J. Physiol. (1983), **336**, *pp.* 433–451
With 9 text-figures
Printed in Great Britain

THE ROLE OF THE ELECTROGENIC SODIUM PUMP IN THE GLUTAMATE AFTERHYPERPOLARIZATION OF FROG SPINAL CORD

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(*Received 26 March 1982*)

SUMMARY

1. Drug responses of isolated hemisected frog spinal cords were examined by means of the sucrose-gap technique. The glutamate-induced depolarizations (glu-d) of motoneurones (recorded from ventral roots), and primary afferents (recorded from dorsal roots), were followed by an afterhyperpolarization (glu-a.h.). The depolarization induced by DL-homocysteic acid (DLH) was only occasionally followed by an afterhyperpolarization (DLH-a.h.).

2. The glu-a.h. on both roots persisted in the presence of tetrodotoxin (TTX, 0·1–1 μM), or Ringer solution containing 10 mM-Mg^{2+}; 0·1 mM-Ca^{2+} or 2 mM-Mn^{2+}; 0·2 mM-Ca^{2+}. This indicated that the response was neither due to the release of endogenous neurally active substances nor to the activation of a Ca^{2+}-sensitive K^+ conductance.

3. The glu-a.h. was reduced or blocked by K^+-free Ringer solution, 3-acetylstrophanthin (3-Ac-Str; 1 μM) or Li^+ ions, and was therefore attributed to the activity of the electrogenic Na^+ pump.

4. The duration of depolarization induced by glu or DLH was increased in the presence of K^+-free Ringer solution, 1 μM 3-Ac-Str or Li ions. It is therefore suggested that the electrogenic Na^+ pump may play a role in limiting the duration of depolarization induced by the action of excitatory amino acids.

5. The re-admission of K^+ ions to preparations which had been incubated in K^+-free Ringer solution produced a transient hyperpolarization (K-a.h.) of the membrane potential of ventral roots which is also attributable to the activation of the electrogenic Na^+ pump.

6. Both the K-a.h. and the glu-a.h. were enhanced in Ca^{2+}-free Ringer solution. It is therefore suggested that the Ca^{2+} ions may modulate the activity of the electrogenic pump in central nervous tissue.

INTRODUCTION

The acidic amino acids, L-glutamic acid (glu) and L-aspartic acid are likely candidates for excitatory neurotransmitters in the central nervous system (C.N.S.)

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(Krnjević, 1974; Watkins & Evans, 1981). In previous experiments on the isolated hemisected amphibian spinal cord it has been noted that the glu-induced depolarization of both motoneurones (recorded from ventral roots) and primary afferents (recorded from dorsal roots) is followed by an afterhyperpolarization (glu-a.h.) (Barker, Nicoll & Padjen, 1975; Constanti & Nistri, 1976; Sonnhof, Graefe & Krumnikl, 1976; Kudo, 1978; Padjen & Smith, 1980*a*, *b*; Padjen & Smith, 1981). The question therefore arises as to whether glu can elicit direct hyperpolarizing responses as has been observed in the nervous system of certain invertebrates (cf. Szczepaniak & Cottrell, 1973) or whether the glu-a.h. is generated by some other mechanism, such as electrogenic Na^+ pumping as originally considered by Zieglgaensberger & Puil (1973). In sympathetic ganglia, the depolarization induced by the nicotinic action of acetylcholine (ACh) is also followed by an afterhyperpolarization (ACh-a.h.) (Pascoe, 1956). Several studies on sympathetic ganglia of various species have indicated that this ACh-a.h. is generated by the activity of the electrogenic Na^+ pump (Brown, Brownstein & Scholfield, 1972; Lees & Wallis, 1974; Libet, Tanaka & Tosaka, 1977; Smith & Weight, 1977). ACh promotes an inward flux of Na^+ ions which accumulate intracellularly. This is thought to activate the Na^+ pump and the consequent electrogenic extrusion of Na^+ ions (Thomas, 1972). This, in turn, would account for the hyperpolarization of membrane potential below its resting level following the action of depolarizing agonists. It should be noted that the ACh-a.h. is quite distinct from the well known atropine-sensitive slow inhibitory post-synaptic potential observed in sympathetic ganglia (cf. Smith & Weight, 1977). The present series of experiments were therefore designed to test whether the glu-a.h. in spinal cord, by analogy with the ACh-a.h. in sympathetic ganglia, is generated by the activity of the electrogenic Na^+ pump. It has already been shown that glu-a.h. in cultured spinal neurones is generated by this mechanism (Ransom, Barker & Nelson, 1975). On the other hand it was recently suggested that glu-a.h. in hippocampal cells is generated by a Ca^{2+}-sensitive K^+ conductance (Nicoll & Alger, 1981). In the present experiments we show that the glu-a.h. of both motoneurones and primary afferents in intact amphibian spinal cord was specifically depressed by Na^+ pump inhibitors but was not depressed by treatments which should antagonize Ca^{2+}-sensitive K^+ conductances. Indeed, removal of extracellular Ca^{2+} ions potentiated the glu-a.h. Possible mechanisms for this potentiation are discussed. Preliminary accounts of some of this work have been presented previously (Padjen & Smith, 1980*a*, *b*).

METHODS

The membrane potential of both dorsal roots (primary afferents) and ventral roots (motoneurones) of isolated hemisected frog (*Rana pipiens*) spinal cords was recorded by means of the sucrose-gap technique as previously described (Barker *et al.* 1975; Padjen & Smith, 1980*c*). In some experiments dorsal roots were stimulated through bipolar platinum electrodes and the resulting synaptic response, the dorsal root–ventral root potential (d.r.–v.r.p.), examined by the sucrose-gap recording. Drugs were applied by superfusion in the compartment bathing the spinal cord via a set of stopcocks or solenoid valves (General Valve). Indirect responses were blocked by the inclusion of tetrodotoxin (TTX; 0·1–1·0 μM) or divalent ions (10 mM-Mg^{2+} or 2 mM-Mn^{2+} with 0·1–0·2 mM-Ca^{2+}) in the Ringer solution (referred to as TTX Ringer solution, Mn^{2+} or Mg^{2+} Ringer solution). Normal Ringer solution used for superfusion (2–4 ml./min; bath volume 0·15 ml.) contained the following (in millimoles/l): NaCl, 115; KCl, 2; $CaCl_2$, 2; HEPES (pH 7·3), 5; D-glucose, 10. K^+-free Ringer

solution had the same composition except KCl was omitted. In the various types of Li^+ Ringer solution, LiCl replaced an equimolar amount of NaCl. Low-Ca^{2+} Ringer solutions were prepared by adding less than 2 mM-$CaCl_2$, e.g. 0·2 mM-$CaCl_2$. Divalent cations were added as $MnSO_4$ or $MgSO_4$ to a final concentration of 2 or 10 mM in Ringer solution. In some experiments extracellular Ca^{2+} levels were adjusted using Ca/EGTA buffer. $CaCl_2$ was replaced with an appropriate mixture of $CaCl_2$ and EGTA so as to obtain the desired free Ca^{2+} concentration, calculated from the graphs of Portzehl, Caldwell & Ruegg (1964). Temperature (usually 14 °C), was maintained within ±0·1 °C using a Peltier device. Permanent records were obtained from a rectilinear pen recorder (Brush model 2400). In some experiments a data aquisition system based on a Z-80 microprocessor was employed. Data were stored on diskettes and permanent records obtained by means of an X–Y plotter. Results were expressed as ±S.E. of means wherever possible. All drugs were purchased from Sigma, St. Louis, MO, U.S.A., except some of the TTX which was from Sankyo, Tokyo, Japan.

