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EGTA AND MOTONEURONAL AFTER-POTENTIALS

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SUMMARY

1. Intracellular iontophoretic injections of EGTA (5–20 nA) into cat spinal motoneurones consistently greatly reduce the amplitude of the delayed after hyperpolarization (a.h.p.) that follows the spike.

2. This effect is accompanied by a large reduction (on average by $\frac{3}{4}$) in the marked increase in input conductance normally associated with the a.h.p.

3. There is also a consistent, though less regular, tendency for the resting input conductance to decrease (on average by $\frac{1}{5}$), as well as some depolarization.

4. Recovery of the a.h.p., the associated conductance increase and the resting conductance is very slow. It is sometimes accelerated by injections of citrate and Cl^-, or Ca^{2+}.

5. Other hyperpolarizing phenomena, such as recurrent or othodromically-evoked i.p.s.p.s, are not depressed by injections of EGTA.

6. When depolarization is minimal, EGTA injections that markedly depress the a.h.p. do not affect the rate of rise or fall of the spike. If, as a result of depolarization, an early a.h.p. is visible, it is patently insensitive to EGTA.

7. The post-spike depolarizing after-potential (delayed depolarization) is not obviously affected by EGTA, apart from the usual diminution seen during depolarization.

8. Since the main action of EGTA is to bind free Ca^{2+}, the marked depression of the a.h.p. indicates that the sharp increase in K conductance which generates the a.h.p. is probably caused by an influx of Ca^{2+} accompanying the action potential. It is suggested that this inward Ca^{2+} current may be manifested in the depolarizing after-potential.

INTRODUCTION

After-potentials were noted by several authors in extracellular studies of antidromic responses of spinal motoneurones (Eccles & Pritchard, 1937; Barron & Matthews, 1938; Gasser, 1939; Renshaw, 1942), but their analysis was complicated by the possibility of synaptic actions due to activity in recurrent branches of motor axons (Renshaw, 1941, 1946). The absence of such collaterals in the brain stem prompted Lorento de Nó's (1947) systematic investigation of hypoglossal

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motoneurones, from which he was able to conclude 'with reasonable assurance' that '... in the somas, the membrane action potential has three parts: a spike, a negative after-potential (residual depolarization) and a positive after-potential (temporary hyperpolarization)'.

This description was essentially confirmed by further extracellular studies in the spinal cord (Brooks, Downman & Eccles, 1950; Lloyd, 1951) and especially by intracellular recording in spinal motoneurones, particularly the extensive studies of Eccles and his collaborators (Brock, Coombs & Eccles, 1952; Coombs, Eccles & Fatt, 1955; Eccles, 1957), as well as those in other laboratories (Granit, Kernell & Smith, 1963; Nelson & Burke, 1967; Calvin & Schwindt, 1972). The after-hyperpolarization (a.h.p.) has been relatively simple to interpret; on the basis of the high associated membrane conductance, a very negative reversal potential, and the effects produced by intracellular injection of various ions, Eccles (1957) ascribed it to a delayed phase of high K^+ conductance (G_K). This interpretation has not been questioned by other observers.

By contrast, nc simple explanation for the negative after-potential has been generally agreed upon. This after-depolarization was considered by Eccles as most likely equivalent to the negative after-potential of peripheral axons (cf. Gasser & Grundfest, 1936; Gasser, 1939), though he did mention the possibility that it might represent a dendritic potential (Brock *et al.* 1952). After noting its marked sensitivity to membrane polarization and tendency to reverse at -60 mV, Eccles (1957) made no suggestion as to the underlying membrane mechanism. Later investigators have tended to describe this as a *delayed depolarization*, more likely to arise in dendrites and to be distinct from a true after-potential (Granit *et al.* 1963; Kernell, 1964; Nelson & Burke, 1967; but cf. Baldissera, 1976).

In the amphibian spinal cord, motoneuronal after-potentials differ somewhat from the above: the spike is immediately followed by a conspicuous early, brief a.h.p. which precedes the delayed depolarization, after which there is only a relatively small, slow a.h.p. (Araki & Otani, 1955; Machne, Fadiga & Brookhart, 1959; Barrett & Barrett, 1976; Magherini, Precht & Schwindt, 1976).

In the light of evidence that intracellular injections of Ca^{2+} increase the G_K of spinal motoneurones, Krnjević & Lisiewicz (1972) speculated that Ca^{2+} entering during the first part of the action potential might be responsible for initiating the delayed phase of high G_K. One way of testing this possibility is to try to abolish the effects of increased internal Ca^{2+} by inactivating free Ca^{2+} with a strong chelator injected intracellularly. The present article is a description of such experiments. Preliminary reports of these results have already appeared (Krnjević, Puil & Werman, 1975; Krnjević, Puil, VanMeter & Werman, 1976).

METHODS

The cats were anaesthetized with a mixture of allobarbitone and urethane (Dial, Ciba Ltd) injected intraperitoneally. After a laminectomy, the exposed lumbo-sacral region of the spinal cord was kept covered with mineral oil, and prevented from cooling by automatically-controlled radiant heat. As a rule, the sixth and seventh lumbar and the first sacral dorsal and ventral roots were cut, and were set up for stimulation. The carotid arterial pressure was monitored continuously; rarely, when necessary, hypotension was countered by a continuous infusion

(I.V.) of saline and noradrenaline. In most instances, the heavy frame in which the cat was mounted provided adequate stability and there was no need for paralyzing agents.

Microelectrodes

The compound glass micropipettes were comparable to those used in the previous study (Krnjević & Lisiewicz, 1972), but they sometimes consisted of four parallel barrels instead of three. The joint tip had a diameter of 2–4 μm. Relatively large tips were preferred, so as to keep barrel resistances reasonably low and thus improve the chances of successful iontophoresis, stable recording and useful resistance measurements. For this reason, the barrel used for recording was most commonly filled with 3 M-KCl. Another channel contained a solution of the K salt of ethylene glycol bis-(β-amino-ethyl ether)-*N*,*N*′-tetra acetic acid (EGTA, Sigma Chemical Company, 0·2 M, pH 6·5–8·5). In the third barrel was a mixture of 1 M-KCl and 1·6 M-K citrate (1 : 1) neutralized to pH 7 (in this solution, owing to the trivalent character of citrate, Cl^- ions account for only about $\frac{1}{8}$ of the total anionic charge); through this channel were applied either brief pulses to measure the cell input resistance (see below) or steady currents to polarize the membrane as desired; in addition, this channel provided a return path for iontophoretic currents ejecting EGTA from the second barrel. The fourth barrel, when used, was filled with solutions of other salts ($CaCl_2$, $MgCl_2$, etc.) or agents, such as 2,4-dinitrophenol, which were tested against EGTA (Krnjević, Puil & Werman, 1978).

Intracellular current injection and iontophoresis

The current injecting circuit converted voltage inputs (steady or in pulses) to corresponding constant current outputs (100 nA/V), the maximum driving voltage (compliance) available at the *output* being $\pm$ 40 V (this unit was designed and built by Mr J. Knowles, in the McIntyre Electronic Workshop, McGill University (see Fig. 7.4 in Kelly, Simmonds & Straughan, 1974). By inverse coupling with a similar unit providing the constant currents used for iontophoresis of EGTA, an identical but opposite current was injected through channel three (citrate-Cl^-), so that normally current flowing to ground through the cell membrane was kept below 0·5 nA. As a further precaution, current flow to the cat's virtual ground was monitored continuously on a polygraph, so that any significant imbalance in this push–pull injection system (such as could arise if the barrel resistance became too high for the compliance of the injecting circuit) would not pass undetected. Voltages proportional to any applied iontophoretic or polarizing currents were also monitored continuously on the polygraph.

Although the input stage of the recording system was 'guarded' and kept close to the microelectrode, and the capacity to ground for various channels was at least partly compensated by positive feedback, the effective input time constant was approximately 100 μsec, sufficiently long to slow down a little the rate of rise of action potentials.

The resting membrane potential was recorded by low gain d.c. amplification and displayed both on an oscilloscope (lower traces) and a penwriter. Action potentials were similarly recorded on the oscilloscope, both directly and after differentiation. About tenfold greater a.c. amplification (time constant > 100 msec) was also used to display after-potentials and synaptic potentials (see upper traces in the Figures).

Cell resistance measurements

For this purpose, the current injecting unit was controlled by a Linc 8 computer, which automatically supplied rectangular pulses of ten evenly graded intensities, within a pre-set range (mainly 0–5 nA, in the hyperpolarizing direction). The pulses usually lasted 20 msec, and they were delivered at a frequency of 2/sec, each group of ten being repeated eight times/min.

The computer recorded the resulting displacements of membrane voltage just before the end of each current pulse, and displayed on-line each set of ten current–voltage points. The corresponding lines-of-best-fit (linear regression) were calculated on-line and also displayed, together with the estimated input resistance and resting potential, as well as their standard errors (eight times/min). The data were also stored on magnetic tape for further analysis, including the calculation of reversal levels for i.p.s.p.s or after-potentials. For controls, similar measurements were repeated after withdrawing the electrode from a cell and used to correct for inadequate bridge balance or coupling artifacts of iontophoresis (see below).

