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INTRACELLULAR FREE MAGNESIUM IN NEURONES OF *HELIX ASPERSA* MEASURED WITH ION-SELECTIVE MICRO-ELECTRODES

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SUMMARY

1. Cytoplasmic free Mg^{2+} concentration, $[Mg^{2+}]_i$, was measured in identified neuronal cell bodies of the suboesophageal ganglia of *Helix aspersa*, using Mg^{2+}-selective micro-electrodes.

2. In calibration solutions, the electrodes showed significant interference from K^+, but not from Na^+, or Ca^{2+}, at concentrations found intracellularly. Therefore, in order to calibrate the electrodes properly, it was necessary first to obtain an accurate value for intracellular free K^+ concentration ($[K^+]_i$). The mean value for $[K^+]_i$ was 91 mM (S.E. of the mean $\pm 2{\cdot}2$ mM, $n = 8$), measured with K^+-sensitive 'liquid ion exchanger micro-electrodes'.

3. In seven experiments, which met stringent criteria for satisfactory impalement and electrode calibration, the mean $[Mg^{2+}]_i$ was 0·66 mM (S.E. of the mean $\pm 0{\cdot}05$ mM).

4. The mean $[Mg^{2+}]_i$ in cells that had spontaneous spike activity was not significantly different from that in quiescent cells.

5. If Mg^{2+} was in electrochemical equilibrium, the ratio $[Mg^{2+}]_i/[Mg^{2+}]_o$ would be about 55. Mg^{2+} is therefore not passively distributed across the neuronal membrane and an outwardly directed extrusion mechanism must exist to keep $[Mg^{2+}]_i$ low and constant, even in cells undergoing spike activity.

INTRODUCTION

Magnesium plays a fundamental role in the regulation of many cellular functions such as protein synthesis and enzyme activation. Amongst the enzymes in which Mg^{2+} acts as an essential co-factor are those concerned with glycolysis, respiration and membrane transport processes, e.g. Na^+ and Ca^{2+} pumps (for references, see Walser, 1967; Wacker, 1969; Günther, 1981; Flatman & Lew, 1981; Schatzman, 1982). Most of the total intracellular magnesium is bound and only a relatively small fraction is free in the cytoplasm.

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Accurate data on the value of cytoplasmic free Mg^{2+} concentration, $[Mg^{2+}]_i$, at rest and under various physiological or experimental conditions, are essential for understanding the distribution of this ion and its transport processes. Equally, the amount of magnesium bound to physiologically relevant binding sites depends on the $[Mg^{2+}]_i$ (and the affinity of the binding sites) and so knowledge of $[Mg^{2+}]_i$ is essential for defining appropriate conditions for investigation of intracellular processes in broken cell preparations, permeabilized cells and subcellular fractions. For biochemical studies, setting the correct Mg^{2+} concentration may be as important as having the right pH, ionic strength and Ca^{2+} concentration.

Total magnesium concentration has been determined in many tissues and cell types (Walser, 1967; Veloso, Guynn, Oskarsson & Veech, 1973; Günther, 1981) including neurones (Baker & Crawford, 1972; Veloso *et al.* 1973; De Weer, 1976; Mullins & Brinley, 1978; Caldwell-Violich & Requena, 1979). However, direct measurements of $[Mg^{2+}]_i$ have been reported in only a few cell types (for references, see Hess, Metzger & Weingart, 1982; Tsien, 1983), and apparently none for neuronal cell bodies. Previous estimates of $[Mg^{2+}]_i$ in neurones have been largely indirect and mostly on squid axons. Veloso *et al.* (1973) estimated the $[Mg^{2+}]_i$ from homogenates of rat brain by two independent methods. The first method involved determination of magnesium-binding properties of the tissue homogenates and from this data and the total magnesium concentration calculated the likely $[Mg^{2+}]_i$ as 0·58 mM. Secondly, using aconitate hydratase activity as a bio-assay, these authors estimated $[Mg^{2+}]_i$ as 1·00 mM. Baker & Crawford (1972) deduced a value of 2–4 mM for the 'concentration of ionized Mg' from measurements of the diffusion coefficient and electrophoretic mobility of a patch of radioactive ^{28}Mg, injected into squid axons. Brinley & Scarpa (1975) found a null-point for the signal from the Mg^{2+}-sensitive dye Eriochrome Blue in squid axons dialysed with solutions containing different Mg^{2+} levels and obtained a value for $[Mg^{2+}]_i$ of 3–3·5 mM. De Weer (1976) used the Mg^{2+} sensitivity of the Na^+ pump in squid axons as a bio-assay for $[Mg^{2+}]_i$ and obtained a similar value of 3–4 mM.

Values similar to those reported for squid axons were found using ion-selective micro-electrodes in vertebrate skeletal and cardiac muscle (Hess *et al.* 1982). However, using a variety of methods, rather lower values, less than 1 mM, have been reported in other vertebrate cells (Flatman, 1980; Rink, Tsien & Pozzan, 1982), and also in skeletal muscle (Gupta & Moore, 1980; Maughan, 1983).