Interpretation of the recorded potentials. The observed potential changes represent an attenuated version of changes in membrane potential of motoneurone cell bodies, when recorded from ventral roots, or intramedullary part of primary afferents, in case of recordings from dorsal roots. Potential difference was measured between the electrode in the central compartment containing spinal cord and the electrodes in the compartments surrounding distal ends of dorsal or ventral roots (cf. Barker *et al.* 1975). Under our recording conditions an upward deflection of the pen represents depolarization. One should also keep in mind that these potentials are average responses of whole population of neuronal elements in the corresponding root.

RESULTS

The glu-a.h. Superfusion of a spinal cord preparation with monosodium glutamate (glu; 1 mM standard concentration), or aspartate, produced a depolarization (glu–d.) of both the ventral and dorsal roots. The depolarization was almost always followed by an afterhyperpolarization (glu-a.h.) on both roots. Fig. 1A illustrates a typical experiment performed on ventral roots. Since the amplitudes of synaptically evoked d.r.–v.r.p. responses were slightly increased during the glu-a.h., it would seem unlikely that this part of the response was generated with a marked increase in membrane conductance. On both roots, both phases of glu response persisted in the presence of Mn^{2+} or Mg^{2+} Ringer solution, treatments that create a very unfavourable situation for neurotransmitter release. It would therefore appear that the recorded responses are not mediated by a Ca^{2+} influx-dependent release of endogenous substances in the spinal cord, but rather by the direct activation of glutamate receptors on primary afferents and motoneurones. Since both the phases of glu responses were present in TTX Ringer solution, the generation of the glu responses was also independent of action potential generation.

The quantitative data on glu responses are summarized in Table 1. The smaller glu-a.h. responses on dorsal roots may be associated with the smaller depolarization produced by acidic amino acids on these roots (cf. Barker *et al.* 1975). The approximate rate constant of the decline of glu-a.h. was determined as the reciprocal of the time taken for the response to decline to 63% of its peak amplitude. There was no significant difference ($0·8 > P > 0·7$) between the rate constant of the responses recorded on ventral roots ($0·51 \pm 0·037$/min, $n = 25$) and that recorded on dorsal roots ($0·49 \pm 0·37$/min, $n = 21$). These rate constants were not altered by the rate of superfusion during the glu-a.h.

The data presented below provide substantial evidence for the hypothesis that the glu-a.h. represents the ouabain-sensitive electrogenic extrusion of Na^+ following glu-induced Na^+ accumulation. It would therefore be expected that large or long

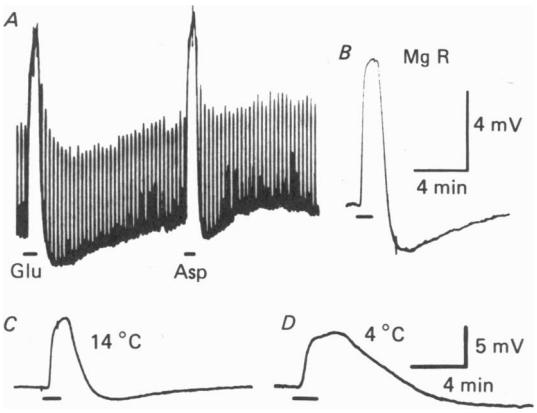

Fig. 1. Effect of high Mg^{2+}/low Ca^{2+} Ringer solution and temperature on glu-a.h. response of ventral roots of frog spinal cord. This figure and all the others are sucrose-gap recordings on a rectilinear chart recorder (except when marked); upward deflexions signify depolarization of motoneurones or primary afferents. *A*, control responses to glutamate (glu, 1 mM) and aspartate (asp, 1 mM) obtained on normal Ringer solution. Note depolarization followed by afterhyperpolarization. Brief upward deflections indicate dorsal root–ventral root potentials (d.r.–v.r.p.) elicited once every 30 sec. Note that d.r.–v.r.p. is not shunted during the glu-a.h.. *B*, response to glu recorded in presence of Ringer solution containing 10 mM-Mg^{2+}/0·1 mM-Ca^{2+}. *C*, response of another preparation to glu (1 mM) in 10 mM-Mg^{2+}/0·1 mM-Ca^{2+} Ringer solution at 14 °C. *D*, same response recorded at 4 °C. Bar indicates duration of exposure to glu, 4 mV/4 min calibration refers to traces *A* and *B*, 5 mV/4 min calibration refers to traces *C* and *D*. Bar indicates duration of exposure to amino acids.

TABLE 1. Means ±S.E. of mean for amplitude of glutamate (1 mM) evoked depolarization (glu-d.), afterhyperpolarization (glu-a.h.) and the rate constant of glu-a.h. on ventral and dorsal roots of isolated frog spinal cord. Figures in brackets indicate the numbers of responses used for respective measurements. All responses obtained in TTX Ringer

	glu-d. mV	glu-a.h mV	glu-a.h. rate constant (per min)
Ventral roots	11·1 ± 0·56 (n = 56)	2·03 ± 0·15 (n = 56)	0·507 ± 0·037 (n = 25)
Dorsal roots	5·66 ± 0·27 (n = 46)	0·78 ± 0·06 (n = 46)	0·492 ± 0·037 (n = 21)

lasting glu depolarizations, in which large quantities of Na^+ would enter the neurones, would be associated with large afterhyperpolarizations. Although there seemed to be a general tendency for larger glu-d. to be associated with larger glu-a.h. responses, no clear-cut statistical correlation existed between the relative sizes of glu-d. to glu-a.h. For their relative areas on ventral roots, $R = 0{\cdot}26$; for relative amplitudes, $R = 0{\cdot}36$. A better correlation existed for both phases of dorsal root responses: for areas, $R = 0{\cdot}73$ and for amplitudes, $R = 0{\cdot}62$.

Effect of temperature. If the glu-a.h. results from an active transport process, it should be more readily inhibited by low temperature than the diffusional processes

such as those underlying the glu depolarization (cf. Puil, 1981). Fig. 1*C* and *D* illustrate the glu depolarization and consequent glu-a.h. recorded at 14 °C and 15 min later at 4 °C. Note that the glu depolarization recorded at the lower temperature is of much longer duration than that recorded at 14 °C. Also in the 4 °C record the glu-a.h. is of slow onset and long duration. The elongation of glu depolarization under these conditions is a similar effect to that seen with the specific Na^+ pump inhibitors described below.