Artifacts of intracellular iontophoresis

There are at least two possible kinds of artifacts which complicate the interpretation of results. The first is a pure electrode artifact, evidently caused by electrical pathways near the electrode tip that are effectively in series with both the recording and the iontophoretic barrels. Such 'coupling' is quite variable, but can be minimized by using relatively large tips. To a large extent, this artifact is similar when recording extracellularly and therefore it can be measured and adequately controlled. Although voltage changes due to 'coupling' could be quite large, they were usually fully reversible and not associated with comparable consistent changes in tip resistance. But even under the best recording conditions, iontophoresis was sometimes associated with a relatively sudden change in tip resistance, to a markedly higher or lower level (cf. Fig. 8). These effects were presumably caused by electrokinetic phenomena at the tip (movements of proteins, membranes, or large ions that tend to 'block' the current path). Some of these may involve movements of Ca^{2+} or other ligands, since tests of some electrodes *in vitro* showed a much greater tendency for the tip resistance to rise during iontophoresis of EGTA into Ca^{2+}-containing (0·1–1·0 mM) saline than into Ca^{2+}-free saline.

Another type of artifact could not be controlled readily. Even when the push–pull injection system was working optimally (judging by the absence of a significant amount of current to ground), some portion of the iontophoretic current sometimes appeared to travel through part of the cell membrane because of corresponding changes in various membrane phenomena (excitability, size or shape of spikes and synaptic potentials). These effects could be distinguished with reasonable certainty from those produced by injected agents, owing to their immediate onset and termination as the iontophoretic currents were switched on and off, as well as their approximately linear relation to the intensity of the applied currents.

RESULTS

Normal features of after-potentials

Since EGTA affected predominantly the hyperpolarizing after-potential (a.h.p.) it is necessary to review the main features of the antidromically-evoked potentials. When the motoneurone was in a relatively good condition (resting potential and spike better than 50 mV) the after-potentials consisted only of a delayed depolarization followed by the a.h.p.

The spike proper lasted 1·0–1·5 msec, to the point on its descending limb (some 2–10 mV above the base line) where rapid deceleration signals the start of the delayed depolarization: this phase of much slower repolarization lasted 2–3 msec and it was smoothly continuous with the subsequent a.h.p. The delayed depolarization sometimes showed a small hump (Fig. 1*B*, *C*; cf. Kernell, 1964). Such a hump became more obvious when a cell was depolarized (Fig. 12*A*–*C*).

The a.h.p. reached its maximum (typically -5 mV) some 8–12 msec from the start of the spike, and it usually lasted at least 50 msec. An a.h.p. was observed in all cases, except when soma-dendritic invasion failed. Though present in most cases, the delayed depolarization was more variable, partly because it is quite sensitive to depolarization (Fig. 12) and partly because it is markedly depressed by i.p.s.p.s (cf. Kernell, 1964; Nelson & Burke, 1967). Thus in the presence of recurrent i.p.s.p.s, the delayed depolarization was much less conspicuous or even appeared totally absent (Fig. 1*F*).

In agreement with the observations of Eccles and his collaborators (Brock *et al.* 1952; Coombs *et al.* 1955; Eccles, 1957), cat motoneurones showed no early a.h.p. under optimal conditions of recording. Only when the resting potential was diminished (whether by articial depolarization or owing to deterioration of the cell) was a

brief, early a.h.p. observed (Figs. 4*B*, *C* and 12), comparable to that seen in amphibian motoneurones (Araki & Otani, 1955).

Injections of EGTA

The principal features of the effects of EGTA are illustrated in Fig. 2. In this experiment, an action potential was evoked by depolarizing current pulses applied through the intracellular three-barrelled electrode. The initial superimposed traces (*A*), recorded during juxta-threshold stimulation, show that most of the post-spike

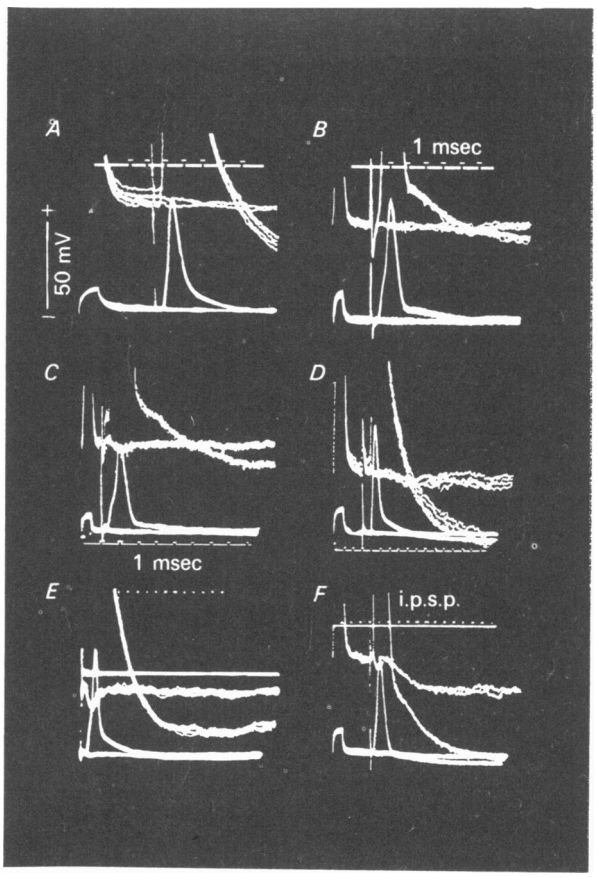

Fig. 1. Antidromic spike and after-potentials of motoneurones recorded during 'threshold-straddling' stimulation of ventral root. In each case several oscilloscope sweeps are superimposed photographically. Lower traces, low gain d.c. amplification; upper traces, high gain a.c. amplification. Corresponding calibrations are given, as well as time calibrations. An electrode resistance monitoring pulse is at extreme left of each trace (except *E*). Note recurrent i.p.s.p. in *F*.

hyperpolarization is a true a.h.p. A push–pull intracellular injection of EGTA (20 nA) was started just after another control record (*B*). Within 15 sec the latency of spike initiation was reduced and the a.h.p. became smaller (*C*), even though the change in latency could not have obscured the peak of the a.h.p. By 30 sec (*D*), the same current pulse evoked a double spike response and a consequently larger a.h.p.

(Ito & Oshima, 1962; Calvin & Schwindt, 1972); owing to the increased excitability, a markedly weaker depolarizing pulse was now adequate to evoke a spike (*E*). Two min after the end of the injection of EGTA (*E*), the excitability remained high and the a.h.p. showed no recovery. Although there was some depression of spike amplitude and increase in its duration, this could have been a secondary effect of a drop in resting potential commonly accompanying EGTA injections (see below), as well as the result of reduced Na efflux (Baker & McNaughton, 1976).

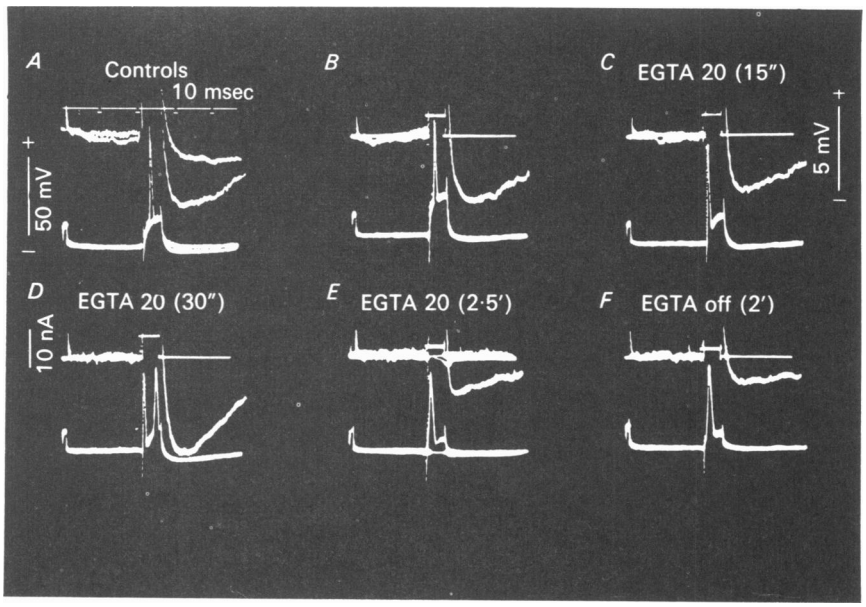

Fig. 2. Effects of EGTA injection on action potential evoked in motoneurone by direct stimulation. In each record, lower traces are at low gain d.c. amplification, upper traces at high gain a.c. amplification. *A*, initial control; depolarizing current injected into cell is straddling threshold. *B*, 5 min later, just before start of EGTA injection. *C*, 15 sec after starting EGTA injection (20 nA). *D*, 15 sec later; *E*, 2 min later. *F*, 2 min after end of EGTA injection. Rectangular pulses in *B–F* monitor depolarizing current.

Depression of the afterhyperpolarization

Practically without exception, intracellular injections of EGTA led to a reduction in the amplitude of the a.h.p. (> sixty trials in > forty motoneurones, in twelve cats). This effect was particularly clear, because it always long outlasted the period of intracellular injection, and therefore could not be an artifact of the iontophoresis (see Methods).

In many cases (as in Fig. 2), the injection of EGTA was followed by some depolarization: usually, such a change in membrane potential enhances the a.h.p. (Coombs *et al.* 1955; Eccles, 1957) and therefore it made the reduction in a.h.p. even more impressive. However, depolarization also tends to depress the spikes, and might conceivably interfere with whatever process initiates the a.h.p., such as Ca^{2+}-influx (Baker, Hodgkin & Ridgway, 1971), although in fact, as Coombs *et al.* (1955) already demonstrated, large depolarizations can enhance the a.h.p. even when depression

of the spike reaches the point where antidromic invasion begins to fail. Hence the striking depression of a.h.p. that could be observed even when depolarization and depression of the spike (and particularly its maximum rate of rise) were minimal is especially convincing evidence that EGTA directly affects the a.h.p. or its underlying mechanism. The two sets of traces (left and right) of Fig. 3 provide three examples of such effects of EGTA, where the marked diminutions of the a.h.p. were not accompanied by any detectable slowing down of the spike (see the time derivative traces marked by arrows).