In the present paper we report the first direct measurement made with Mg^{2+}-selective micro-electrodes in a neuronal cell body. Non-marine molluscan nerve cell bodies were chosen because their extracellular and intracellular fluid compositions are closer to those of vertebrates than of squids. These cells were also selected because of the relative ease of using ion-selective micro-electrodes and because a great deal of information on intracellular ion activities and basic neuronal membrane properties have been obtained in this preparation (e.g. Thomas, 1980; Adams, Smith & Thompson, 1980; Alvarez-Leefmans, Rink & Tsien, 1981*a*). The Mg^{2+}-selective micro-electrodes used contained a sensor based on the neutral ligand ETH-1117 (Lanter, Erne, Ammann & Simon, 1980). Brief accounts of some of these findings have been previously presented (Alvarez-Leefmans & Gamiño, 1982; Alvarez-Leefmans, Gamiño, & Rink, 1983; Gamiño & Alvarez-Leefmans, 1983).

METHODS

Experiments were done with neurones 1F, 2F, 19F, 77F, 79F or 1D (Kerkut, Lambert, Gayton, Loker & Walker, 1975) of the left and right parietal ganglia of *Helix aspersa*. The freshly collected snails were kept in plastic boxes and fed lettuce until the day of the experiment. The circum-oesophageal ganglia were removed and pinned to a Sylgard dish. The thick external layer of connective tissue which surrounds the ganglia was removed with fine scissors under a dissecting microscope. The remaining fine inner connective tissue layer was usually softened with a brief exposure to protease V (Sigma) and then a small area of this layer was carefully removed from over a few cells with fine Castroviejo scissors and sharpened forceps. The ganglia were transilluminated by means of a dry dark-field condenser (Leitz, D 0·80–0·95) and viewed through a dissecting microscope. There was no obvious difference between the cells from ganglia treated with protease and the untreated cells. Results were accepted only when the electrical properties of a cell corresponded to those usually found in previous studies (Alvarez-Leefmans *et al.* 1981*a*) and by other workers (Kerkut *et al.* 1975) for that particular cell. Measurements of intracellular free K^+ concentration ($[K^+]_i$) were done during February and March, and of $[Mg^{2+}]_i$ in March, April, August and September.

Solutions. The basic bathing solution contained (mM): NaCl, 80; KCl, 4; $CaCl_2$, 4·5; $MgCl_2$, 5; Na HEPES, 5; pH 7·5, equilibrated with air. This is similar to the solution used by Kerkut & Thomas (1965) and resembles the composition of the haemolymph of hydrated snails (Burton, 1975). However, the $CaCl_2$ in our solution was 4·5 mM instead of the usual 7 mM because this is the value of the free Ca^{2+} concentration measured in the haemolymph using Ca^{2+}-selective micro-electrodes (F. J. Alvarez-Leefmans & T. J. Rink, unpublished data). All experiments were done at room temperature (20–24·5 °C).

Electrodes. The bath reference electrode was either a Ag/AgCl pellet, when Cl^- was kept constant, or an agar bridge made out of a Polythene tube containing 3 M-KCl gelled in agar. Micro-electrodes for recording membrane potential (voltage micro-electrodes) were filled with 3 M-KCl and had resistances from 5 to 20 MΩ. Experiments in which the voltage reference micro-electrodes developed at some stage tip potentials of more than 1 mV were rejected.

Liquid membrane ion-selective micro-electrodes (ISE) were prepared following a method similar to that described previously (Alvarez-Leefmans *et al.* 1981*a*) except that the tips of the micropipettes were not broken back but left intact. In brief, thin-walled borosilicate capillary tubing (TW-150, WPI, New Haven, CT) of outer diameter 1·5 mm and inner diameter 1·05 mm was cleaned with nitric acid and ethanol. The tubes were drawn into micropipettes on a Brown–Flaming puller model P-77 (Sutter Instrument Co., San Francisco, CA) similar to those used as voltage micro-electrodes. They were siliconized at 200 °C with tri-*N*-butylchlorosilane vapour as described by Tsien & Rink (1980). Micropipettes were backfilled with 0·1 M-KCl applying air pressure through a syringe connected to the back end via flexible tubing. Under observation through a dissecting microscope, the electrode tip was introduced into a droplet of ion-selective sensor held in a fire-polished glass capillary. The sensor was sucked into the electrode tip by partial vacuum from the syringe to get a column of about 200 μm.

K^+-selective micro-electrodes were prepared with Corning 477317 K^+ liquid ion exchanger while Mg^{2+}-selective micro-electrodes were prepared with the liquid sensor based on the neutral synthetic ion carrier ETH 1117 (Lanter *et al.* 1980). To avoid capacitative artifacts during solution changes, in some experiments the shank of the electrodes was painted to within approximately 0·5 mm of the tip with silver conductive paint (Tsien & Rink, 1980).

Calibration and recording procedures. The ISE potential (V_{ISE}) was monitored through one of the probes of a WP-Instruments F-223A electrometer (New Haven, CT). The potential on the voltage micro-electrode (V_m) was recorded using a WP-Instruments M-707 electrometer and subtracted electronically from V_{ISE} to give the 'differential signal' $V_{ISE} - V_m$ which indicated the free ion (I) level. Signals were displayed on digital voltmeters and recorded on a multichannel pen recorder (Watanabe P.O.C. MC-6612). The ISE were calibrated using the same electrometers, in solutions held in a small glass funnel, the stem of which was bent to form an N-shaped siphon trap (Tsien & Rink, 1980). The funnel was held by Plasticine to the inner wall of a beaker which was placed very close to the experimental bath, inside a Faraday cage. Standard bathing solution was then squirted into the funnel and the potentials of the ISE and the voltage micro-electrode with respect to the reference electrode were backed off to zero. Therefore, by convention, the potential of either

the preparation chamber or the calibrating funnel filled with standard bathing solution was zero for both the ISE and the voltage micro-electrode. Once these potentials were stable, the potentials in the calibration solutions were then recorded. Calibrations for ascending and descending concentrations of the primary ion were obtained. Then, the voltage micro-electrode, the ISE and the reference bath electrode were all transferred to the preparation chamber. The ganglion was continuously superfused and therefore the reference potential was measured in fresh Ringer solution before impalement and after withdrawal of the electrodes.