Effect of K^+-free Ringer solution. In most tissues, the ouabain-sensitive electrogenic extrusion is absolutely dependent upon the presence of K^+ ions in the extracellular fluid (Rang & Ritchie, 1968; Thomas, 1972). Thus, if glu-a.h. represents the activity of the electrogenic pump it should be blocked by K^+-free Ringer solution. A typical experiment testing this question is illustrated in Fig. 2. In this preparation, simultaneous recordings were made from both ventral and dorsal roots and the response to glu compared with that to DL-homocysteic acid (DLH). Control responses on ventral roots to both drugs are followed by afterhyperpolarizations. The hyperpolarizing response following depolarization induced by DLH (Fig. 2), as well as by *N*-methyl-D,L-aspartate (not shown), was observed only in some preparations. Introduction of K^+-free Ringer solution promotes a hyperpolarization as would be expected from the Nernstian relationship. Application of glu in K^+-free Ringer solution produces a large (cf. Evans, Francis & Watkins, 1977) and prolonged depolarization but the glu-a.h. is inhibited. On re-introduction of normal Ringer solution, a large hyperpolarization of membrane potential occurs before a slow repolarization returns the membrane to its resting level. This K^+-activated hyperpolarization (K-a.h.), elicited following the removal of glu presumably reflects Na^+ pump activation and the electrogenic extrusion of the Na^+ load accumulated in the preceding glu depolarization. If the preparation is incubated in K^+-free Ringer solution and then K^+ is re-introduced without an interposing glu depolarization a small K-a.h. still occurs (Fig. 2*C*). This response which has been observed in several other tissues (e.g. Rang & Ritchie, 1968; Akasu & Koketsu, 1976) is attributed to the electrogenic Na^+ pump extruding the Na^+ load which has accumulated during Na^+ pump inhibition in K^+-free Ringer solution. The afterhyperpolarization induced by DLH (DLH-a.h.) was also inhibited in K^+-free Ringer solution, and a large hyperpolarization occured when K^+ was re-introduced (Fig. 2*D*).

The right hand traces in Fig. 2 illustrate the glu and DLH responses of a dorsal root. On this root there is no DLH-a.h. The membrane potential of dorsal roots seems to be much more sensitive to extracellular K^+ ion concentration in that the K^+ removal caused significantly greater hyperpolarization than on ventral roots (Fig. 2*F versus* Fig. 2*B*). In the K^+-free Ringer solution, the depolarizations induced by both glu and DLH are substantially reduced in amplitude and the glu-a.h. is blocked. Re-introduction of normal Ringer solution following glu fails to produce a K-a.h. on dorsal roots even following presumed Na^+ loading by glu or DLH depolarizations in K^+-free Ringer solution.

A similar experiment on the glu-a.h. of ventral roots is illustrated in Fig. 3. In this experiment the original data records were stored in the microcomputer memory and replotted superimposed for more accurate comparison. The original data records, glu depolarization with glu-a.h. (1), glu depolarization in K^+-free Ringer solution with

subsequent re-introduction of normal Ringer solution (2) and control effect of K^+-free Ringer solution (3) are illustrated in Fig. 3*A*. In the top panel of Fig. 3*B* records (1) and (2) are superimposed. From this figure the increased duration of the glu depolarization in K^+-free Ringer solution is evident. The amplitude of the post-glu K-a.h. trace (2) and the glu-a.h. trace (1) may be seen to be quite similar, presumably

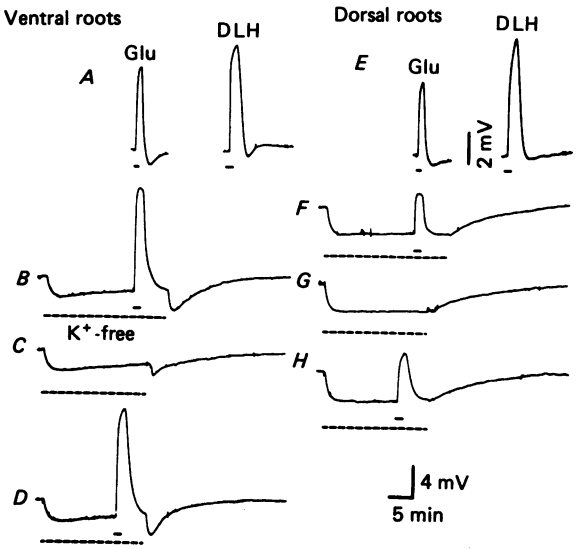

Fig. 2. Effect of K^+-free Ringer solution (K^+-free) on glu-a.h. and DLH-a.h. of dorsal and ventral roots of frog spinal cord. Left hand records ventral roots; right hand records dorsal roots. Traces *A* to *D* obtained consecutively from same preparation at same time as traces *E* to *H*. *A*, control responses of ventral roots to glu (1 mM) and DLH (50 μM). *B*, effect of K^+-free Ringer solution on membrane potential and response to glu. Note initial hyperpolarization of membrane potential, blockade of glu-a.h., increment of duration of glu depolarization, and K^+-activated hyperpolarization (K-a.h.) when superfusion with normal Ringer solution is resumed. *C*, control effect of K^+-free Ringer solution on ventral roots applied for the same time as in *B* and *D*. Note hyperpolarization of membrane potential and small K-a.h. when superfusion with normal Ringer solution is resumed. *D*, effect of K^+-free Ringer solution on membrane potential and response to DLH. Note blockade of DLH-a.h. and large K-a.h. following return to normal Ringer solution. *E*. Control responses of dorsal roots to glu and DLH. *E*, effect of K^+-free Ringer solution on membrane potential and response to glu. Note pronounced initial hyperpolarization of membrane potential, blockade of glu-a.h. apparent absence of K-a.h. on return to normal Ringer solution. *G*, control effect of K^+-free Ringer solution on dorsal roots applied for the same time as in *F* and *H*. *H*, effect of K^+-free Ringer solution on membrane potential and response to DLH. Dashed lines indicate duration of exposure to K^+-free Ringer solution, bar indicates duration of exposure to glu or DLH. 4 mV/5 min calibration applies to ventral root responses, voltage calibration for dorsal root responses is 2 mV as indicated (time calibration as for ventral roots). Ringer solution contained 0·2 μM-TTX.

because they reflect the extrusion of similar Na^+ loads. In the lower panel of Fig. 3*B*, the K-a.h. produced by a period of incubation in K^+-free Ringer solution (3) is compared with trace (2). The hyperpolarizing response in the latter case is greatly enhanced.

Thus in general, the effect of K^+-free Ringer solution is consistent with the

hypothesis that the glu depolarization involves an influx of Na^+ ions which are extruded electrogenically thereby generating the glu-a.h.. The increased duration of the glu depolarization in K^+-free Ringer solution was similar to the effect of low temperature.

It should be noted that in some experiments an early enhancement of the glu-a.h. was observed in K^+-free Ringer solution or Ringer solution containing only 0·1 mM-K^+. Since the response to GABA was also transiently increased at this time the enhancement could have been due to Na^+ pumping occuring across an increased membrane resistance.

