressed that whole nerve trunks were used in these experiments and it was not possible to determine whether glutamate was transported into both axons and surrounding Schwann cells or into only one of these compartments.

As glutamate uptake increased linearly for over 1 h an accurate measure of influx was obtained by determining uptake over a 5- to 10-min period. The influx was expressed as mmoles of glutamate per kg nerve (excluding extracellular fluid which constitutes 30% of the wet weight of blotted nerves[5]) per h.

The influx was very dependent on external Na^+. In the absence of added external Na^+, influx increased linearly with external glutamate concentration over the range 0.2 to 100 mM. At all glutamate concentrations, the influx in the absence of external Na^+ was less than that in its presence. This Na^+-insensitive influx – which amounted to 0.130 ± 0.006 mmole/kg nerve per h per mM increase in external glutamate concentration in fed crabs and approximately half that in starved crabs – has been deducted from the total glutamate influx observed with external Na^+ and the difference termed the Na^+-sensitive influx. Figs. 1a and 1b show the dependence of the Na^+-sensitive glutamate influx on external Na^+. The most striking feature of the curves in Fig. 1a is their definite sigmoid character. The Lineweaver-Burk analysis, shown in Fig. 1b, reveals that the reciprocal of the Na^+-sensitive influx is a linear function of $[Na^+]_0^{-2}$ which suggests that two Na^+ may act as co-substrates with glutamate.

Two other possible explanations of the sigmoid activation curves are: (1) Choline$^+$ which was used as a substitute for external Na^+ may have some inhibitory action which becomes increasingly apparent at low Na^+, high choline$^+$ concentrations. This is unlikely as similar experiments in which sucrose was used to replace external Na^+ gave results which were not significantly different from those of the middle curve in Fig. 1a. (2) Under conditions of low external Na^+ when the glutamate influx is reduced there is usually a rise in intracellular ionized Ca^{2+} due to an increase in Ca^{2+} influx and a decrease in Ca^{2+} efflux[6,7]. Since high levels of Ca^{2+} are known to have adverse effects on several transport systems, the possibility that intracellular Ca^{2+} influenced the shape of the activation curves was examined by repeating several of the experiments in Ca^{2+}-free sea water containing 1 mM EGTA. The open squares in Fig. 1b represent these experiments; the results do not show any significant deviations from the observations with Ca^{2+}-containing sea waters. The conclusion from these experiments is that Na^+-sensitive glutamate influx requires two Na^+. This would be strengthened if a coupling constant of 2 was found to relate Na^+ influx and Na^+-sensitive glutamate influx. However, determination of the coupling constant is extremely difficult experimentally as the total Na^+ influx in crab nerve is at least two orders of magnitude greater than the maximum Na^+-sensitive glutamate influx. The Na^+ requirements of this glutamate uptake system in crab nerve are strikingly similar to those of the glycine transport system described by Vidaver[8] in pigeon red cells.