ISE resistances were measured by passing a current pulse of 1 pA while the electrode tips were in the standard bathing solution. For K^+-selective micro-electrodes, resistances ranged from 3·6 to 31×10^9 Ω, while for Mg^{2+}-selective micro-electrodes they ranged from 15 to 40×10^9 Ω.

TABLE 1. Composition of standard calibration solutions for K^+-selective micro-electrodes

Concentration (mM)			
K^+	Na^+	Cl^-	$HEPES^-$
70	40	107·5	5
80	30	107·5	5
90	20	107·5	5
100	10	107·5	5
110	2·5	110·0	5
120	2·5	120·0	5

The final pH was 7·5.

Calibration solutions. All the solutions used in the present work were prepared with glass-double-distilled water which, in addition, was deionized by passing it through a Milli-Q water purification system (Millipore Co., Bedford, MA), until an electrical resistance of 18 MΩ cm was achieved.

The K^+-selective micro-electrodes were calibrated in mixed solutions containing different free K^+ concentrations, whose compositions are shown in Table 1. The ionic strength was kept constant for the solutions whose K^+ concentration was in the range between 70 and 100 mM, and was slightly higher in those containing 110 and 120 mM of K^+. The electrodes gave virtually Nernstian responses between 70 and 120 mM-free K^+ concentration, which is the range expected for $[K^+]_i$. The Nernstian behaviour of the electrodes in the mixed calibration solutions was expected since the reported selectivity coefficient K^{pot}_{KNa} is 0·0141 (Oehme & Simon, 1976). (This coefficient is denoted by a symbol K^{pot}_{ij}, which expresses the relative contributions of the primary ion i and an interfering ion j to the potential.) This implies that the K^+-selective micro-electrodes have a selectivity for K^+ over Na^+ of 70:1. It was found that adding 1–3 mM-$MgCl_2$ or $MgSO_4$ to the calibration solutions produced no detectable response in the K^+-selective micro-electrode. This is also consistent with the reported selectivity coefficient K^{pot}_{KMg} of 0·0022 (Oehme & Simon, 1976).

Mg^{2+}-selective micro-electrodes show negligible interference from Na^+, Ca^{2+} and H^+ at the free concentrations of these cations in the cytoplasm (Lanter *et al.* 1980; Hess *et al.* 1982). However, these electrodes show considerable interference from K^+ at the levels in which this cation is usually present in living cells (Lanter *et al.* 1980; Hess *et al.* 1982). The composition of the solutions used to calibrate the Mg^{2+}-selective micro-electrodes was based on the value found for $[K^+]_i$ in the neurones used in the present study (see below).

The convention used in the present study for reporting an intracellular level of either Mg^{2+} or K^+ is to denote it by the free concentration that in the appropriate calibration solutions would produce the same differential signal ($V_{ISE} - V_m$). The rationale behind this convention is discussed in a previous paper (Alvarez-Leefmans *et al.* 1981*a*), and more extensively in a recent review (Tsien, 1983). This is the convention usually used in measurements of $[Ca^{2+}]_i$.

Chemicals. KCl, NaCl, $CaCl_2$, $MgCl_2$ and $MgSO_4$ were spectroscopically pure (Baker or Merck). HEPES came from Hopkins & Williams or Sigma and Tri-*N*-butylchlorosilane (Pfaltz & Bauer). Corning 477317 K^+-ligand ion exchanger was a gift from Professor R. Llinás. Mg^{2+} liquid sensor was a gift from Professor W. Simon and Dr D. Ammann. Silver conductive paint Electrodag 416, came from Acheson Colloids Co., Port Huron, MI, U.S.A.

RESULTS

The general experimental procedures and precautions followed for measuring either $[K^+]_i$ or $[Mg^{2+}]_i$ were essentially the same as those previously described (Alvarez-Leefmans, Rink & Tsien, 1981*a*, *b*) and which are partly illustrated in Figs. 1 and 3. First the ISE was calibrated in solutions of ascending and descending concentrations of the ion to be measured (only the ascending part of the pre-calibration is shown in Figs. 1 and 3) and then this electrode and the voltage micro-electrode were positioned in the bathing solution and the potentials with respect to the bath reference electrode were backed off to zero. The neurones were impaled first with the voltage micro-electrode and after a few minutes the ISE was introduced. This allowed assessment of possible damage produced by the second impalement. Sometimes this second impalement produced virtually no displacement of the membrane potential; sometimes there was a depolarization which was only transient (as happened in the cell shown in Fig. 1 and as illustrated in Fig. 3), or was maintained indicating slight reversible or irreversible damage respectively.