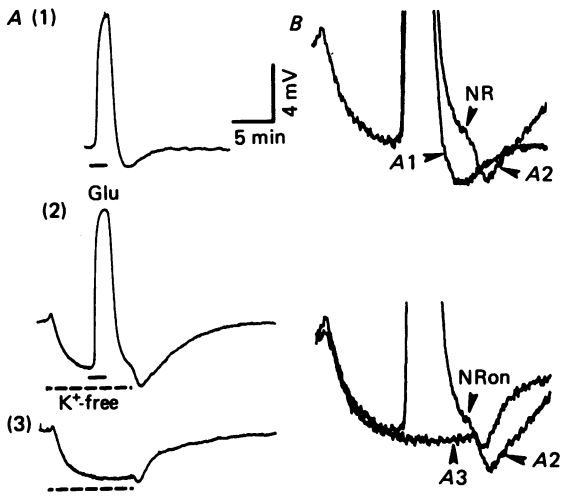

Fig. 3. Effect of K^+-free Ringer solution on glu-a.h. of ventral roots of frog spinal cord. *A*: (1), control response to 1 mM-glu; (2), effect of K^+-free Ringer solution on response to glu; (3), control effect of K^+-free Ringer solution on membrane potential. Original records from rectilinear pen recorder. *B*, superimposed traces from *A*. Upper trace: glu-a.h. from *A*1 and K-a.h. response following glu depolarization from *A*2. Note increased duration of glu depolarization in K^+-free Ringer solution and that the glu-a.h. and the post glu K-a.h. are of similar amplitude and duration. Lower trace: K-a.h. response following glu depolarization from *A* 2 and K-a.h. from *A* 3. Note that post-glu K-a.h. is larger and of longer duration than normal K-a.h. Dashed lines in *A* represent duration of exposure to K^+-free Ringer solution, bar indicates duration of exposure to glu. 5 min/4 mV calibration applies to *A*. Ringer solution contained 0·5 μM-TTX.

Further investigation of the K-a.h. Following a period of incubation in K^+-free Ringer solution, the transient introduction of normal Ringer solution may be used to produce a short and readily studied response (cf. Akasu & Koketsu, 1976). If this response results from the K^+-induced extrusion of accumulated Na^+ via the Na^+ pump, then the response should depend on the length of incubation in K^+-free Ringer solution. The Na^+ load would be expected to progressively increase following prolonged incubation in K^+-free Ringer solution.

Fig. 6*A* (top trace ventral root) illustrates the effect of superfusing ventral roots with K^+-free Ringer solution and briefly re-introducing normal Ringer solution for 2 min periods. As would be expected, removal of extracellular K^+ promotes a hyperpolarization. Subsequent brief re-introductions of normal Ringer solution cause

hyperpolarizing responses which progressively increase with time. This is to be expected if the K-a.h. results from the activation of Na^+ extrusion. The amplitude of K-a.h. is of course impaired by the simultaneous membrane depolarization caused by elevating extracellular K^+. This effect must be overcome in order for any hyperpolarization to appear. In an attempt to eliminate this depolarizing influence

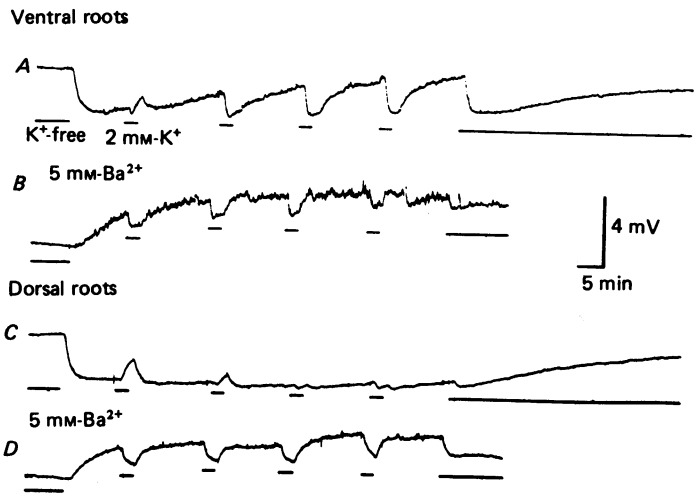

Fig. 4. Effect of 5 mM-$BaCl_2$ on K-a.h. and other K^+ induced membrane potential changes on dorsal and ventral roots of frog spinal cord. Records *A* and *C*, *B* and *D* recorded simultaneously from ventral and dorsal roots of the same preparation in TTX Ringer solution. *A*: ventral roots control effect of K^+-free Ringer solution and brief re-introductions of normal (2 mM-K^+) Ringer solution. Note initial hyperpolarization induced by K^+-free Ringer solution, biphasic response to first transient re-introduction of 2 mM-K^+ followed by progressively increasing K-a.h. responses to successive exposures to 2 mM-K^+ and large K-a.h. response upon return to normal Ringer solution. *B*, Effect of K^+-free Ringer solution in the presence of 5 mM-Ba^{2+}. Note depolarization of membrane potential induced by K^+-free Ringer solution, and hyperpolarizing responses to all subsequent transient exposures to 2 mM-K^+. *C*; dorsal roots; control effect of K^+-free Ringer solution and brief re-introductions of normal 2 mM-K^+-free Ringer solution. Note initial hyperpolarization of membrane potential and greater tendency towards depolarizing responses to subsequent transient exposures to normal 2 mM-K^+-free Ringer solution. Final washout of K^+-free Ringer solution is associated with a small K-a.h. *D*: dorsal root; effect of K^+-free Ringer solution in presence of 5 mM-ba^{2+}. Note depolarization of membrane potential induced by K^+-free Ringer solution and hyperpolarizing responses to all subsequent transient exposures to normal Ringer solution and to K^+-free Ringer solution washout. 5 min/4 mV calibration applies to all records. Lines indicate periods of superfusion of preparation with normal Ringer solution containing 0·2 mM-TTX; preparation exposed to K^+-free-TTX Ringer solution at other times.

of increasing extracellular K^+, the K-a.h. was examined in the presence of 5 mM-Ba^{2+}. This ion reduces K^+ permeability in a variety of systems (e.g. Krnjević, Pumain & Renaud, 1971; Standen & Stanfield, 1978) and may thereby reduce the depolarizing component of the response induced by elevation of extracellular K^+ (cf. Sjodin & Ortiz, 1975). Application of K^+-free Ringer solution in the presence of 5 mM-Ba^{2+} produced a slowly developing depolarization of membrane potential and the initial

K-a.h. produced under these conditions was much larger than that obtained in control (K^+-free) Ringer solution (Fig. 4*B*). Subsequent K-a.h. responses were however little larger than those observed in the absence of Ba^{2+}.

As mentioned above, the K-a.h. was absent or only very small on dorsal roots under normal conditions (Fig. 2, Fig. 4*C*). During superfusion with 5 mM-Ba^{2+}, however,

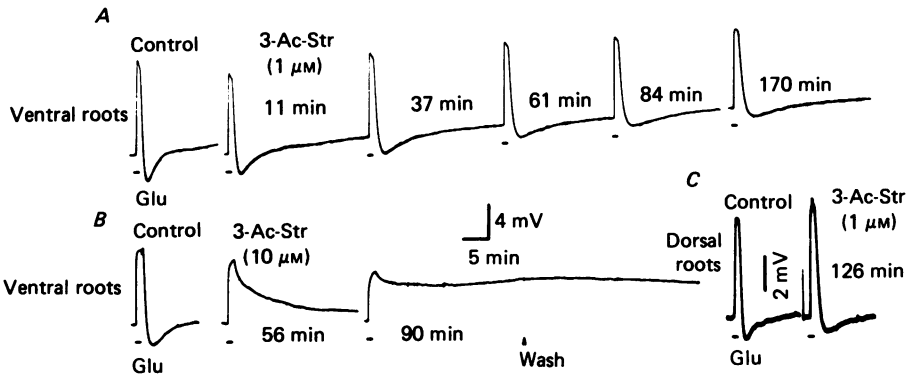

Fig. 5. Effect of 3-acetylstophanthin (3-AC-Str) on responses of ventral and dorsal roots of frog spinal cord to glu. Numbers beside traces indicate duration in minutes of exposure to drugs. *A*, effect of 1 μM-3-AC-Str on ventral root responses. Note progressive reduction of glu-a.h. amplitude and increase in duration. *B*, effect of 10 μM-3-AC-Str on glu responses of ventral roots. *C*, effect of 1 μM-3-AC-Str on glu responses of dorsal roots. Wash indicates removal of glycoside. 5 min/4 mV calibration applies to ventral root responses, 2 mV calibration applies to dorsal root responses. Bar indicates duration of exposure to glu. Ringer solution contained TTX (0·2 μM).