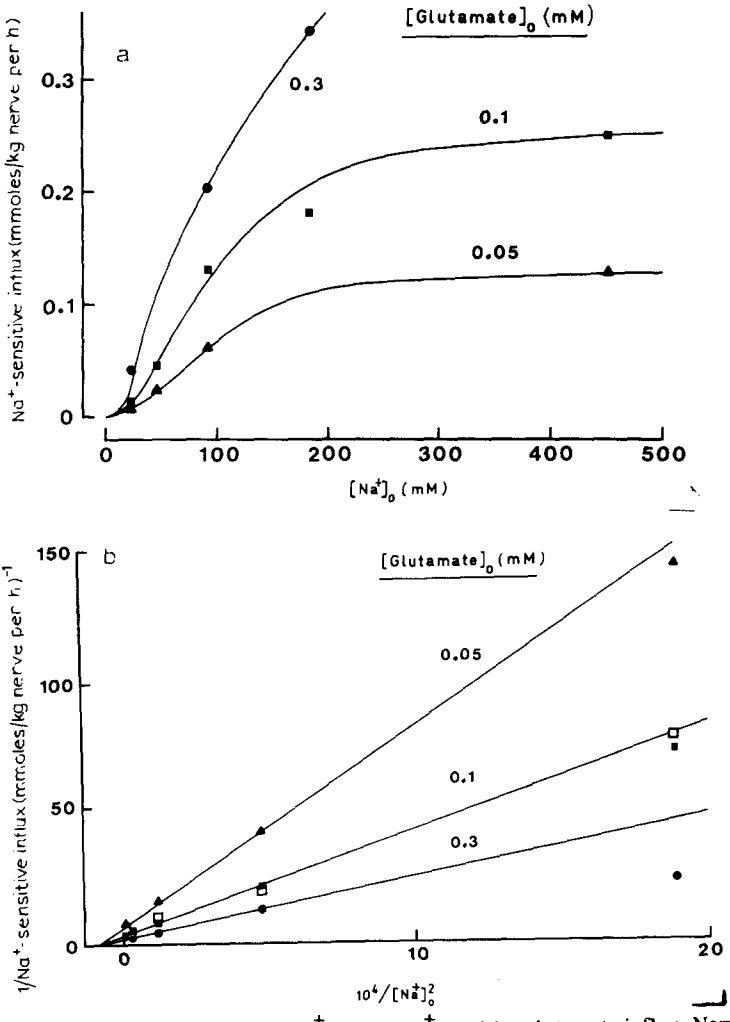

Fig. 1. The effect of external Na^+ on the Na^+-sensitive glutamate influx. Nerves kept in 10 mM K^+ (Na^+) artificial sea water were blotted on filter paper and transferred to test solutions containing $5 \cdot 10^5$–$6 \cdot 10^5$ counts/min per ml of L-[U-^{14}C]glutamate and the external concentrations of Na^+ and unlabelled L-glutamate indicated above. K^+ was constant at 10 mM and choline$^+$ replaced external Na^+. The solid symbols represent results with test solutions containing 11 mM Ca^{2+} while the hollow squares in Fig. 1b are points obtained with Ca^{2+}-free solutions containing 1mM EGTA. The concentration of external Na^+ required for half-maximal influx was approximately 90 mM.

There is some evidence that external K^+ acts as a competitive inhibitor of Na^+ in several Na^+-sensitive transport systems[9,10], but the glycine system in pigeon red cells exhibits no response to changes in the concentration of external K^+ (ref. 8). Fig. 2a shows that the Na^+-sensitive glutamate influx in crab nerve is sensitive to the level of external K^+ and that K^+ and Na^+ have opposite effects on the influx. Furthermore, the Lineweaver-Burk analysis, shown in Fig. 2b, and the corresponding Dixon plot (not shown) both indicate that K^+ is a competitive inhibitor of Na^+. It is also of interest