As a rule we tested that both electrodes were inside the same cell. This was done visually and by passing a depolarizing current pulse of 1×10^{-9} A through the voltage micro-electrode and observing the resulting deflexion on the ISE, as shown in Figs. 1 and 3. No attempt was made to balance the bridge circuit when current was passed through the voltage micro-electrode. This explains why a voltage deflexion was also recorded in the differential trace ($V_{ISE} - V_m$), as can also be seen in Figs. 1 and 3. In cells which were spontaneously active (e.g. cells 1F and 2F) this procedure of passing a depolarizing current pulse also served the purpose of producing a burst of action potentials which was usually followed by a long-lasting hyperpolarization (Moreton, 1972) which allowed a period of 'silence' during which measurements could be accurately made.

After the differential signal between the ISE and the voltage electrode had stabilized, to give a measure of the basal free level of either Mg^{2+} or K^+ concentration, changes in the bath composition could be made. Finally, the electrodes were withdrawn from the cell when the ganglion was superfused with the standard solution ('snail Ringer'; SR) and their potentials recorded in the bath for a few minutes to check for any drift. Then a post-impalement calibration was performed, as illustrated in Figs. 1 and 3 (only the descending parts of post-calibrations are shown).

Cells 1F, 2F and 19F are spontaneously active neurones which usually fire bursts of action potentials which are followed by slow hyperpolarizations. There were fluctuations in the differential signal during each burst, due to the slow response of the ISE relative to the voltage micro-electrode (e.g. Figs. 1 and 4). Measurements of $[K^+]_i$ or $[Mg^{2+}]_i$ were made on these active cells only during the silent periods resulting from either naturally occurring post-burst hyperpolarizations or those induced by passing depolarizing current pulses as already mentioned. Both conditions are illustrated in Fig. 1.

Measurement of basal free K^+ concentration

Fig. 1 shows an example of a typical experiment for measuring $[K^+]_i$. The bottom trace shows the potential recorded by the voltage reference micro-electrodes (V_m) a few minutes after impalement of cell 1F. A burst of action potentials followed by a

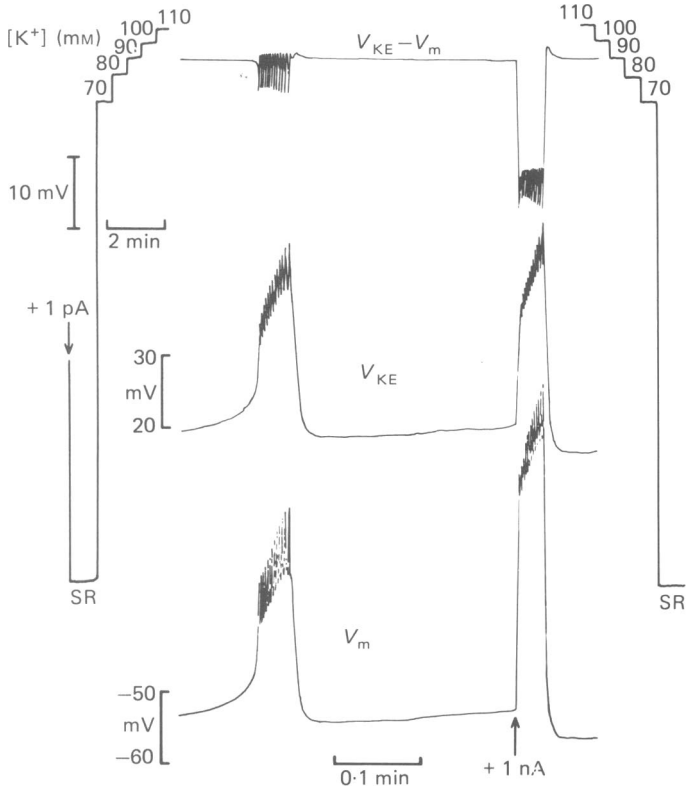

Fig. 1. Example of a measurement of $[K^+]_i$ in cell 1F. Shown on the left is the pre-impalement calibration record for the K^+-selective micro-electrode (differential signal). The resistance of the electrode was measured by passing a current of +1 pA through the K^+-selective micro-electrode while its tip was in contact with the bathing solution (SR). The intracellular reference micro-electrode recording, V_m, is shown in the lower trace. The simultaneous recording from the K^+-selective micro-electrode V_{KE}, is shown in the middle trace and the differential signal, $V_{KE}-V_m$, is shown in the top trace, the latter indicating the $[K^+]_i$. Comparing the differential signal, $V_{KE}-V_m$, in the bathing solution with that in the cell shows the same potential drop as that between the bathing solution and the calibration solution containing 90 mM-free K^+ concentration. A depolarizing current pulse of +1 nA was passed through the V_m recording micro-electrode to test that both electrodes were inside the same cell and to measure its input resistance. No attempt was made to balance the bridge as can be seen in the signal displacement of the $V_{KE}-V_m$ trace. On the right is the post-impalement calibration.

long-lasting hyperpolarization can be observed. The spikes appear truncated in this and other records shown in this paper (Fig. 4) due to the relatively slow pen response. However, they were usually monitored with an oscilloscope and had overshoots of 20–40 mV. The middle trace shows the potential recorded by the K^+-selective micro-electrode (V_{KE}) and the top trace shows the differential signal ($V_{KE}-V_m$), which indicates $[K^+]_i$. Comparing the differential signal in the bathing solution (SR) with that in the cell shows the same potential drop as that between the SR and a calibration solution containing 90 mM-K^+. In eight experiments of this type, in which the K^+-selective micro-electrode gave Nernstian responses in the range between 70

and 110 mM-K^+, before and after impalement, the mean intracellular K^+ activity corresponded to the activity of 91·0 mM-free K^+ concentration in the calibrating solutions (S.E. of the mean ± 2·2 mM, range 85–102·4 mM, n = eight cells).