K-a.h. responses were readily recorded on dorsal roots. On both roots application of K^+-free Ringer solution in the presence of Ba^{2+} produced a depolarization of membrane potential (Fig. 4*D*). As noted above, the membrane potential of dorsal roots seemed much more sensitive to changes in extracellular K^+ than did that of ventral roots.

Effect of cardiac glycosides. The Na^+ pump and hence any electrophysiological changes resulting from its electrogenic nature are blocked by cardiac glycosides such as ouabain (Rang & Ritchie, 1968; Brown *et al.* 1972; Lees & Wallis, 1974; Smith & Weight, 1977). Some typical data records, illustrating the reduction of the glu-a.h. by a fast acting cardiac glycoside, 3-acetylstrophanthin (3-Ac-Str) are illustrated in Fig. 5. Note the progressive reduction in amplitude and decrease of the rate constant of the glu-a.h. of ventral roots during prolonged incubation in 1 μM-3-Ac-Str (Fig. 5*A*). As with other manipulations which inhibit the glu-a.h. the width of the depolarizing phase of the response is increased and decay slowed down. However, the amplitude of the glu depolarization even after 150 min in 3-Ac-Str (1 μM) is not significantly decreased. The glu-a.h. of dorsalroots (Fig. 5*C*) seems to be somewhat less sensitive to the action of 3-Ac-Str than does that of ventral roots. Fig. 5*B* illustrates the effect of a higher dose of 3-Ac-Str (10 μM) on the glu-a.h. of ventral roots. The glu-a.h. is completely eliminated after 56 min incubation in this concentration of glycoside. The glu depolarization is greatly prolonged at this time.

After 90 min incubation, the amplitude of the glu-d is so long that no repolarization is apparent in the record.

Effect of Li^+ ions. Li^+ will to some extent, substitute for Na^+ ions in drug-induced or active conductance changes (cf. Koketsu & Yamamoto, 1974) but will not substitute for Na^+ at the internal Na^+ ion recognition site of the Na^+ pump, i.e. Li^+ is not transported outward by the Na^+ pump (Keynes & Swan, 1959). Li^+ ions were therefore used to test the hypothesis that the glu-a.h. resulted from the activity of the electrogenic Na^+ pump. Isotonic substitution of Li^+ for Na^+ in the Ringer solution promoted a pronounced and prolonged depolarization of the resting potential of both dorsal and ventral roots. It was therefore not practical to examine drug responses under these conditions. Instead, preparations were examined in normal (TTX) Ringer and the depolarizing dose of glu administered in Ringer solution containing 50% (57·5 mM) or 25% (28·7 mM) Li^+; both drugs were then washed out with normal Ringer solution. In all experiments, the effect of the brief administration of a control dose of Li^+ Ringer solution was also examined. This test application of Li^+ produced a dose-dependent hyperpolarization followed by depolarization on the ventral roots and a pronounced depolarization on the dorsal roots (Li^+ response). When glu was administered at the same time as 57·5 mM- or 28·7 mM-Li^+ Ringer the glu-a.h. was reduced or blocked on both ventral and dorsal roots. The experiment illustrated in Fig. 6 shows that the effect was dose-dependent in that a more complete block of the glu-a.h. was observed in the Ringer solution containing the higher proportion of Li^+ ions. The glu-a.h. recovered when glu was readministered in normal Ringer solution.

Examination of the control records for the effects of short applications of Li^+ Ringer solution (Fig. 6*B* and E) shows that the elimination of the glu-a.h. on both roots is unlikely to result from the algebraic subtraction of these Li^+ responses from the glu-a.h.. Little or no depression of the amplitude of the glu depolarization by Li^+ was observed on ventral roots (Fig. 6*A*). As with other processes which inhibit the glu-a.h., the glu depolarization was increased in duration in the presence of Li^+ Ringer solution. In the experiment illustrated in Fig. 6, the effect of Li^+ on the glu response was compared to its effect on the response to DLH. In this preparation no DLH-a.h. was observed but the presence of Li^+ ions tended to broaden the response of both ventral and dorsal roots (Fig. 6*C* and *F*) in a fashion similar to the glu response (Fig. 6*A* and *D*). On dorsal roots the DLH depolarization was potentiated by Li^+. One possible explanation for this result could be that the DLH depolarization and the Li^+ response summed algebraically (Fig. 6*E* and *F*).

Effect of the removal of extracellular Ca^{2+} ions. Intense neuronal activity in cultured spinal neurones (Ransom *et al.* 1975) or amphibian sympathetic ganglion cells (Minota, 1974) as well as glu-induced depolarization of hippocampal CA1 cells (Nicoll & Alger, 1981) is followed by an afterhyperpolarization. Each of these responses are attenuated by Ca^{2+} channel blocker and/or by the exclusion of Ca^{2+} ions from the bathing medium. They have therefore been attributed at least in part, to the activation of Ca^{2+}-sensitive K^+ conductances (Meech, 1978). Ca^{2+} ions are thought to enter during intense neuronal activity and this may activate the increased K^+ conductance associated with the afterhyperpolarization. Since glu is thought to promote influx of both Ca^{2+} and Na^+ ions (Buehrle, Richter & Sonnhoff, 1978; Padjen

& Smith, 1980*a*, 1981; Pumain & Heinemann, 1981) it was of interest to test whether a Ca^{2+} activated K^+ conductance contributed to the glu-a.h. (Ransom *et al.* 1975; Nicoll & Alger, 1981). The glu depolarization and glu-a.h. were therefore examined in Ca^{2+}-free Ringer solution. Extracellular Ca^{2+} levels were adjusted by superfusion with a Ca/EGTA buffer (Portzehl *et al.* 1964).