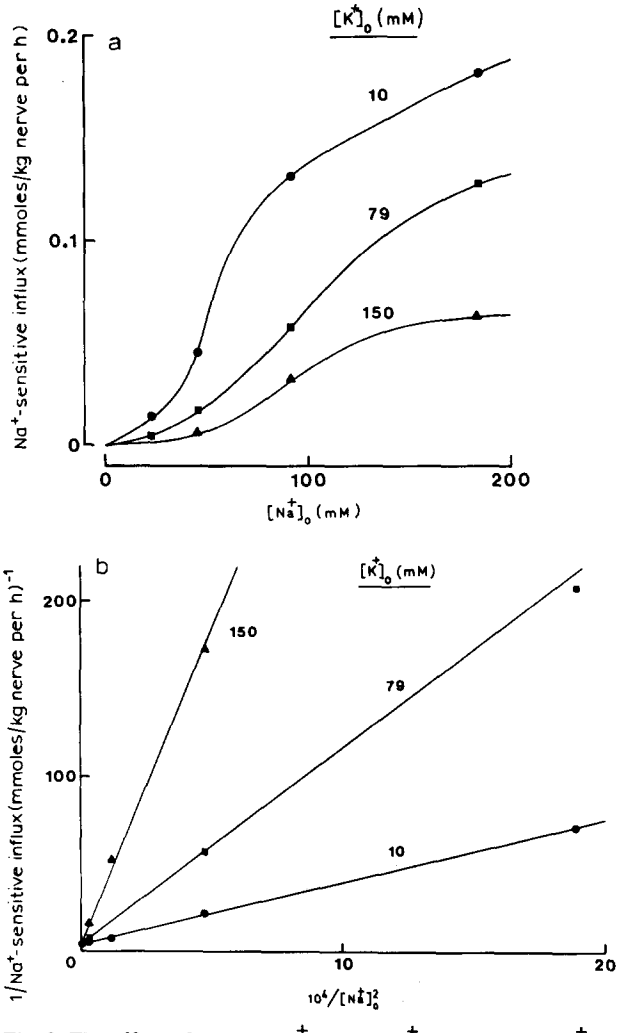

Fig. 2. The effect of external K^+ on the Na^+ activation of Na^+-sensitive glutamate influx. Test solutions contained 0.1 mM L-[U-^{14}C]glutamate (specific activity, $5 \cdot 10^3$–$6 \cdot 10^3$ counts/min per nmole) and the concentrations of external Na^+ and K^+ indicated above. Choline$^+$ was used to maintain isotonicity.

to note that the Na^+-insensitive glutamate influx does not respond to changes in external K^+ (Table I).

The specificity of the uptake process for glutamate was examined in several experiments in which the nerves were exposed to glutamate in the presence of a 10-fold excess of various amino acids and analogues. Table II summarises the results which are graded so that the strongest inhibitor of glutamate influx appears at the top of the list. Amino acids which inhibit the total influx usually decrease the Na^+-insensitive component but to a lesser degree. Exceptions are L-(γ-methyl)glutamate and norvaline both of which affect the Na^+-insensitive component to a slightly greater degree than the

total influx. It should be stressed that the Na^+-insensitive influx that would be generated by the concentration of glutamate that was present in 10 mM K^+ (Na^+) artificial sea water (0.2 mM) was estimated to be only 4% of the total influx so that inhibitions of the total influx were due almost entirely to interference with the Na^+-sensitive component.

TABLE I

THE EFFECT OF EXTERNAL K^+ ON THE Na^+-INSENSITIVE GLUTAMATE INFLUX

Conditions were identical to those in Figs. 2a and 2b except that external Na^+ was completely replaced by choline$^+$. The uptakes are expressed as means ± S.E. with the number of experiments in parentheses.

External glutamate (mM)	*Influx (mmoles/kg nerve per h) in Na^+-free sea water containing K^+ at*		
	10 mM	*79 mM*	*150 mM*
0.05	0.007 ± 0.002 (3)	0.006 ± 0.001 (3)	0.008 ± 0.001 (3)
0.10	0.012 ± 0.001 (6)	0.013 ± 0.001 (15)	0.012 ± 0.001 (11)
0.30	0.031 ± 0.001 (13)	0.038 ± 0.002 (6)	0.044 ± 0.003 (3)

TABLE II

THE EFFECT OF OTHER AMINO ACIDS AND ANALOGUES ON GLUTAMATE INFLUX

Nerves kept in 10 mM K^+ (Na^+) artificial sea water were blotted on filter paper and placed in test solutions consisting of 10 mM K^+ (Na^+) artificial sea water or 10 mM K^+ (choline$^+$) artificial sea water, L-glutamate, L-[U-^{14}C]glutamate ($5 \cdot 10^5$-$6 \cdot 10^5$ counts/min per ml) and the various compounds listed below. These additions were present at 2 mM in 10 mM K^+ (Na^+) artificial sea water or 20 mM in 10 mM K^+ (choline$^+$) artificial sea water. DL mixtures were present at twice the concentrations of L isomers. In the absence of additions, glutamate influx in 10 mM K^+ (Na^+) artificial sea water was 0.400 ± 0.044 (18 experiments) mmole/kg nerve per h and that in 10 mM K^+ (choline$^+$) artificial sea water was 0.146 ± 0.008 (15 experiments) mmole/kg nerve per h. The experiments were performed on unfed animals so Na^+-insensitive component is approximately half of its normal value. Na^+-insensitive influx at an external glutamate concentration of 0.2 mM can be calculated on the assumption that the influx in Na^+-free sea water is linear.