Only two measurements of $[K^+]_i$ in giant neurones of non-marine molluscs appear to be published in the literature; those of Kostyuk, Sorokina & Kholodova (1969), and Thomas (1980) (for reviews, see Lev & Armstrong, 1975; Walker & Brown, 1977). The mean intracellular K^+ activity reported by Kostyuk *et al.* (1969) in unidentified nerve cells of *Helix pomatia* was 73 mM and in *Planorbis corneus* it was 39 mM. From analytical concentrations of total K they claimed to have calculated an apparent mean intracellular activity coefficient of 0·78 for *Helix* and 0·73 for *Planorbis*, thus giving a mean $[K^+]_i$ of 93·6 mM for *H. pomatia* and 53·4 mM for *Planorbis*. Therefore, the mean $[K^+]_i$ they reported for *H. pomatia* is close to the one found in the present study, but considerably higher than the one they published for *Planorbis* cells. Kostyuk *et al.* (1969) used protruding-tip-type micro-electrodes made with K^+-sensitive glass (KABS 20-9-5). The length of the uninsulated protruding part of their cation-selective glass micro-electrodes was between 5 and 20 μm. This means that the insulating glass tip plus the protruding part of the K^+-sensitive glass electrode must penetrate the neurone to ensure that all the ion-selective glass is intracellular. It follows that the possibility of cell damage or artifactual measurements, the latter due to incomplete penetration of the K^+-sensitive glass, cannot be excluded in the results of Kostyuk *et al.* (1969). The other measurement reported was that of Thomas (1980) and it was obtained from the soma of neurone 1F of *H. aspersa*. He quoted a value of 100 mM for $[K^+]_i$. Unfortunately, the methods were not specified. The value we measure (91 mM) is close to the 92·9 mM calculated by Moreton (1968) from the dependence of the membrane potential on external $[K^+]$.

Calibration and properties of Mg^{2+}-selective micro-electrodes

Having obtained a value for the $[K^+]_i$, it was now possible to calibrate correctly the Mg^{2+}-selective micro-electrodes in solutions mimicking the intracellular cationic environment of the cells used in this study. Therefore the Mg^{2+}-selective micro-electrodes were calibrated in solutions containing (mM): KCl, 91; NaCl, 5; Na HEPES, 5; pH 7·5 and variable concentrations of $MgSO_4$ (0, 0·2, 0·5, 1·0, 2·0, 5·0, 10·0). In some experiments $MgCl_2$ was used instead of $MgSO_4$ in the calibration solutions. Mg^{2+} activity was measured in both types of solutions and found to be the same in the relevant range for the present experiments (0·2–2·5 mM). Fig. 2 shows a typical calibration curve of a Mg^{2+}-selective micro-electrode. The traces in the top part of Fig. 2 are the differential signal between the Mg^{2+}-selective micro-electrode (V_{MgE}) and the voltage reference micro-electrode (V_m). The Mg^{2+}-selective electrode response in the calibration solutions, although sub-Nernstian between 0·2 and 10 mM-Mg^{2+}, was adequate for intracellular measurements. The deviation from ideally Nernstian behaviour is mainly due to K^+ interference (Lanter *et al.* 1980; Hess *et al.* 1982). Responses to all Mg^{2+} concentrations tested were very similar between different electrodes, and quite stable with time and after cell penetrations. For instance, the mean differential voltage responses to changes in Mg^{2+} concentration at 23·4 ± 0·2 °C (S.E. of the mean), in twenty-seven calibrations of fourteen electrodes used to obtain the measurements reported in this work were (mean ± S.E. of the mean):