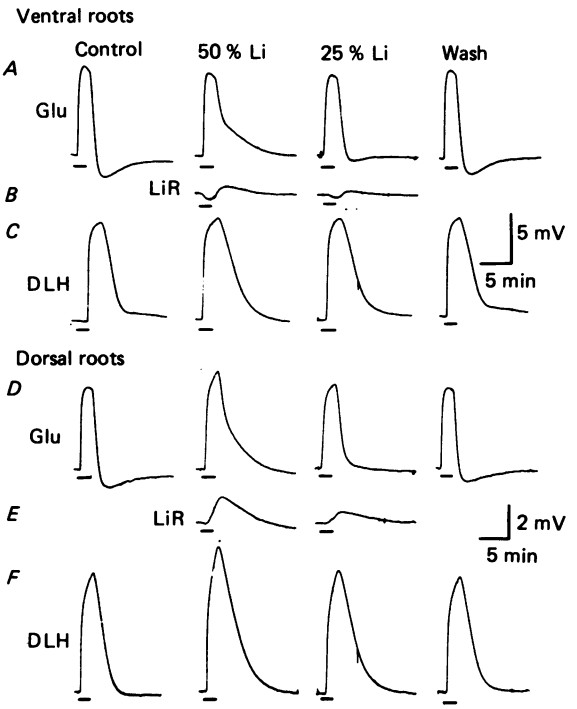

Fig. 6. Effect of Li^+ Ringer solution (LiR) on responses of ventral and dorsal roots to glu and DLH. Responses in *A*, *B* and *C* on ventral roots recorded simultaneously with responses in *D*, *E* and *F* on dorsal roots. *A*: ventral roots control response to 1 mM-glu and responses obtained to glu applied in Ringer solution containing 57·5 mM-or 28·7 mM-Li^+. Note depression of glu-a.h. especially by higher dose of Li^+. *B*: ventral roots; control responses to brief exposures to 57·5 mM-or 28·7 mM-LiR. *C*: ventral roots control response to 75 μM-DLH and responses obtained to DLH applied in Ringer solution containing 57·5 or 28·7 mM-Li^+. Note elongation of response in LiR. *D*, effect of 57·5 and 28·7 mM-LiR on dorsal root responses to glu. *E*, control responses of dorsal roots to brief exposures to 57·5 or 28·7 mM-LiR. *F*, effect of LiR on dorsal roots responses to DLH. Bars indicate duration of exposure to glu, DLH, glu and Li^+, DLH and Li^+ or Li^+. 5 min/5 mV calibration applies to ventral root responses, 5 min/2 mV calibration applies to dorsal root responses. Ringer solution contained 0·2 μM-TTX.

Lowering extracellular Ca^{2+} tended to potentiate the glu-a.h. of both ventral (Fig. 7) and dorsal roots (Fig. 8). Maximum potentiation was obtained at extracellular Ca^{2+} concentrations of 1 μM (mean amplitude = 164 ± 14 % control on ventral roots, $n = 5$; 155 ± 15 % on dorsal roots, $n = 5$). In Fig. 7 the effect of a Ca/EGTA buffer (1 μM Ca^{2+}) on glu-a.h. is compared with its effect on the DLH-a.h. and the response of ventral roots to 8 mM-K^+. Both the DLH-a.h. and glu-a.h. are enhanced with

consequent shortening of the depolarizing phases of the responses. The amplitude of the depolarization to 8 mM-K^+ is slightly increased. This may be interpreted as an indication of increased membrane resistance in low-Ca^{2+} Ringer solution. Essentially the same results are observed in Fig. 8. These responses were obtained simultaneously from the dorsal roots of the preparation illustrated in Fig. 7.

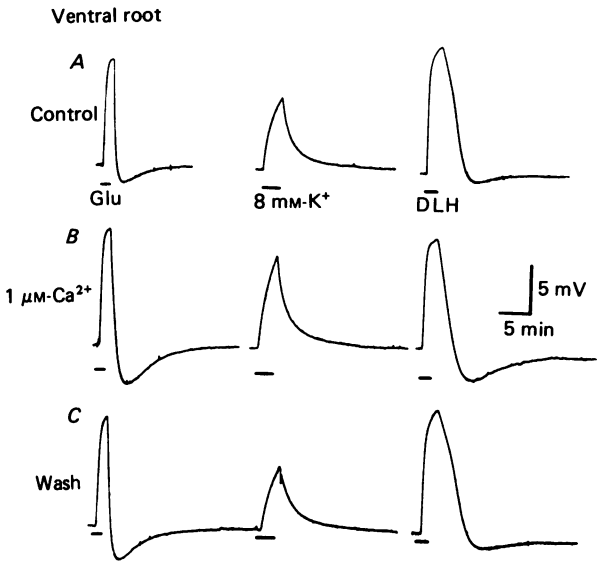

Fig. 7. Effect of low-Ca^{2+} Ringer solution on responses of ventral roots to glu, 8 mM-K^+ and DLH. *A*, control resonses to glu (1 mM), 8 mM-K^+ and DLH (50 μM). *B*, responses recorded in the presence of Ringer solution containing 0·9 mM-Ca^{2+}/1 mM-EGTA (1 μM-Ca^{2+}). Note enhancement of glu-a.h., DLH-a.h. and response to 8 mM-K^+. *C*, Recovery of responses following return to normal Ringer solution. Ringer solution contained TTX (0·2 μM). Bar under traces indicates duration of exposure to drugs. Calibration 5 mV/5 min.

With lower values of extracellular Ca^{2+} (< 1 μM Ca^{2+}) or with long periods (> 2 hr) of incubation at 1 μM-Ca^{2+} there was a tendency for depression of the glu-a.h. (mean amplitude = 66 ± 29% of control on dorsal roots, $n = 5$; 75 ± 37% of control on ventral roots, $n = 5$). The Ca^{2+} antagonist, cobalt (3–5 mM) reduced the amplitude of the glu-a.h. in three out of five preparations. Ba^{2+} (2–5 mM), on the other hand, usually increased the amplitude of the glu-a.h.

Reduction of extracellular Ca^{2+} ion concentration not only tended to enhance the glu-a.h. but also tended to enhance the K-a.h. A typical experiment is illustrated in Fig. 9. Fig. 9*A* illustrates the effect of K^+-free Ringer solution on ventral roots and the subsequent transient re-introduction of normal Ringer solution containing 2 mM-K^+. There is an initial hyperpolarization and a progressive increase in the amplitude of successive K-a.h. responses. In the second part of the experiment performed in Ca^{2+}-free Ringer solution and illustrated in Fig. 9*B* the K-a.h. responses obtained at similar time intervals to those in Fig. 9*A* are relatively larger.

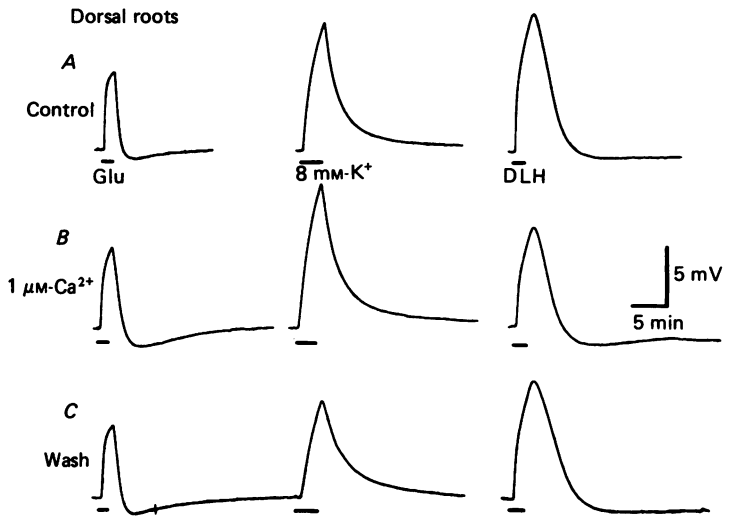

Fig. 8. Effects of low-Ca^{2+} Ringer solution on responses of dorsal roots to glu. DLH and 8 mM-K^+. Same preparation as Fig. 7 responses recorded simultaneously from dorsal rather than ventral roots. Note enhancement of glu-a.h., DLH-a.h. and response to 8 mM-K^+. Calibration 5 mV/5 min.