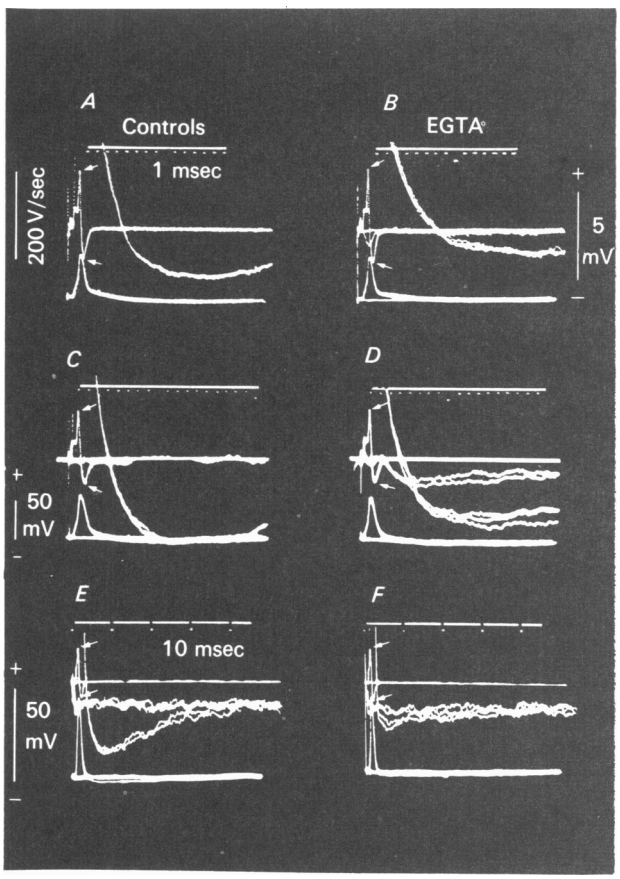

Fig. 3. Intracellular EGTA injections greatly reduce a.h.p. in three different spinal motoneurones excited antidromically, without altering rate of rise or fall of spike. There are four sets of traces in each record: lowest, shows spike at very low d.c. amplification; central shows same signal at high gain a.c. amplification, as well as another trace showing time derivative of action potential (its positive and negative peaks are indicated by arrows); uppermost trace gives time calibration. *A*, *C*, *D*, initial controls; corresponding record at right shows effect of EGTA injection. *B*, 15 sec after end of 1 min injection of EGTA (40 nA). *D*, 30 sec after start of EGTA injection (40 nA); note that a recurrent i.p.s.p. becomes evident. *F*: 30 sec after end of 1 min injection of EGTA (40 nA).

In these tests, as throughout the experiments, the antidromically-evoked a.h.p. was distinguished from recurrent i.p.s.p.s (Eccles, Fatt & Koketsu, 1954) by 'threshold-straddling' stimulation of the ventral roots (Fig. 1*F*; cf. Coombs *et al.* 1955; Eccles, 1957). This procedure,

however, does not totally guarantee the exclusion of such i.p.s.p.s. Thus in Fig. 3*C* it failed to reveal a small i.p.s.p., possibly because the latter was at the same potential as the resting membrane owing to Cl^- leakage from the electrode; however, the recurrent i.p.s.p. became evident after the injection of EGTA (Fig. 3*D*), presumably as a result, of some depolarization, and also perhaps of reduction of shunting by the membrane K^+ conductance (G_K) (see below).

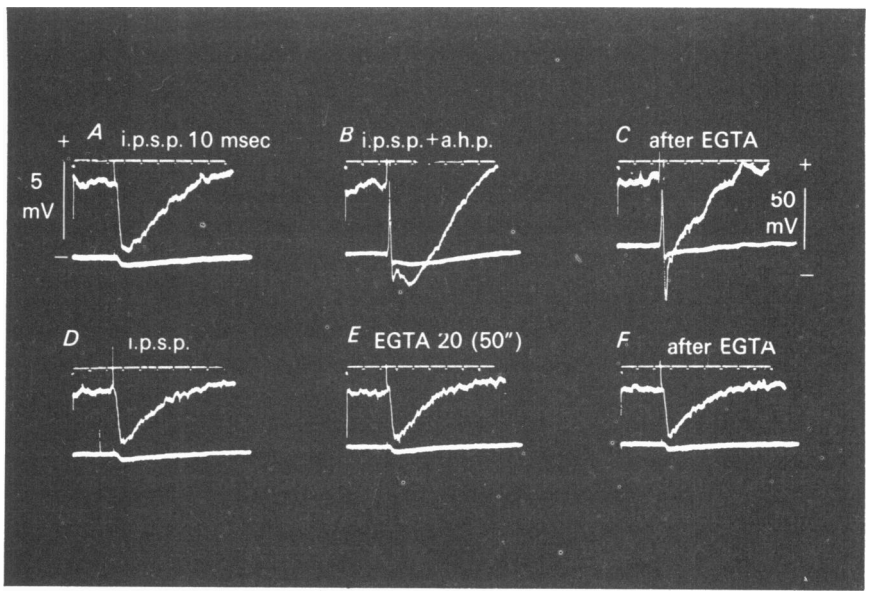

Fig. 4. Selective block by EGTA of a.h.p., but not i.p.s.p., evoked in lumbar 6 motoneurone by antidromic stimulation. In each case the same signal is displayed at low gain d.c. amplification (lower) and high gain a.c. amplification (upper). *A*, 'subthreshold' stimulus evoked only a recurrent i.p.s.p. *B*, stronger antidromic stimulus evokes both early and late a.h.p., as well as i.p.s.p. *C*, same, 5 sec after end of 35 sec injection of EGTA (20 nA); only i.p.s.p. and early a.h.p. are left. *D–E*, another series from same motoneurone recorded 3 min later while evoking only i.p.s.p. *D*, control. *E*, after 50 sec of EGTA injection (20 nA). *F*, 5 sec after end of injection of EGTA, which lasted 1 min.

Effect of EGTA on other hyperpolarizing responses

EGTA does not interfere with all *hyperpolarizing* responses of motoneurones. This was shown by comparing simultaneously the a.h.p. and various types of i.p.s.p.s.

A relative insensitivity of recurrent i.p.s.p.s has already been suggested by the traces of Fig. 3*C*, *D*. A more systematic demonstration is given in Fig. 4. Ventral root stimulation evoked a large hyperpolarizing i.p.s.p. (*A*), on which were superimposed two further hyperpolarizing responses when the ventral root stimulus reached the threshold for the axon of this motoneurone (*B*): an early brief one at the end of the spike, similar to that seen in amphibian motoneurones (exceptionally visible here because of a relatively poor resting potential), and a slow a.h.p., with the usual long duration and a maximum characteristically later than that of the i.p.s.p. When EGTA was injected, there was some depolarization but the late a.h.p. largely disappeared; by contrast, the i.p.s.p. remained, and the early hyperpolarizing transient was enhanced (*C*). A further large injection of EGTA (Fig. 4*D–F*) failed to induce any obvious changes in the i.p.s.p. (now evoked alone, by weaker stimuli).

Other tests of the same kind were performed while evoking i.p.s.p.s orthodromically (by dorsal root stimulation). Thus in Fig. 5, an i.p.s.p. was observed in a sacral 1 motoneurone, on stimulating the lumbar 6 dorsal root. Initially (inset trace *A*), this i.p.s.p. was much smaller than the a.h.p. evoked by antidromic stimulation of the sacral 1 ventral root; but the injection of EGTA almost abolished the a.h.p., whereas the i.p.s.p. became larger owing to the concomitant small depolarization (inset traces *B* and *C*; for time course of changes, see graph below).

The consistent failure to depress i.p.s.p.s or hyperpolarizing responses evoked by inward current pulses, provides strong evidence that EGTA does not interfere with hyperpolarizing responses indiscriminately, but rather inactivates the a.h.p. specifically.

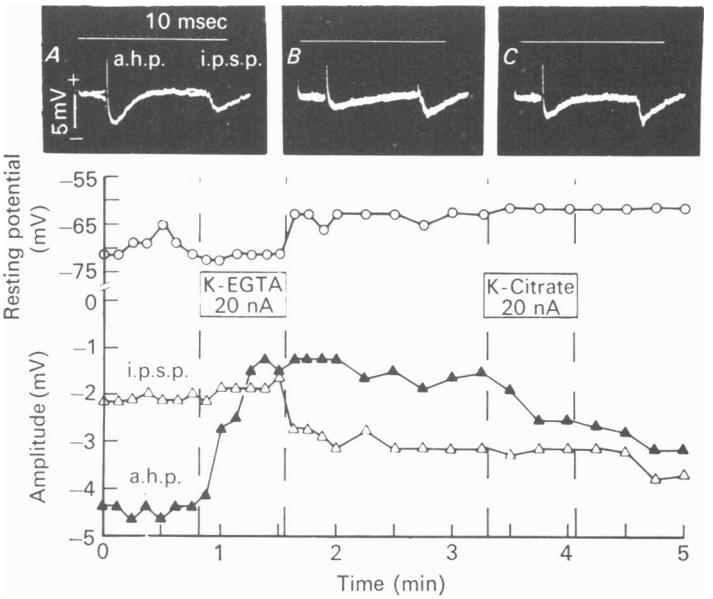

Fig. 5. Time course of selective depression of a.h.p. by intracellular injection of EGTA into sacral 1 motoneurone. Antidromic stimulation of ventral root evoked a spike and a.h.p. (first response in inset traces), and orthodromic stimulation of lumbar 6 dorsal root evoked an i.p.s.p. (second response). *A*, initial controls; only bottom portion of spike is visible in these high gain a.c. records. *B*, 20 sec after end of intracellular injection of EGTA (20 nA, for 50 sec). *C*, 45 sec after end of 20 nA citrate injection, lasting 1 min. In graph, open circles plot resting potential, open triangles amplitude of i.p.s.p., and closed triangles, that of a.h.p.