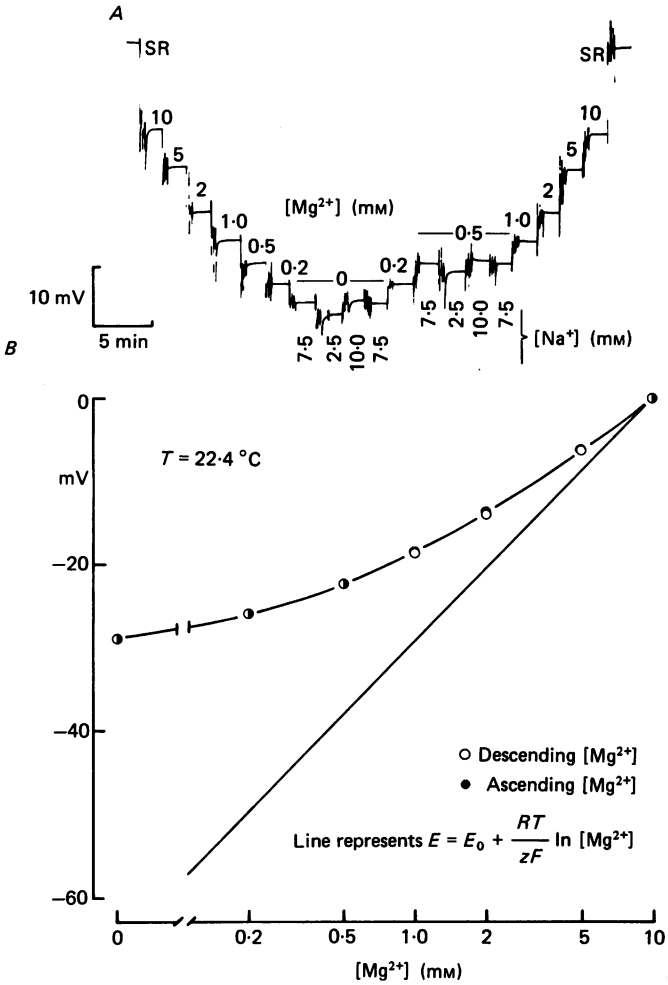

Fig. 2. Calibration of a Mg^{2+}-selective micro-electrode and Na^+-interference test. *A* shows a calibration recording following the procedure outlined in the text. The numbers give the Mg^{2+} and Na^+ concentrations in mM as indicated. In *B*, the differential signal is plotted against Mg^{2+} concentration. The open circles represent the descending calibration, and the filled circles the ascending calibration for the solutions with 7·5 mM-Na^+. The straight line shows the ideal Nernstian response. E is the electrode potential; E_0 is a constant reference potential; R, T, F and z have their normal meaning.

16·1 ± 0·4 mV between 10 and 1 mM-Mg^{2+}; 3·6 ± 0·1 mV between 1 and 0·5 mM-Mg^{2+} and 5·5 ± 0·2 mV between 0·5 and nominally 0 mM-Mg^{2+}.

Another intracellular monovalent cation which might interfere with the Mg^{2+}-selective micro-electrode response is Na^+. The reported selectivity coefficient, $K^{pot}_{MgNa} = 0{\cdot}0794$ (Lanter *et al.* 1980), implies that the electrode is 12·6 times more sensitive to Mg^{2+} than to Na^+. The basal free Na^+ concentration in the cytoplasm of *Helix* neurones is 6–7·5 mM (Thomas, 1980; F. J. Alvarez-Leefmans, unpublished observations). Therefore, possible interference from Na^+ was taken into account when

calibrating the Mg^{2+}-selective micro-electrodes in solutions containing a fixed background Na^+ concentration of 7·5 mM. It was desirable, however, to have some way of assessing potential Na^+ interference on the Mg^{2+}-selective micro-electrodes, within the limits in which $[Na^+]_i$ is expected to vary under the experimental conditions in which these cells were maintained. The most suitable way of assessing any interference is by changing the free concentration of the interfering ion (in this case Na^+) in the virtual absence of the primary ion (in this case Mg^{2+}). This severe test was done with three different Mg^{2+}-selective micro-electrodes, one of which is illustrated in Fig. 2. With no Mg^{2+} in the calibration solution, increasing the Na^+ concentration from 2·5 to 7·5 mM produced a mean voltage displacement of the Mg^{2+}-selective electrode of $1·73 \pm 0·08$ mV (S.E. of the mean). Going from 7·5 to 10 mM-Na^+ gave a response of $0·96 \pm 0·10$ mV (S.E. of the mean). In the presence of 0·5 mM-Mg^{2+} in the calibration solution, increasing the Na^+ concentration from 2·5 to 7·5 mM gave a mean voltage displacement of 1·2 mV, and going from 7·5 to 10 mM gave only $0·50 \pm 0·12$ mV (S.E. of the mean). Therefore, the Na^+-interference tests show that changing the Na^+ concentration in the calibration solutions within the limits at which this monovalent cation is expected to be in the cytoplasm, produced negligible interference, when the background Mg^{2+} concentration in the calibration solutions is 0·5 mM (the lowest level of free $[Mg^{2+}]_i$ found in this study).

Although the Mg^{2+}-selective electrodes are not very selective between Mg^{2+} and Ca^{2+}, interference from Ca^{2+} ($K^{pot}_{MgCa} = 12·6$) is expected to be unimportant in the cytoplasm since the mean $[Ca^{2+}]_i$ in *Helix* neurones is only 170 nM (Alvarez-Leefmans *et al.* 1981*a*). We tested the response of the Mg^{2+}-selective micro-electrodes to each of the standard calibration solutions containing 0, 0·5 and 1·0 mM-Mg^{2+}, to which variable amounts of K_2EGTA (0, 10, 20 and 40 μM) had been added. No response of the Mg^{2+}-selective micro-electrodes could be detected when the concentration of K_2EGTA was changed from 0 to 40 μM, keeping constant the background Mg^{2+} concentration at 0·5 or 1·0 mM. In the calibration solution to which Mg^{2+} was not added (i.e. the solution having a concentration of nominally 0 Mg^{2+}), the response of the electrode between a solution containing no EGTA and the one having 40 μM-EGTA was only 0·4 mV. This is in agreement with Hess *et al.* (1982) who have shown that increasing the Ca^{2+} concentration in the calibration solutions from 100 nM to 10 μM produces no significant effect on Mg^{2+}-electrode responses. Similarly, pH changes in the physiological range do not give significant interference (Hess *et al.* 1982; Lanter *et al.* 1980).