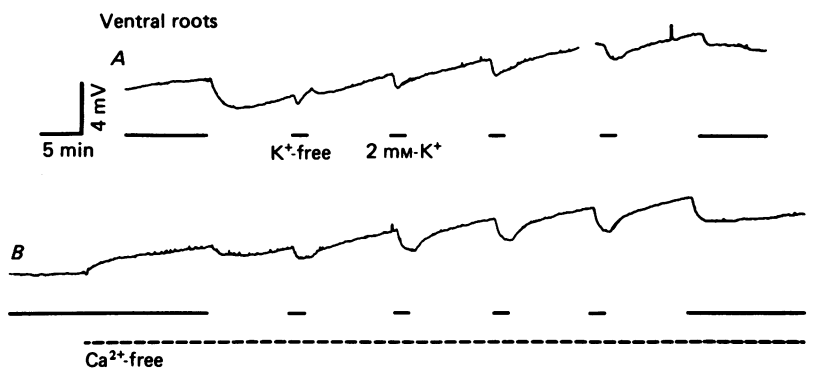

Fig. 9. Effect of Ca^{2+}-free Ringer solution on the K-a.h. and other K^+ induced membrane potential changes on ventral roots of frog spinal cord. *A*: Ventral roots; control effect of K^+-free Ringer solution and brief reintroductions of normal 2 mM-K^+-free Ringer solution. Note initial hyperpolarization induced by K^+-free Ringer solution followed by progressively increasing K-a.h. responses to successive exposures to 2 mM-K^+ Ringer solution. *B*, effect of 2 mM-K^+ Ringer solution in presence of Ca^{2+}-K^+ Ringer solution. Note larger K-a.h. responses observed in this medium. Calibration 4 mV/5 min. Line indicates periods of superfusion with normal or Ca^{2+}-K^+ Ringer containing 0·2 μM-TTX. Preparations exposed to K^+-free/TTX or K^+-free Ca^{2+}-free-TTX Ringer solution at all other times. Duration of exposure to Ca^{2+}-free/K^+-free or Ca^{2+}-free/2 mM-K^+ Ringer solution indicated by dashed line.

DISCUSSION

In the present experiments we have demonstrated the specific inhibition of the glu-a.h. in frog spinal cord by agents known to block the activity of the electrogenic Na^+ pump. The glu-a.h. was not blocked by Ca^{2+}-free Ringer solution nor by high Mg^{2+}/low Ca^{2+} Ringer solution nor by Mn^{2+} Ringer solution. These results suggest that glu-a.h. was not mediated by a Ca^{2+}-sensitive potassium conductance as has been proposed for a glu-a.h. in hippocampal CA1 neurones (Nicoll & Alger, 1981). The present results thus demonstrate the existence of an electrogenic Na^+ pump in C.N.S. tissue and that the glu-a.h. results from the activation of such a pump. The K-a.h. observed when K^+ is re-administered to preparations pre-incubated in K^+-free Ringer solution is also attributable to the activation of the electrogenic Na^+ pump. The rate constant of the glu-a.h. which is related to the rate of Na^+ extrusion (Brown *et al.* 1972) was 0·51/min on ventral roots and 0·49/min on dorsal roots. Since there was little significant difference in the rate constant for the glu-a.h. on the two roots ($0·8 > P > 0·7$) this may imply that similar rates of pumping occur at both sites. The absolute quantity of Na^+ entering dorsal roots is probably less than that entering ventral roots because the peak amplitude of the glu depolarization and glu-a.h. are smaller on dorsal roots (cf. Brown *et al.* 1972). This smaller influx of Na^+ may produce a similar intracellular accumulation on dorsal roots compared with ventral roots because of the low volume to surface area ratio of primary afferents compared to motoneurones. A similar rate of pumping may then result on both roots if the internal Na^+ sites of the Na^+ pump are saturated. The rate of pumping in these C.N.S. tissues is considerably greater than that measured in peripheral amphibian tissues. In sympathetic ganglia the rate constant for the Na^+ pump mediated ACh-a.h. following the depolarizing action of ACh (Smith & Weight, 1977) was 0·18/min (Smith & Weight, unpublished observations). For the ACh induced depolarization and subsequent ACh-a.h. in rat sympathetic ganglia, Brown *et al.* (1972) suggested that the maximum hyperpolarization should be directly proportional to the initial increment in the internal Na^+ and that the area under the hyperpolarization time curve should be proportional to the total amount of Na^+ extruded. Since the amount of Na^+ entering the neurones should be related to the initial depolarization, the relative amplitude of the depolarizing and hyperpolarizing phase of responses should be well correlated. This was not the case in the present experiments; the poor correlation may be explained if a slow washout of glu follows prolonged incubation with drug and shunts potential changes arising from pump currents in the glu-a.h. (cf. Brown *et al.* 1972). It is well known that the potential changes resulting from electrogenic pumping depend on resting membrane resistance (Rang & Ritchie, 1968).

Although the ionic mechanism of glu depolarization is not clear, participation of Na^+ fluxes have been implied (cf. Krnjević, 1974; Puil, 1981). Our results indirectly support such a notion. It was interesting to note that amino acids such as DLH and *N*-methyl-D,L-aspartate exhibited a Na^+ pump mediated afterhyperpolarization. This could imply that although these substances may act on a different receptor from glu (Watkins & Evans, 1981) the depolarization induced may well involve an influx of Na^+ which is subsequently electrogenically extruded producing the hyperpolarizing response.

The cardiac glycoside 3-acetyl-strophanthin progressively decreased the rate constant and thus progressively slowed the rate of Na^+ pumping in successive glu-a.h. responses (Fig. 5). The glu-a.h. on dorsal roots was generally less susceptible to the action of cardiac glycoside than that response on ventral roots. One explanation for this result could be that the Na–ATPase of dorsal roots is simply less susceptible to inhibition by glycosides. It is well known that the lipid and protein content of the cell membrane can influence the sensitivity of the ATPase to ouabain (Lelievre, Zachowski, Charlemagne, Laget & Paraf, 1979), thus a different chemical composition of the cell membranes of ventral compared with dorsal roots could readily explain the observed difference in sensitivity to 3-acetyl-strophanthin. Alternatively, it may be that the glu-a.h. of dorsal and ventral roots were generated by slightly different mechanisms. It is possible that glu depolarization of dorsal roots is an indirect response resulting from glu-induced potassium release from surrounding neuronal elements (Evans, 1980). The glu-a.h. may then also result from an (indirect) mechanism such as the depletion of extracellular K^+ following Na^+ pumping in neuronal elements other than the dorsal roots themselves (cf. Krnjević & Morris, 1975; Davidoff and Hackman, 1980). This explanation is somewhat unlikely since although the glu-induced depolarization of dorsal roots is associated with an increase in extracellular K^+ (Kudo, 1978; Evans, 1980) no reduction of extracellular K^+ in dorsal horn appears to be associated with the glu-a.h. (Fig. 3 in Evans 1980; but cf. Sonnhof & Buehrle, 1980, for ventral horn measurements). Furthermore Ba^{2+}, which can completely block the potential changes evoked by increasing extracellular K^+ levels, has only a small effect on the glu depolarization and usually enhances the glu-a.h. of dorsal roots (Padjen & Smith, 1981).