After hyperpolarization conductance change

EGTA could reduce the a.h.p. by suppressing either the underlying conductance increase, widely accepted as due to a rise in G_K (Coombs *et al.* 1955; Eccles, 1957; Machne *et al.* 1959; Kernell, 1964; Calvin & Schwindt, 1972; Baldissera & Gustafsson, 1974) or the electrochemical gradient generating the efflux of K^+; the latter might occur if EGTA lowered the internal K^+ activity by binding K^+ ions, driving them out of the cell, or slowing down Na–K pumping, or by raising the external K^+.

Measurements of membrane resistance and of the conductance increase during the a.h.p. were performed as described in the Methods, and illustrated in Fig. 6. The

upper records show separately pairs of similar resistance-measuring hyperpolarizing pulses (*A*) and an antidromic spike and a.h.p. (*B*), in each case with low gain d.c. and high gain a.c. traces displayed below and above respectively. In the lower records (*C*, *D*) the resistance-measuring pulses are combined with the same evoked potentials: the first pulse precedes the antidromic spike, and therefore monitors the 'resting' input resistance, while the second measures the resistance shortly after the maximum of the a.h.p. (the injected currents are shown in the uppermost traces). In traces *C*, the sharp reduction in pulse amplitude (and time constant) during the

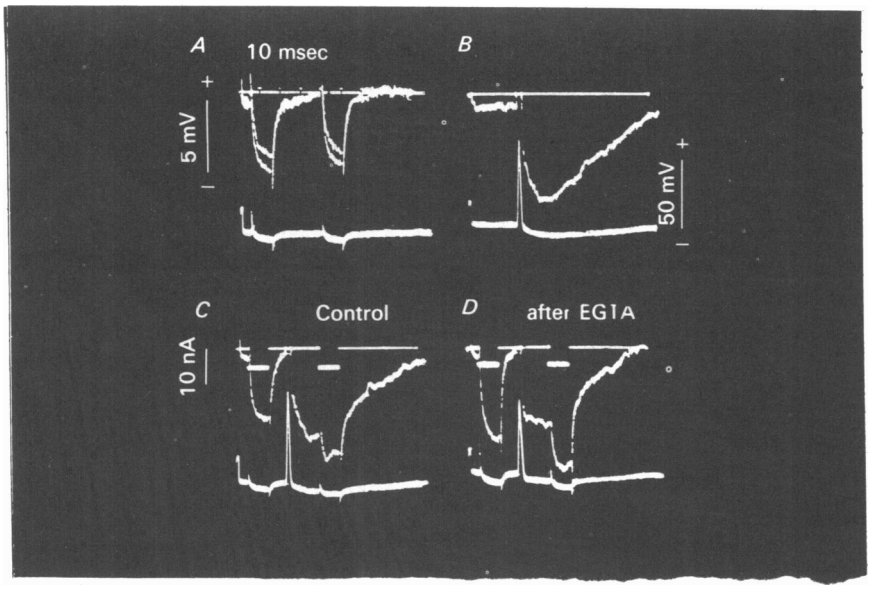

Fig. 6 EGTA increases resting membrane resistance and reduces conductance increase associated with a.h.p. In each record, lower traces were obtained at low gain d.c. amplification, upper traces at high gain d.c. amplification. *A*, two 10 sec intracellular current pulses evoked comparable hyperpolarizations in resting state. *B*, spike and a.h.p. evoked by antidromic stimulus. *C*, intracellular hyperpolarizing current pulses (monitored on uppermost trace) reveal increase in conductance during a.h.p. *D*, 1 min after end of injection of EGTA (10 nA; for 1 min).

a.h.p. confirms previous evidence of a high a.h.p. conductance (Eccles, 1957; Ito & Oshima, 1962; Baldissera & Gustafsson, 1974). Systematic measurements on nineteen motoneurones in six cats, indicated increases in conductance during the a.h.p. (before deliberate injection of EGTA) varying widely between +12 and +140%, with an average of +58% (Table 1).

In the experiment demonstrated in Fig. 6, after an injection of EGTA (*D*), slightly smaller current pulses evoked a first voltage pulse that was both greater and increased more slowly, reflecting a rise in 'resting' resistance, and a much increased second pulse, indicating a correspondingly large relative rise in resistance during the a.h.p. Thus, the diminished amplitude of the a.h.p. in *D* can probably be fully explained by the reduced increase in conductance (this was halved, changing from +120% in the control period, to +62% after EGTA).

More complete voltage/current data, obtained from two other cells by the same

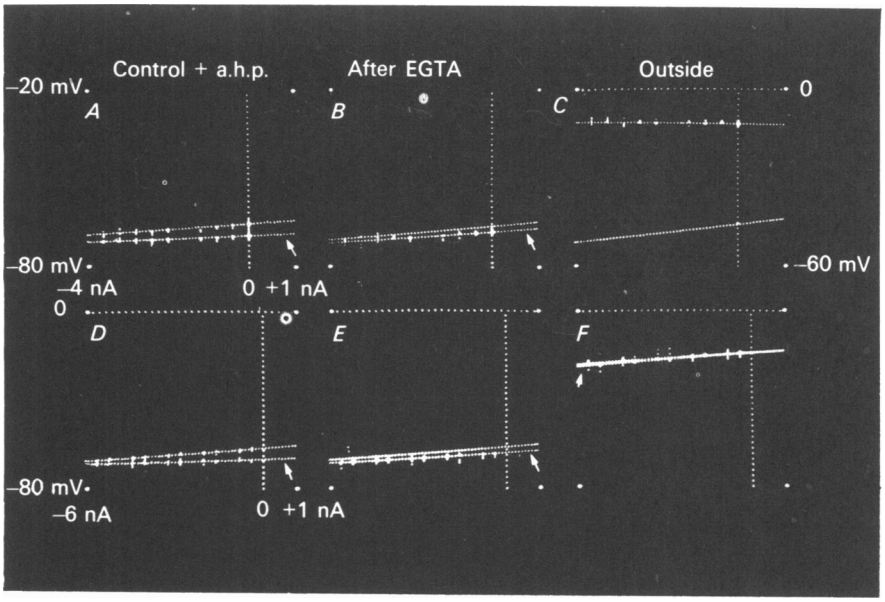

Fig. 7. Current–voltage relations showing depression by EGTA of a.h.p. and associated conductance changes. In *A*, *B*, *D* and *E*, current–voltage points and/or corresponding lines-of-best-fit for resting membrane are above and for a.h.p. below (marked by arrows). *A* and *D* are initial controls from two separate motoneurones. *B* and *E*, during injections of EGTA (5 nA in *B*, 10 nA in *E*). *C*, *F*, extracellular control runs (in *C*, final intracellular data are also shown for comparison); lines-of-best-fit for resting and a.h.p. points are indistinguishable in *C*. Note the change in voltage scales. In *F*, resting and a.h.p. points give slightly different lines-of-best-fit. In these and other comparable plots (cf. Fig. 11), reference points for voltage and current scales are indicated by four dots, one at each corner. Where voltage and/or current scales are not given, they are the same as in plot at left.

TABLE 1. Effects of intramotoneuronal injections of EGTA on resting input conductance (G_{rest}), increase in conductance near peak of a.h.p. (ΔG_{ahp}) and increase in conductance near peak of i.p.s.p. (ΔG_{ipsp}); latter was evoked by stimulating ventral or dorsal roots. Table summarizes observations made in six cats, after injecting EGTA iontophoretically with currents of mostly 5–20 nA, for about 1 min. Since repeated injections tend to produce a much smaller further change (see Table 2), especially with regard to ΔG_{ahp} corresponding data obtained after first injection of EGTA are shown separately. s: change statistically highly significant ($P < 0{\cdot}001$); n.s.: not significant ($P > 0{\cdot}1$).

	Initial controls			After EGTA injection, percentage change in			
	G_{rest} (μmho)	ΔG_{ahp} (%)	ΔG_{ipsp} (%)	G_{rest}	ΔG_{ahp} (all data)	ΔG_{ahp} (after first EGTA injection)	ΔG_{ipsp}
Mean	1·04	+57·7	+62·0	−19·5	−65·2	−74·3	+67·9
S.D.	0·51	35	56·0	19·6	33·4	19·5	231
No. of tests	20	19	10	35	29	18	15
No. of cells	20	19	10	20	18	18	10
				(s)	(s)	(s)	(n.s.)

technique, and showing similar changes are illustrated in Fig. 7 (*A–C* and *D–F*). In each display, the two sets of points and lines are groups of voltage/current points recorded in the 'resting' state and near the maximum of the a.h.p., as well as the corresponding lines-of-best-fit (a.h.p. data are marked by an arrow). The initially marked differences between these slopes (*A* and *D*) are clearly much reduced after injections of EGTA (*B* and *E*).