Measurement of $[Mg^{2+}]_i$

Fig. 3 shows an example of a typical experiment for measuring $[Mg^{2+}]_i$. In this case the cell impaled was 79F, a neurone which does not fire action potentials unless it is depolarized. However, these cells show a background activity consisting of long-lasting subthreshold fluctuations in membrane potential, probably slow synaptic potentials, as can be observed in the record. At the time marked by the arrow, the Mg^{2+}-sensitive electrode was introduced, producing a transient reversible depolarization of the cell membrane potential. The differential record ($V_{MgE} - V_m$) shows a fairly stable reading of $[Mg^{2+}]_i$ which in this case corresponded to 0·64 mM (the mean value

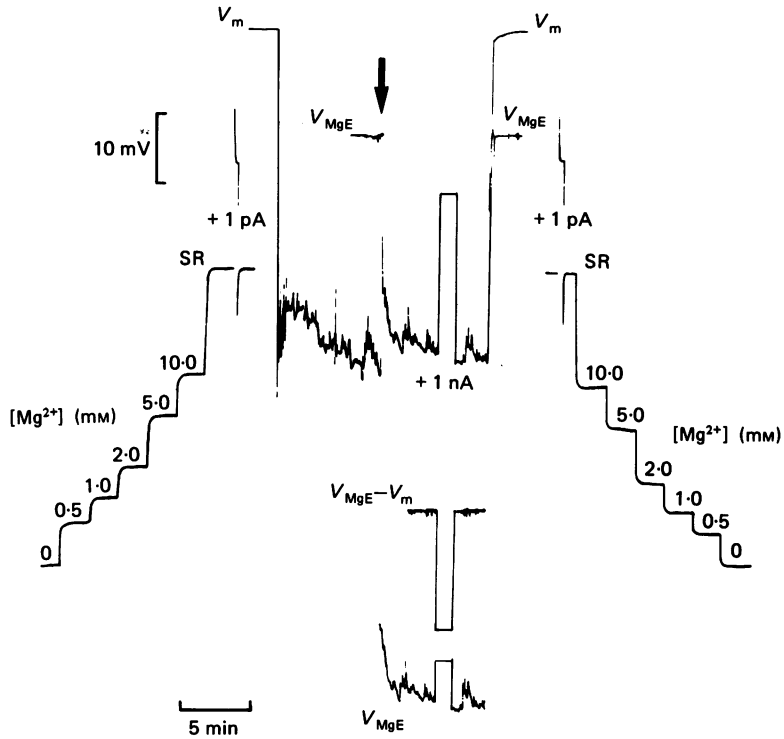

Fig. 3. Measurement of $[Mg^{2+}]_i$ in cell 79F. On the extreme left and right are shown the ascending calibration of the Mg^{2+}-electrode before impalement, and the descending calibration after withdrawal. SR denotes the bathing solution ('snail Ringer'). Note that differential signal ($V_{MgE} - V_m$) in the bathing medium in the chamber is adjusted to this level. V_m is the potential recorded through the voltage reference micro-electrode and V_{MgE} that in the Mg^{2+} electrode. The arrow indicates the time at which the cell was penetrated with the Mg^{2+}-selective micro-electrode. The +1 pA pulses were applied to measure the ISE resistance. The +1 nA pulse was applied through the voltage electrode, when both electrodes were inserted into the cell. No attempt was made to balance the bridge and this is why the differential signal $V_{MgE} - V_m$ shows a voltage displacement during the pulse. For other details, see text.

obtained from two pre-impalement and two post-impalement calibrations). A depolarizing test pulse of 1×10^{-9} A passed through the voltage micro-electrode confirmed that, as seen through the microscope, both electrodes were in the same cell. The voltage deflexion in the Mg^{2+}-selective micro-electrode (V_{MgE}) indicated an input impedance of *ca.* 6·5 MΩ. From more than fifteen satisfactory penetrations, seven were selected which fulfilled the following stringent criteria for cell viability and electrode performance: (i) the resting potential was more negative than −45 mV (actual mean ± S.E. of the mean was −52·4 ± 3 mV), and was not at all or only transiently perturbed by the second impalement; (ii) the differential signal drifted less than 5 mV/h (actual mean ± S.E. of the mean was 1·4 ± 0·7 mV/h); (iii) the Mg^{2+}-electrode signals in the calibration solutions were adequate, responding always below 0·5 mM-Mg^{2+}, before and after impalement. For these neurones the mean intracellular Mg^{2+} activity corresponded to the activity of 0·66 mM-free Mg^{2+}

concentration in the calibrating solutions (S.E. of the mean $\pm$ 0·05 mM, range 0·5–0·87 mM). Other impalements which did not fulfil completely one of the above criteria (e.g. drift greater than 5 mV/h) but allowed a measure of $[Mg^{2+}]_i$ to be made in cells with resting potentials more negative than -45 mV gave a mean $[Mg^{2+}]_i$ of 0·85 mM (S.E. of the mean $\pm$ 0·14 mM, range 0·53–1·29 mM, $n = 7$). The $[Mg^{2+}]_i$ in these cells was not significantly different ($P > 0·05$) to that found in the first group. The mean $[Mg^{2+}]_i$ for all fourteen measurements was 0·76 $\pm$ 0·06 mM (S.E. of the mean).