Addition	*% Inhibition of*	
	Influx from 0.2 mM L-glutamate in 10 mM K^+ (Na^+) artificial sea water	*Influx from 2.0 mM L-glutamate in 10 mM K^+ (choline$^+$) artificial sea water*
L-Cysteic acid	94.1	−4.5
L-Aspartic acid	89.6	32.6
L-Leucine	54.2	38.4
L-Phenylalanine	51.8	35.8
Glycine	46.3	8.9
L-Alanine	45.8	28.3
L-α-Aminoisobutyric acid	33.5	12.9
Taurine	32.4	6.9
L-(γ-Methyl)glutamic acid	25.6	35.8
L-Glutamine	21.5	17.9
n-Valeric acid	20.5	−12.0
DL-Norvaline	15.8	19.6
L-α-Aminobutyric acid	12.7	30.4
L-Lysine	3.5	−12.0
γ-Aminobutyric acid	−7.0	−7.5

Several conclusions concerning the structural requirements of the Na^+-sensitive system can be formulated on the basis of the inhibitory patterns. For example, since glutamine L-(γ-methyl)glutamate, norvaline and α-aminobutyric acid do not inhibit uptake extensively it seems likely that the Na^+-sensitive system requires the presence of a free carboxyl group in the C-5 position. The strong interactions of aspartic and cysteic acids suggest that acidic groups in the C-4 position are also acceptable. The lack of inhibition by γ-aminobutyric acid may reflect a requirement for the C-1 carboxyl group. Judging from the degree to which acidic amino acids inhibit the glutamate influx, it seems likely that they and glutamate are handled predominantly by the same system. The Na^+-insensitive component is more difficult to inhibit: at least 60% of the influx in 10 mM K^+ ($choline^+$) artificial sea water could not be reduced even in the presence of those compounds which strongly inhibited the Na^+-sensitive component. This difference between the two components in addition to their differing responses to changes in external K^+ (Fig. 1 and Table I) and glutamate suggests that the Na^+-sensitive and Na^+-insensitive uptakes may reflect two separate systems and not merely different operational modes of a single system.

As the glycine system in pigeon red cells is the only other amino acid transport system known to require two Na^+ as co-substrates[8,11], a brief general comparison with glutamate transport in crab nerve is of interest. Kinetic analysis suggests that the Na^+-sensitive influx in both tissues procedes *via* a similar mechanism. For instance, when the data in Fig. 1b is re-arranged so that the reciprocal of the external glutamate concentration appears on the abscissa, it becomes apparent that only the K_m for glutamate is altered by the progressive replacement of external Na^+ by $choline^+$. On the basis of a similar observation for glycine transport in pigeon red cells, Vidaver[8] proposed a kinetic mechanism requiring the obligatory combination of two Na^+ with the amino acid carrier prior to the attachment of the amino acid itself. Our results, although entirely consistent with the model, indicate that K^+ is an additional factor which must be considered in the crab nerve system. By re-arrangement of the data in Fig. 2b, it can be seen that both the reciprocal of the Na^+-sensitive influx and the external concentration of Na^+ required for half maximal influx are linear functions of $[K^+]_o^2$. These features require modification of the model to include the competitive displacement of two Na^+ from the carrier by two K^+ and the inability of the K^+-loaded carrier to bind glutamate.

The following equilibrium equations can be written for the reactions taking place during Na^+-sensitive glutamate influx.

$$C + Na^+ \rightleftharpoons CNa \quad (1)$$
$$CNa + Na^+ \rightleftharpoons CNa_2 \quad (2)$$
$$C + K^+ \rightleftharpoons CK \quad (3)$$
$$CK + K^+ \rightleftharpoons CK_2 \quad (4)$$
$$CNa_2 + \text{glutamate} \rightleftharpoons CNa_2\ \text{glutamate} \quad (5)$$
$$CNa_2\ \text{glutamate} \dashrightarrow \text{influx} \quad (6)$$

If K_1, K_2, K_3, K_4 and K_{Glu} are the respective equilibrium dissociation constants for Eqns. 1-5 and C is the amount of free carrier at the external surface of the membrane, provided the total amount of carrier is constant the equation describing the Na^+-sensitive glutamate influx is

$$v = \frac{v_{max}}{\frac{K_{Glu}}{[Glu]}\left[\frac{K_1 K_2}{[Na^+]^2}\left(\frac{[K^+]^2}{K_3 K_4} + \frac{[K^+]}{K_3} + 1\right) + \frac{K_2}{[Na^+]} + 1\right] + 1} \quad (7)$$

where v and v_{max} are the Na^+-sensitive glutamate influx and its maximum value; $[Na^+]$, $[K^+]$ and [Glu] are the concentrations of external Na^+, K^+ and glutamate, respectively. This equation was used to calculate the smooth curves drawn through the experimental points in Figs. 1a and 2a.

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