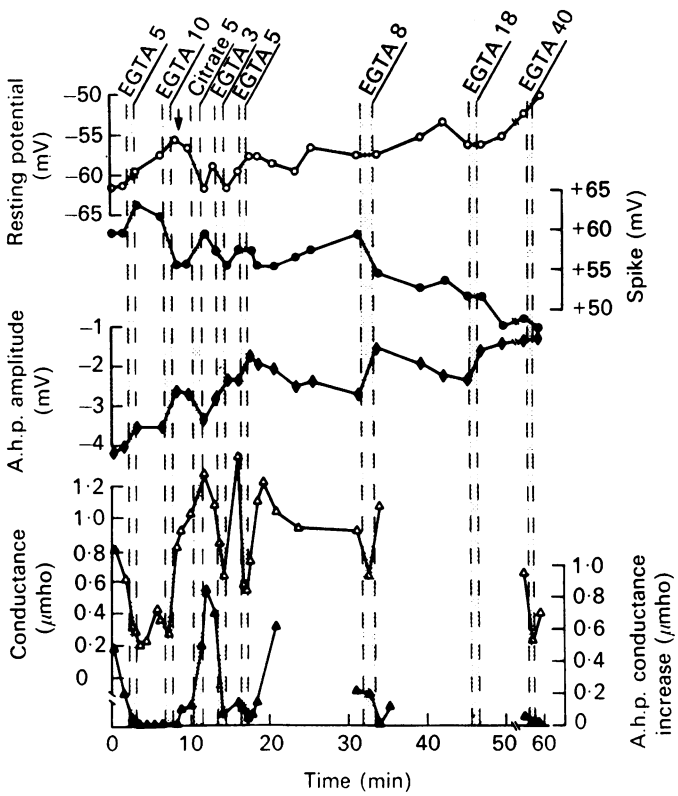

Fig. 8. Graph showing effects of several intracellular injections of K-EGTA and one of K-citrate on resting potential (open circles), antidromic spike amplitude (filled circles), a.h.p. amplitude (filled diamonds), resting conductance (open triangles) and increase in conductance near peak of a.h.p. (filled diamonds), all recorded from same spinal motoneurone. Estimates of resting conductance and increase during a.h.p. were obtained by technique illustrated in Figs. 6 and 7. Push–pull iontophoretic currents used for injections are indicated in nA. At arrow, 2 nA braking current was begun and maintained subsequently.

In twenty-six out of twenty-nine tests (90 %) of the action of EGTA on eighteen motoneurones (in six different experiments) there was a clear reduction in a.h.p. conductance increase: in only one instance did there appear to be a greater increase of conductance, and in two cases there was no obvious change. The data obtained in these experiments are summarized in Table 1. The mean alteration in a.h.p. conductance increase observed just after the twenty-nine injections of EGTA was a reduction by −65 %. The effects of repeated injections of EGTA soon approached a maximum (cf. Table 2); therefore Table 1 also gives the mean reduction (74 %) seen

after the first injection into any one cell. One can see also in Table 1 that the comparable increase in i.p.s.p. conductance showed no consistent change after EGTA.

Quantity of EGTA needed and the time course of the effect

In initial experiments, large amounts of EGTA were injected (with iontophoretic currents of 40–80 nA, or even more): but it soon became apparent that even without deliberate injections, there was a general tendency for a.h.p.s to diminish progressively. The small amount of EGTA leaking spontaneously from electrodes was probably sufficient to depress a.h.p.s. This leakage may have accounted for our initial impression that the effects of EGTA were largely irreversible (Krnjević *et al.* 1975). Subsequently we reduced this continuous efflux by the application of a small retaining current.

TABLE 2. Effect of three successive intracellular injections of EGTA (each lasting about 1 min) on resting input conductance (G_{rest}), conductance near peak of a.h.p. (G_{ahp}) and percentage increase in conductance during a.h.p. (ΔG_{ahp}) measured in same motoneurone. Control data were obtained just before injections, which were separated by intervals of 15–20 min. Percentage change in G_{rest} following EGTA release with reference to preceding control is also indicated (ΔG_{rest}).

Controls			EGTA	After EGTA injection			
G_{rest} (μmho)	G_{ahp} (μmho)	ΔG_{ahp} (%)	current (nA)	G_{rest} (μmho)	ΔG_{rest} (%)	ΔG_{ahp} (μmho)	ΔG_{ahp} (%)
1·08	1·93	79	5	0·62	−42	0·69	+10
0·90	1·12	24	10	0·63	−30	0·62	−2
0·62	0·66	6	20	0·51	−18	0·55	+8

The beneficial effect of such a braking current can be seen in Fig. 8; this graph shows the 'resting' potential, the magnitude of the spike, and the a.h.p., as well as the input conductance increase associated with the a.h.p. All were recorded from one motoneurone over a period of one hour, during which several intracellular injections of EGTA were made. Initially, in the absence of braking current, the a.h.p. was diminishing spontaneously, but this trend became less conspicuous after the start of 2 nA of retaining current (at *arrow*; see also changes in 'resting' potential). The effects of subsequent injections of EGTA showed a clear, though slow spontaneous recovery.

This figure also demonstrates that injections of EGTA by only 3 nA had an unmistakable depressant effect on the a.h.p.; and indeed, the depression appeared to reach (or approach) a maximum after an injection at 8 nA; an 18 nA injection produced no greater change (but there was no recovery); and even a 40 nA injection was hardly more effective, although the a.h.p. amplitude had not fallen below $\frac{1}{4}$ of its initial value. In general, in all our experiments, the reduction of the a.h.p. never exceeded a maximum of 75–80 % (Figs. 2, 3, 5 and 10). Comparable data in Table 2 show that repeated and increasing injections of EGTA failed to produce correspondingly greater depressions of the a.h.p. conductance change; but the resting conductance was progressively lowered. Thus, it appears that the a.h.p. conductance change is particularly sensitive to EGTA, but the effect of the latter rapidly reaches saturation.

Even in the presence of a retaining current, the rates of recovery of the a.h.p. were quite slow (in the order of 0·1 mV/min). However, recovery was sometimes accelerated by certain procedures, such as intracellular injections of anionic currents of citrate-Cl^- or injections of Ca^{2+}.

Injections of 'anionic' currents or Ca^{2+}

In three experiments, a reversed 'push–pull' current was injected into eight motoneurones as a control for the injections of EGTA: that is, current was made to flow from the barrel containing K-EGTA to the indifferent channel. This would effectively release K-citrate, which is a very much weaker chelator, as well as a

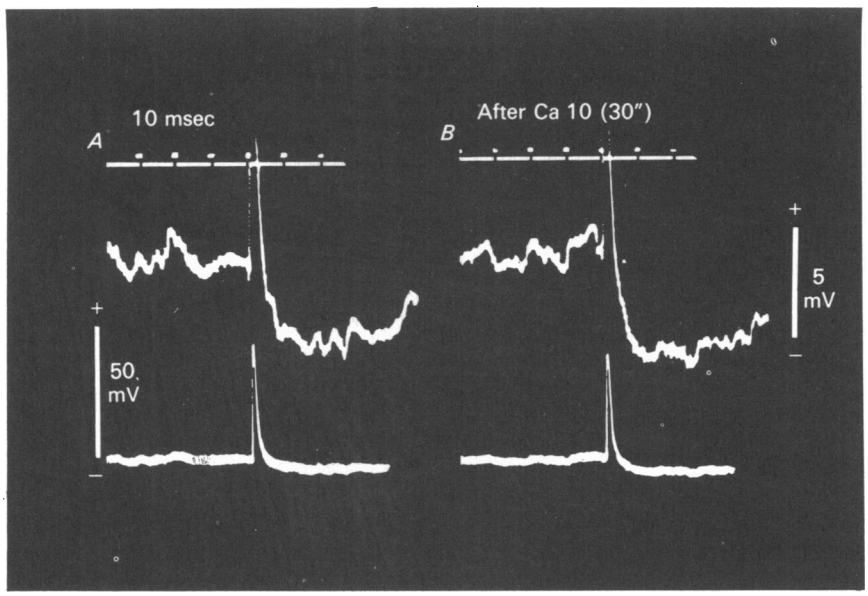

Fig. 9. Intracellular injection of Ca^{2+} enhances a.h.p. evoked by antidromic activation of L7 motoneurone. *A*, control. *B*, 5 sec after end of calcium injection (10 nA, for 30 sec).

small amount of Cl^- (see Methods). In seven cases out of ten this appeared to accelerate the rate of recovery of the a.h.p., for example in Fig. 5 and possibly in Fig. 8, where the conductance increase in particular became much more striking (though this must have been at least partly due to an electrode artifact). The average increase in a.h.p. conductance change was by 79%. In only one instance did we record a diminished a.h.p. conductance change. By contrast, the most common effect of such injections on i.p.s.p.s was to *reduce* the conductance change (by a mean of nearly 30%). This may be ascribed to the substitution of internal Cl^- by non-permeant citrate anions.

Intracellular injections of Ca^{2+}, with currents of about 10 nA, which are near the minimum required to produce regular changes in motoneuronal resistance (Krnjević & Lisiewicz, 1972), sometimes increased the amplitude of a.h.p.s (Fig. 9). However, a similar injection of Ca^{2+} into another cell, *during an injection of EGTA*, was hardly effective, and indeed, did not prevent further depression of the a.h.p. by EGTA

(Fig. 10*B–D*). But another injection of Ca^{2+}, after the end of the long release of EGTA, accelerated the recovery of the a.h.p. (Fig. 10*E*, *F*). Especially when injections of Ca^{2+} were not preceded by deliberate injections of EGTA, the most common effect was to *reduce* the increase in a.h.p. conductance, possibly by occlusion, since Ca^{2+} tends to raise the K conductance (Krnjević & Lisiewicz, 1972). Precise quantification of these observations was made difficult by large variations in tip resistance when both Ca^{2+} and EGTA were released from the same electrode. It has also been found (Krnjević, Puil & Werman, 1978), that the recovery of depressed a.h.p.s can be speeded up by applications of 2,4-dinitrophenol, presumably because these also raise free $[Ca^{2+}]_i$.