A possible explanation for the depolarization induced by K^+-free Ringer solution in 5 mM-Ba^{2+} could be that the Na^+ pump normally contributes to motoneurone (ventral root) resting potential and that removal of K^+ causes a removal of this hyperpolarizing influence. The depolarization is only seen in the presence of Ba^{2+} which masks the tendency for the membrane to hyperpolarize as K^+ is removed. The potentiation of K-a.h. in Ba^{2+} may also be related to Ba^{2+} blockade of K^+ channels resulting in (1) removal of K^+-evoked depolarization and (2) increased potential change due to pumping across the increased membrane resistance (see above).

Alternatively, if Ba^{2+} and K^+ compete for the same binding sites (Standen & Stanfield, 1978) and Ba^{2+} blocks K^+ channels, lowering of extracellular K^+ may intensify Ba^{2+} blockade and the resulting depolarization may result from an increased blockade of K^+ channels. Similarly, if K^+ displaces Ba^{2+} this depolarizing effect of Ba^{2+} may be reversed. In the situation illustrated in Figs. 4*B* and *D*, short applications of 2 mM-K^+ Ringer solution to K^+-free/5 mM-Ba^{2+} Ringer may transiently displace Ba^{2+} and thereby produce transient increases in K^+ conductance. The resulting hyperpolarization would sum with that resulting from Na^+ pump activation and a potentiation of the over-all K-a.h. in Ba^{2+} would be observed. This possibility cannot be excluded on the basis of the present data. The lack of progressive enhancement of successive K-a.h. responses recorded in the presence of Ba^{2+} may occur because (i) this ion has an inhibitory action on the Na^+ pump, or (ii) that Ba^{2+}–Na^+ exchange can maintain intracellular Na^{2+} at low levels, or (iii) Ba^{2+} is toxic. Ba^{2+} treatment also revealed the K-a.h. on dorsal roots, possibly because Ba^{2+} blocked the depolarizing action of potassium.

In addition to maintaining the electrochemical gradients, upon which all neuronal activity ultimately depends, the Na^+ pump has been proposed to be involved in several modulatory processes in the nervous system. For example, Van Essen (1973)

has suggested that the Na^+ pump-mediated hyperpolarization following trains of impulses may restrict action potential propagation at branch points in complex neuronal networks. Krnjević & Morris (1975) have suggested that the Na^+ pump mediated uptake of K^+ from extracellular space may reduce the concentration of this ion below resting levels thereby promoting hyperpolarization of primary afferent fibres in mammalian nervous system. A possible role for Na^+ pump mediated hyperpolarizations in restricting the detonation of seizure-like discharges has been suggested (Kuno, Miyahara & Weakly, 1970). In the present experiments it was noted that procedures which inhibited the Na^+ pump increased the duration of depolarizing responses to acidic amino acids. Glu, DLH and *N*-methyl-D,L-aspartate presumably cause depolarization by some mechanism involving a Na^+ influx (cf. Puil, 1981). The duration of this depolarization could thus be restricted by activation of the Na^+ pump which would tend to generate a progressively increasing hyperpolarizing influence which would subtract algebraically from any depolarizing response produced by the drug. It is possible that inhibition of amino acid uptake by Na^+ pump inhibitors (Evans, *et al.* 1977) may play a role in the prolongation of responses recorded under conditions where the pump is blocked. It should be noted that both DLH and *N*-methyl-DL-aspartate are poor substrates for the uptake system (Stallcup, Bullock & Baetge, 1979) so the prolongation of responses to these substances is unlikely to be the result of changes in uptake. Thus the Na^+ pump potentials described in the present study may well have a physiological role in the normal operation of the nervous system in that they may limit the duration of depolarizing responses. A similar role for the electrogenic Na^+ pump has been suggested in the termination of the acetylcholine response in smooth muscle (Bolton, 1973).

In TTX-blocked preparations, removal of extracellular Ca^{2+} enhanced the amplitude of the glu-a.h. The glu-a.h. could also be recorded in Ringer solution containing 20 mM-Mg^{2+}/0·2 mM-Ca^{2+} or 2 mM-Mn^{2+}/0·2 mM-Ca^{2+}. Each of these situations is very unfavourable for neurotransmitter release (Katz, 1969), and it is unlikely that the glu-a.h. reflects the glu induced release of hyperpolarizing substances. It is conceivable therefore that the glu-a.h. may reflect the existence of hyperpolarizing glu receptors. Since the glu-a.h. is mediated by activation of the Na^+ pump, this would imply that glu receptor would have to be intimately associated with the Na, K-ATPase. To the best of our knowledge, there are no reports of Na, K-ATPase activation by glu in cell free systems, and this possibility would therefore seem to be rather unlikely.

Three possible mechanisms may be advanced for the enhancement of Na^+ pump mediated potentials in Ca^{2+}-free Ringer solution. Firstly, in spinal cord the resting membrane potential and thus presumably the resting K^+ conductance appears to be Ca^{2+}-dependent (Krnjević, Puil & Werman, 1978; Krnjević, Lamour, MacDonald & Nistri, 1979; Padjen & Smith, 1981). Ca^{2+} removal could thus promote a decrease of resting K^+ conductance so that activation of Na^+ pumping would be associated with a larger change of potential (cf. Rang & Ritchie, 1968). This conclusion is supported by the observation in Figs. 7 and 8 that an increment of the glu-a.h. is associated with an enhancement of the response to 8 mM-K^+ which may indicate an increase in membrane resistance. Secondly, from a more biochemical stand-point an apparent change in membrane resistance may reflect the solubilization of membrane proteins and consequent changes in lipid microenvironment and hence the activity of the Na, K-ATPase enzyme (Lelievre *et al.* 1979, Kimelberg, 1976). Thirdly, Lindenmayer & Schwartz (1975) have suggested that the Na^+ pump can function in an electroneutral mode exchanging one Ca^{2+} and one K^+ for three Na^+. If such an electroneutral exchange contributes to part of the Na^+ extrusion in normal Ringer solution the

potential change produced would be smaller than that expected if all extrusion of Na^+ took place by the electrogenic exchange of two K^+ for three Na^+. On the other hand, if extracellular Ca^{2+} is removed, all extrusion of Na^+ would be expected to perform by the electrogenic mechanism (exchange of two K^+ for three Na^+). This could therefore explain the greater membrane hyperpolarization associated with Na^+ pump activity in Ca^{2+}-free Ringer solution. It is entirely possible that all three of the proposed mechanisms could contribute to the enhancement of Na^+ pump responses in low Ca^{2+} Ringer solution.

Drastic changes in extracellular Ca^{2+} are known to occur during seizure activity (Heinemann, Lux & Gutnick, 1977; Krnjević, Morris & Reiffenstein, 1980; Heinemann & Pumain, 1981) and since such changes may modulate the electrogenicity of the Na^+ pump a better understanding of such phenomena may be pertinent to the understanding of convulsive disorders.

The support of the MRC of Canada and a travel grant from the Alberta Heritage Fund for Medical Research are gratefully acknowledged. A.L.P. holds a MRC Scholarship. Support for P.A.S. was from an MRC grant to Dr B. Collier. We thank Dr K. Krnjević for critical reading of an earlier version of the manuscript.

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