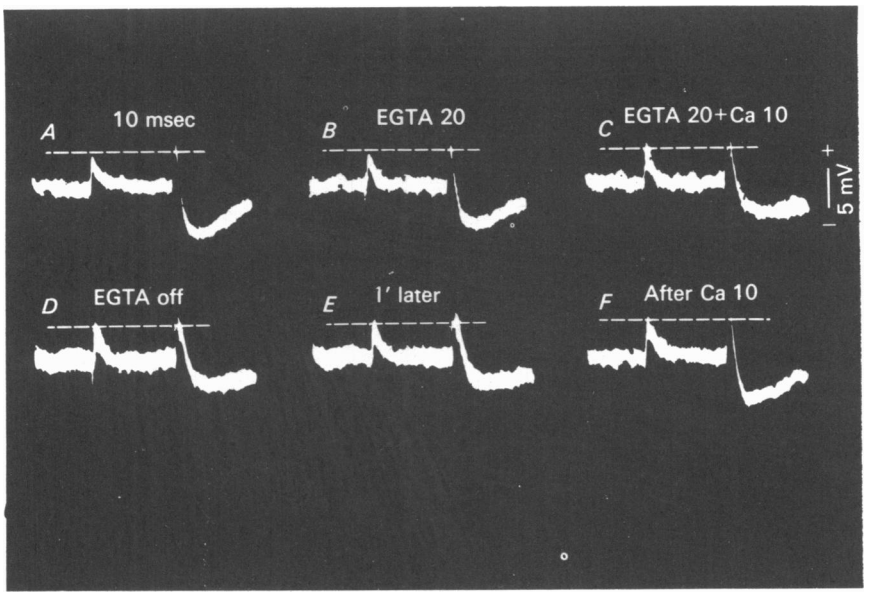

Fig. 10. Interactions of intracellular injections of EGTA and Ca^{2+} in a sacral 1 motoneurone. Each record shows first, an e.p.s.p. evoked by stimulation of S1 dorsal root, followed by antidromic spike and a.h.p. (high gain, a.c. recording). *A*, initial control. *B*, after 30 sec of EGTA injection (20 nA). *C*, during continued EGTA injection, 40 sec after start of injection of Ca^{2+} (10 nA). *D*, 10 sec after end of prolonged EGTA injection (lasting 2 min). *E*, 1 min later. *F*, after end of 1 min injection of Ca^{2+}.

Other effects of EGTA

On resting potential and resistance. As already pointed out, EGTA injections tended to raise the motoneuronal input resistance (see Fig. 6). This effect is illustrated more systematically in Fig. 11, with data from three different motoneurones (*A–C*, *D–F* and *G–I*). In each series, the voltage–current points at the left are initial control values, with corresponding lines-of-best-fit; those in the middle are values obtained during an injection of EGTA (with the initial control lines also displayed for reference), and at the right are data recorded during a similar injection of EGTA *outside* the cell. The latter consistently showed no comparable increase, but even a decrease in apparent resistance (*C*). Increases in input resistance are also evident in Figs. 7 and 8, though in the latter this effect is largely masked by large shifts in apparent resistance

probably due to artifacts. In twenty motoneurones, twenty-nine out of thirty-five injections (83 %) of EGTA were followed by a rise in resistance. As shown in Table 1, the mean change was an increase of 20 %. Repeated injections had a cumulative effect that showed little saturation (Fig. 8, Table 2).

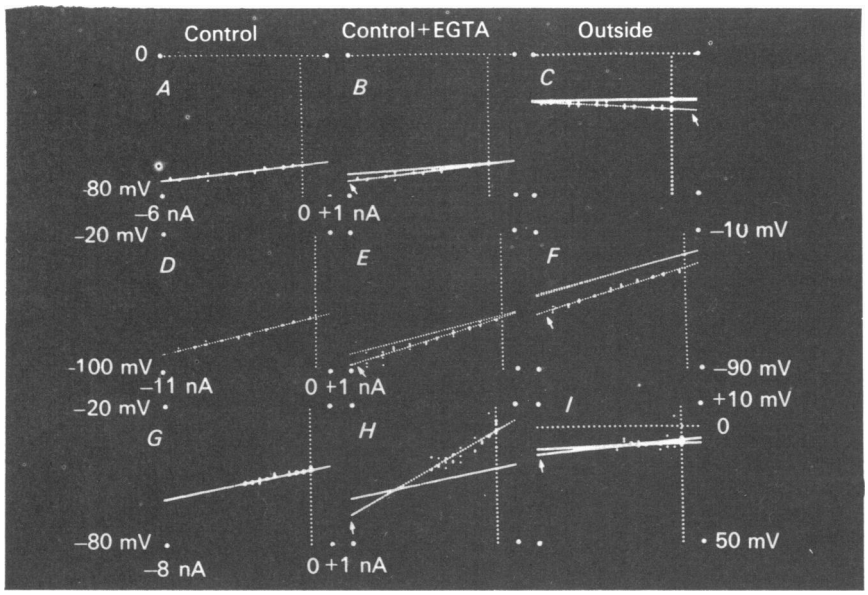

Fig. 11. Current–voltage relations from three separate motoneurones showing rise in input resistance and depolarization evoked by intracellular injection of EGTA. *A*, *D* and *G*, control data with lines-of-best-fit. *B*, *E* and *H*, control lines-of-best-fit (unmarked) and current–voltage data recorded during injections of EGTA (marked by arrows). Iontophoretic injections were 20 nA, 20 nA and 40 nA in *B*, *E* and *H* respectively. *C*, *F* and *I*: control runs performed extracellularly: arrows mark data obtained during identical EGTA injections, the other lines being pre- and post-injection controls. Where voltage and/or current scales are not indicated, they are the same as for plots at left.

Coupling artifacts cause greater problems in the analysis of changes in resting potential. Thus, the traces in Fig. 11*E* at first sight seem to indicate a hyperpolarizing action of EGTA. However, the extracellular control runs often showed quite substantial negative shifts (*C*, *F*). If allowance is made for these coupling artifacts, the true intracellular change is clearly a depolarization. Generally, there was a depolarizing trend (see also Figs. 5, 6), but this was quite variable, and not readily quantified owing to the coupling artifacts.

The spike and other evoked potentials. As a rule EGTA showed little effect on spike potentials of cells with stable resting potentials. In particular, the rate of decay of the spike, displayed more directly by the time-derivative trace (see Fig. 3), showed no deceleration, even when the a.h.p. was markedly reduced. On those occasions when the spike was followed by a brief phase of hyperpolarization (clearly distinct from the a.h.p.) it was not only *not* depressed by EGTA, but rather was usually enhanced, presumably owing to further depolarization (Fig. 4*B*, *C*).

Of course, when depolarization was more pronounced, the spikes became not only smaller (Figs. 2, 6), but also appreciably slower (Fig. 12); but there was no selective depression of the rate of decay of the spike (as compared to its rate of rise).

Similarly, we observed no consistent alteration in the delayed depolarization that could not be ascribed simply to a corresponding change in resting potential. Thus, the depression of the delayed depolarization in Fig. 12 (*A–D*), as a result of large and repeated injections of EGTA, can be accounted for by the accompanying depolarization; it is fully comparable to the effect of artificial depolarization illustrated in the lower traces (*E–H*) (cf. Kernell, 1964; Nelson & Burke, 1967).

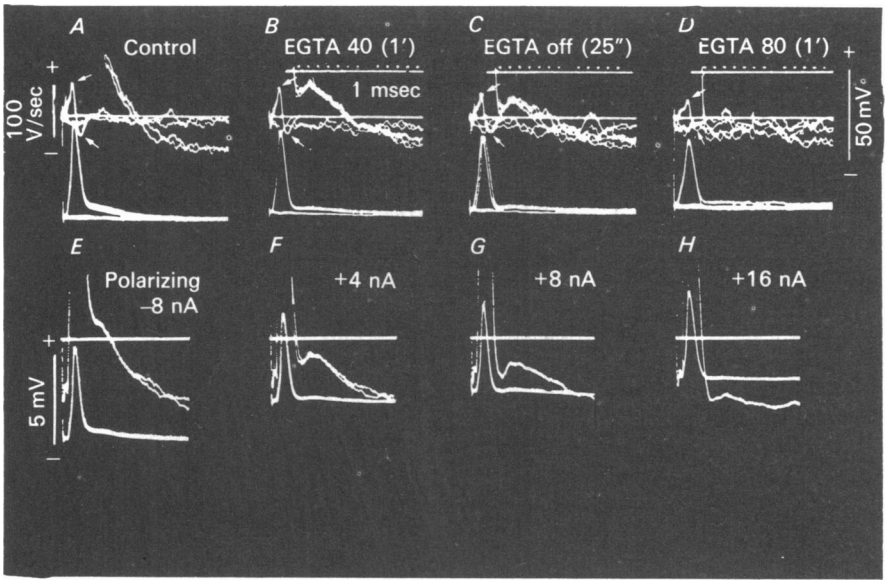

Fig. 12. Both EGTA and artificial polarization can reduce the delayed depolarization. In all cases lowest traces are low gain d.c. records and higher traces the same at high gain a.c. amplification. In *A–D*, a further trace also displays the time derivative of the action potential (this trace and the solid line in *E–H* provide a fixed reference level for d.c. changes). *A*, control. *B*, 1 min after start of injection of EGTA (40 nA). *C*, 25 sec after end of EGTA injection. *D*, 3 min later, 1 min after start of another EGTA injection (80 nA). *E–H* show effect of hyperpolarizing (*E*) or depolarizing (*F–H*) currents on delayed depolarization recorded in another motoneurone; strength of polarizing current is indicated in each trace.

Several attempts to elicit clear changes in e.p.s.p.s (evoked either by dorsal root stimulation, or in a more discriminating fashion by stimulating individual nerves in the hind limbs) produced somewhat inconclusive results. The short-lasting, low threshold e.p.s.p. illustrated in Fig. 10 showed some facilitation after the injection of EGTA that can be attributed to increase in input resistance. A potentiating action was also suggested by the appearance of spikes in several instances, but whether this was mainly due to a lowering of firing threshold (by depolarization) or an enhancement of the e.p.s.p. could not be determined.

Observations in 'neuroglia'

EGTA was injected also into ten cells classed as probably 'neuroglia' on the basis of their high and stable resting potentials, inexcitability in response to intracellular stimulation, and absence of spontaneous or evoked synaptic potentials. Four of these cells responded repeatedly to the injection of EGTA with a rise in input resistance, and more variable changes in potential. The other six cells showed either no consistent effects, or, in two cases, a reproducible *fall* in input resistance.

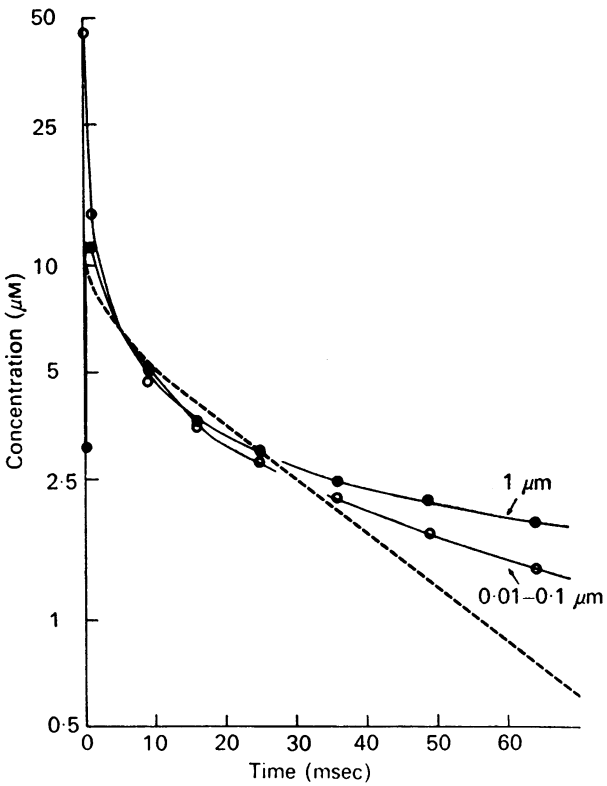

Fig. 13. Comparison of time-course of a.h.p. conductance increase and that of theoretical change in intracellular Ca^{2+}. Typical a.h.p. conductance change, as recorded in motoneurones by Baldissera & Gustafsson (1974), is indicated by dotted line. Changes in Ca^{2+} concentration at various distances from instantaneous spherical source (5 pmole/cm^2, see text) are shown by circles (filled, at 1 μm; open, at 0·01–0·1 μm).

DISCUSSION

EGTA and K conductance

The most important direct action of EGTA is to bind Ca^{2+} (Portzehl, Caldwell & Rüegg, 1964; Baker *et al.* 1971). Some other significant effects cannot be excluded, such as an interference with Na–K pumping (Baker & McNaughton, 1976; Logen & O'Donovan, 1976). Although these may contribute to some of the observed changes (for example in resting potential and spike amplitude), it is difficult to see how they would explain the conspicuous reduction in the increased conductance during the

a.h.p.; especially since any significant binding of the other internal divalent cation, Mg^{2+}, should produce opposite effects (Krnjević, Puil & Werman, 1976*a*). Our results therefore provide substantial evidence that, as previously suggested (Krnjević & Lisiewicz, 1972), an influx of Ca accompanying the action potential causes a significant temporary increase in motoneuronal G_K, but the high G_K of the falling phase of the spike is evidently not generated in this way, neither in cat motoneurones nor in other neurones that have been investigated, such as squid axons (Begenisich & Lynch, 1974; Meech, 1974*a*), Aplysia cells (Meech, 1974*b*) and frog motoneurones (Barrett & Barrett, 1976).

Two kinds of K channels

At least two kinds of K channels can be distinguished in cat motoneurones. The first population is activated by intracellular Ca (Krnjević & Lisiewicz, 1972). It is also revealed by the depressant effect of EGTA (especially on the slow a.h.p.) and evidently includes K channels that contribute significantly to the resting membrane conductance (cf. the 20% mean fall in input conductance that follows injections of EGTA; a comparable effect was seen in some Aplysia cells by Parnas & Strumwasser, 1974). These channels are *not* conspicuously sensitive to intracellular TEA or ACh (Krnjević *et al.* 1976*b*), but their activation can be depressed by intracellular Mg, possibly because the latter competes with Ca (Krnjević *et al.*, 1976*a*). *Extracellular* applications of ACh also appear to reduce the activation of these channels, but through what mechanism is not clear.

The second kind of K channels is quite insensitive to intracellular injections of EGTA or Mg, and therefore presumably is not activated by internal Ca. These channels, which include the voltage-sensitive K conductance responsible for the falling phase of the spike, are readily blocked by intracellular TEA or ACh (Krnjević *et al.* 1976*b*; see also Coombs *et al.* 1955; Krnjević, Pumain & Renaud, 1971; Shapovalov & Kurchavy, 1974).

The above observations thus fully agree with those of Barrett & Barrett (1976) on frog motoneurones *in vitro*; of particular relevance is their complementary demonstration that the slow a.h.p. is abolished by the removal of extracellular Ca.

Significance of Ca-activated K channels

A comparable phenomenon of post-activity hyperpolarization, due to high G_K induced by Ca influx, appears to be present in a variety of excitable cells (e.g. Brown & Brown, 1973; Nishi & North, 1973; Meech, 1974*c*; Minota, 1974; Mounier & Vassort, 1975; Vassort, 1975; Bassingthwaighte, Fry & McGuigan, 1976; Fink & Lüttgau, 1976; Clusin & Bennett, 1977). It may well be a very widespread intrinsic mechanism for limiting the duration of excitatory processes.

The rapid depression of the a.h.p. by EGTA may indicate that its K channels include a particularly high proportion of Ca-sensitive channels, or that these may have a relatively low affinity for Ca and therefore cannot readily compete with EGTA. The opposite appears to be true for the K channels responsible for the resting G_K. The presence of high-affinity Ca-dependent channels in resting motoneurones is in keeping with the suggestion that variations in intracellular free Ca may establish

a significant and relatively direct link between metabolic activity and neuronal excitability (Krnjević & Lisiewicz, 1972).

Interaction between EGTA and $[Ca]_i$

If one assumes that a typical motoneurone has a volume of some 100 pl. (from the data given by Lux, Schubert & Kreutzberg, 1970), 20 nA injections might be expected to lead to an intracellular accumulation of EGTA at the rate of at least 0·1 mM/sec. So after a 1 min injection, in the absence of any marked outward leakage or of any significant internal uptake (neither of which seems likely), the cell would have a cytoplasmic concentration of EGTA well over 1 mM. Assuming an internal pH 6·6–7·1 and therefore an apparent association constant for Ca-EGTA of about $10^{-6\cdot4}$ (Portzehl *et al.* 1964), this concentration of EGTA would prevent free Ca from rising above 1 μM, unless enough Ca entered the cell to reduce free EGTA significantly. We have no information about the presumed influx of Ca during an action potential. However, an upper limit is obtained by assuming that the action potential is generated only by influx of Ca. Thus, for a 120 mV spike, and a membrane capacitance of 6 μF/cm^2 (Eccles, 1957), the Ca influx would approach 5 pmole/cm^2. The resulting increase in cytoplasmic Ca can be estimated as follows:

If the motoneurone is approximated to a sphere, one can calculate the expected internal concentration at a given distance from the surface membrane at various times after the Ca influx, assumed to be instantaneous at time zero. The appropriate equation (for an 'instantaneous spherical source') is given by Carslaw & Jaeger (1959) as:

$$c = \frac{Q'}{8\pi r r' (\pi D t)^{\frac{1}{2}}} \left[e^{-(r-r')^2/4Dt} - e^{-(r+r')^2/4Dt} \right] \quad (1)$$

where D is the diffusion coefficient and c is the concentration in mole/cm^3 at a point r cm from the centre of the sphere, at time t; the source being situated at the surface of the sphere ($r = r'$), and its strength $Q' = 4\pi r'^2 Q$, where Q is the influx per unit area. Therefore,

$$c = \frac{r' \times 0{\cdot}282Q}{r(Dt)^{\frac{1}{2}}} \left[e^{-(r-r')^2/4Dt} - e^{-(r+r')^2/4Dt} \right] \quad (2)$$

Taking D as 10^{-5} cm^2/sec, and an upper limit of 5 p-mole/cm^2 for Q (cf. above), eq. (2) becomes:

$$c = \frac{14{\cdot}1}{t^{\frac{1}{2}}} \cdot \frac{r'}{r} \left[e^{-(r-r')^2/4t} - e^{-(r+r')^2/4t} \right] \quad (3)$$

the concentration being now in μM, r and r' in μm, and t in msec.

Fig. 13 gives the changes in internal concentration near the surface of a sphere with $r' = 20$ μm, the closed circles at a distance of 1 μm from the surface, the open circles at distances of 0·01–0·1 μm from the surface (these are identical for $t > 0{\cdot}1$ msec). The dashed line indicates for comparison the normalized time course of conductance change during the a.h.p., observed in cat spinal motoneurones by Baldissera & Gustafsson (1974; from their Fig. 4*A*).

Several conclusions can be drawn from Fig. 13. The first is that during the portion of the a.h.p. for which there is reliable information (that is for $t > 1$ msec), the rate of change of concentration is approximately the same at distances ranging between 0·01 and 1 μm from the source. The peak concentration is very sensitive to distance since at 0·1 μm the peak is 14 μM (closed circles), whereas at 0·1 μm it exceeds 46 μM and at 0·01 μm it is over 140 μM, but the high peaks expected at very short distances are so brief that they are unlikely to be of significance for the prolonged a.h.p. (unless one postulates a long-lasting gating action, see Heyer & Lux (1976)). If moreover it is realized that these values are based on a maximal estimate of Ca, influx (which may be exaggerated by a factor of as much as 1000, since according to the observations of Dambach & Erulkar (1973) and Barrett & Barrett (1976), Ca contributes negligibly

to the frog motoneuronal action potential, while in the squid axon, Hodgkin & Keynes (1957) measured an additional Ca influx of only 5 fmoles/cm^2 impulse) then these calculations suggest that the Ca needed for G_K activation can hardly be much greater than 10^{-6} M, and indeed may well be much less. This conclusion is therefore more in keeping with Meech's (1974*a*) observations on molluscan neurones, according to which a rise in G_K is induced when $[Ca]_i$ reaches 10^{-6} M, than with Lew & Ferreira's (1976) recent estimate of 10^{-3} M for the K_m of K flux activation by $[Ca]_i$ in red blood cells.

A final point of interest in Fig. 13 is that, while the rate of decay of concentration roughly matches the conductance change for times between 1 and 35 msec, there is a marked discrepancy for longer times. One possible explanation is that rapid sequestration of Ca (Rose & Loewenstein, 1975; Baker & Schlaepfer, 1975), which is absent from the simple diffusion model, would accelerate the fall in $[Ca]_i$ and therefore in conductance. An alternative is that, like some other actions of Ca (e.g. Dodge & Rahaminoff, 1967), this also requires the cooperative action of two or more ions of Ca, in which case the conductance change may decay more rapidly than $[Ca]_i$.

Nature of EGTA-insensitive component of a.h.p.

Even the largest, repeated injections of EGTA did not always abolish the a.h.p. A residual a.h.p. amounting to at least 20% of the original was consistently observed (see Figs. 2, 3, 5 and 7). This may indicate that the Ca-induced high G_K is not the only significant factor involved in the generation of the a.h.p. This is probably not a recurrent i.p.s.p. since residual a.h.p.s were evident even when i.p.s.p.s were markedly reversed by Cl^- leakage. A more likely explanation is that the voltage-dependent high G_K (delayed rectification) persists for 10 or more msec after the end of the action potential, long enough to overlap with the slower, Ca-activated process. There is another possibility: if the internal surface of the membrane is negatively charged (Chandler, Hodgkin & Meves, 1965), anions would tend to be at a lower concentration in the electric double-layer than in the bulk of the cytoplasm (Davies & Rideal, 1961); a multivalent anion, such as EGTA, may thus be prevented from reaching the high concentration required at the site where Ca presumably activates G_K (especially since a lower surface pH would further diminish EGTA's chelating action).

The assumption made here, that G_K is raised by the increase in $[Ca]_i$, consequent on a spike-linked influx of Ca, is similar to that of Meech & Standen (1975) in their analysis of the late outward current in snail neurones. In both cases it is based on previous evidence that a rise in G_K is evoked by injections of Ca into the same neurones. However, there are some complicating features: in spinal motoneurones, such injections do not usually cause a sharp hyperpolarization (Krnjević & Lisiewicz, 1972); and even in snail neurones, there is evidence of a biphasic action (Meech, 1974*a*). High levels of $[Ca]_i$ may therefore activate less selective channels, or a mixture of both Na^+ and K^+ channels. Alternatively, the relatively slow iontophoretic injections may initiate a release of protons from mitochondria, thus leading to a secondary depolarizing effect (Meech & Thomas, 1977). On the other hand, Heyer and Lux (1976) found that intracellular injections of Ca^{2+} *depressed* the delayed outward current in snail cells; and, on this basis (as well as some other less direct evidence) they concluded that G_K is activated by the transmembrane Ca *current* but inactivated by $[Ca]_i$. It is difficult to reconcile this scheme with the marked depression of a.h.p.s caused by internal EGTA, which should enhance I_{Ca} by making the inward gradient for Ca^{2+} even steeper. The critical feature may well be the amount and distribution of Ca injected intracellularly. As shown in Fig. 9, an injection of Ca can sometimes potentiate the a.h.p., presumably by permitting a greater increment of $[Ca]_i$ following a spike. The range of $[Ca]_i$ over which this is seen may be quite narrow for several reasons: a probable rise in 'resting' G_K would tend to occlude the a.h.p.; the relation between $[Ca]_i$ and G_K may be very non-linear; and as mentioned above, another conductance (such as G_{Na}) may also be activated, either directly or indirectly.

Duration of action of EGTA

The absence of recovery from the effects of EGTA injections in the initial experiments may have been due to leakage of EGTA from the microelectrode. When a 2 nA braking current was applied continually, recovery was commonly seen, though often only partial and quite slow. For example, in Fig. 8, the a.h.p. took about 6 min to recover from a 90 sec injection of EGTA (5 nA). Since EGTA is unlikely to be rapidly removed, its inactivation may depend upon binding to Ca.

Assuming again a transport number of 0·2, this would correspond to a total intracellular injection of 0·2 pmole of EGTA. If the recovery of the a.h.p. in 6 min corresponds to the time taken for an equivalent amount of Ca to enter the cell (0·4 pmole, all of which is assumed to be chelated by EGTA), the rate of Ca^{2+} influx caused by continual antidromic stimulation (at 2/sec) would have been 1·2 pmole/sec. For a surface area of 10^{-4} cm^2 (Lux *et al.* 1970), this is equivalent to an influx of 12 $pmole.cm^{-2}.sec^{-1}$. The agreement with the maximum Ca influx postulated per impulse (6 pmole against 5 $pmole/cm^2$) is almost certainly fortuitous. Nor is it likely that the spontaneous influx is vastly greater than in the squid axon ($< 0{\cdot}1$ $pmole.cm^{-2}.sec^{-1}$ according to Hodgkin & Keynes (1957) and Rojas & Taylor (1975)), unless there was significant damage to the cell membrane and a corresponding large influx of Ca (Hodgkin & Keynes, 1957); the fact that the neurone remained excitable is not necessarily strong evidence against membrane damage, since the EGTA may have protected the cell against its worst consequence, a rapid rise in free $[Ca]_i$.

An alternative possibility is that EGTA may have captured Ca from internal binding sites of lower affinity, including mitochondria (though an opposite trend was observed in Aplysia neurones by Meech (1974*b*)): its rate of inactivation may therefore not reflect the influx of external Ca.

It is of interest that the rate of recovery of the a.h.p. was accelerated by injecting other anions, indicating that EGTA may be electrophoresed out of the cytoplasm by this procedure.

Delayed depolarization

Some differences between amphibian and cat motoneuronal after-potentials would be explained if the delayed depolarization is generated by an inward current of Ca. Although the delayed depolarization evidently lasts well beyond the peak of delayed rectification, there is probably overlap of the two processes (for a comparable overlap of Ca and K currents, see Eckert & Lux (1976) and Heyer & Lux (1976)): hence, in cats, where the Ca influx may be relatively important (as suggested by the large slow a.h.p.), the early outward current of K would be partly masked by the inward current of Ca; by contrast, in frogs, where the slow a.h.p. is small and therefore suggests a rather minor Ca influx, the delayed rectification is able to generate an overt early a.h.p. This explanation, rather than an alternative one involving a slower onset of G_{Ca}, is supported by the variability of the reversal potential for the early a.h.p., which indicates the involvement of more than one ionic conductance (Magherini *et al.* 1976). A mixture of G_K and G_{Ca} would account for the relatively positive reversal level of the delayed depolarization in cats (Fig. 12; Eccles, 1957, p. 83). More direct evidence that it is related to Ca^{2+} influx is provided by Barrett & Barrett's (1976) illustrations of the effects of Ca removal (or the application of Mn^{2+}), which consistently appear to abolish the delayed depolarization together with the slow a.h.p. (see their Figs. 2–4).

These features of the delayed depolarization agree well with descriptions of the

slow Ca^{2+} current in molluscan neurones (Baker *et al.* 1971; Meech & Standen, 1975; Eckert & Lux, 1976). The delayed depolarization also markedly resembles the after-depolarizations recorded in mammalian motor or sensory axons (Coombs *et al.* 1955; Eccles & Krnjević, 1959); the reason why the latter are not followed by large a.h.p.s. may be that axons have few Ca-activated K^+ channels (cf. Meech & Standen, 1975). One can conclude that the delayed depolarization is likely to be a true depolarizing after-potential, generated by Ca influx.

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Note added in proof. More recent experiments, also on spinal motoneurones in cats, have failed to demonstrate any clear inhibition of the delayed depolarization by extracellular Co^{2+} or Mn^{2+}, and therefore cast some doubt on the suggestion that delayed depolarization reflects an inward Ca current (J. F. MacDonald, K. Krnjević & A. Nistri (1978), *Can. J. Physiol. Pharmacol.*, in the Press).

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