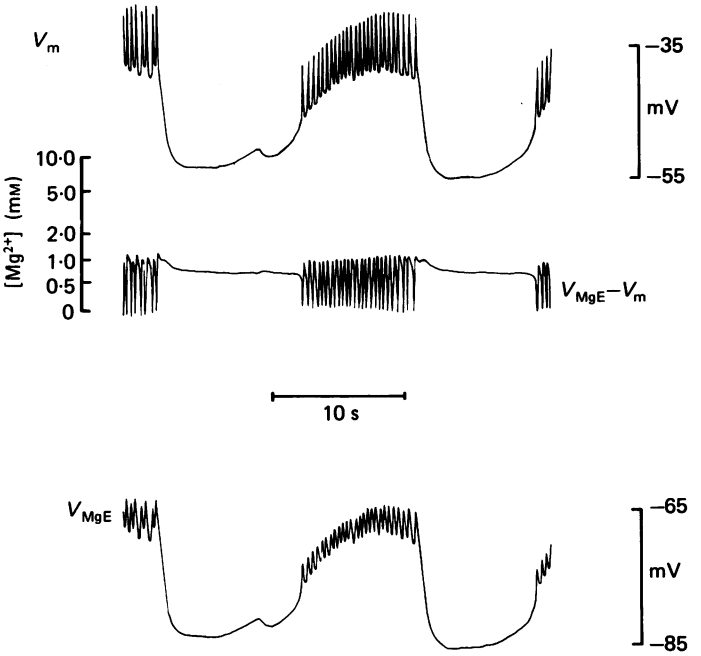

Fig. 4. Measurement of $[Mg^{2+}]_i$ in cell 19F. A section of the record is shown, during the period that both electrodes were in the cell. The middle trace is the differential signal ($V_{MgE} - V_m$), with the appropriate calibration shown on the left. The spikes on the differential trace are artifacts caused by the much longer time constant of the Mg^{2+} electrode compared with the voltage reference micro-electrode.

A few measurements were made on the effect of changing the $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$. Although this is a subject which needs studying in much more detail, in four cells we tested the effect of increasing $[Mg^{2+}]_o$ from 5 to 10 mM. 10 min after superfusion of the preparation with the high $[Mg^{2+}]_o$ we found no significant changes in the basal levels of intracellular free Mg^{2+}. This indicates that membrane sealing around the electrode was adequate.

$[Mg^{2+}]_i$ *in spiking neurones*

There are reports in the literature suggesting a tetrodotoxin (TTX)- and tetraethylammonium (TEA)-resistant Mg^{2+} entry during nervous activity in squid axons, which is blocked by Mn^{2+} (Baker & Crawford, 1972; Rojas & Taylor, 1975; Caldwell-Violich & Requena, 1979). Therefore, we thought it would be interesting to see whether there was any difference in the basal intracellular free levels of Mg^{2+}

between neurones undergoing spontaneous spike activity and those which are not spontaneously active. Fig. 4 shows an example of a bursting cell (in this case, cell 19F). The $[Mg^{2+}]_i$ could be reliably measured only during the silent period, i.e. during the slow hyperpolarization, and at the time when a steady value could be read. In this particular cell, the differential record during the silent period indicated an $[Mg^{2+}]_i$ of 0·6 mM. The basal $[Mg^{2+}]_i$ in six quiescent cells was $0·71 \pm 0·12$ mM (mean $\pm$ S.E. of the mean, range 0·50–1·29 mM) while that of cells showing sustained bursting activity was $0·80 \pm 0·07$ mM (mean $\pm$ S.E. of the mean, range 0·58–1·15 mM, $n = 8$). Although the spiking cells showed a mean $[Mg^{2+}]_i$ slightly higher than that in silent cells, the difference between the means was not statistically significant. Although these experiments do not rule out the possibility that Mg^{2+} enters during action potentials, they suggest that if it does, these neurones have efficient mechanisms to keep their $[Mg^{2+}]_i$ quite stable.

DISCUSSION

The response of our Mg^{2+}-selective micro-electrodes in the calibration solutions indicates that they can measure free Mg^{2+} concentrations to below 0·2 mM in the presence of the intracellularly measured levels of H^+, Ca^{2+}, K^+ and Na^+. Therefore, the values of $[Mg^{2+}]_i$ reported in the present paper should not have been limited by the response of the electrodes or by interferences from intracellular cations. A problem with ISE techniques is that the readings represent only the free-ion level at the tip of the electrode. We think our readings of $[Mg^{2+}]_i$ (and $[K^+]_i$) are representative of the levels present in the bulk of the cytoplasm for the following reasons: (i) the small dispersion around the mean of measurements made in different neurones; (ii) the absence of evidence suggesting Mg^{2+} (or K^+) gradients inside cells in basal conditions, e.g. no alteration in the readings of the electrodes when inserted more deeply into the impaled cells; (iii) the relatively high cytoplasmic mobility of K^+ and to a lesser extent Mg^{2+} (Kushmerick & Podolsky, 1969; Baker & Crawford, 1972; Edzes & Berendsen, 1975) which further suggests that $[Mg^{2+}]_i$ (or $[K^+]_i$) gradients, if any, would not persist over the time course of our measurements.

Basal intracellular free Mg^{2+}

The values obtained in the present study derived from careful measurements made in fourteen cells range from 0·5 to 1·29 mM with a mean of $0·76 \pm 0·06$ mM (S.E. of the mean). The mean $[Mg^{2+}]_i$ for seven neurones which fulfilled stringent criteria for cell viability and electrode performance although lower ($0·66 \pm 0·05$ mM, range 0·5–0·87 mM) was not significantly different from that in the rest of the cells. These values are considerably lower than those found in cardiac and skeletal muscle fibres (Hess *et al.* 1982) using micro-electrodes made with the same Mg^{2+} sensor used in the present study. We do not know if this discrepancy represents a genuine difference between vertebrate muscle and molluscan neurones, or if it can be attributed to unknown interference from K^+ and Na^+, the intracellular free concentrations of which were assumed but not measured in the experiments of Hess *et al.* (1982).

At present the intracellular free Mg^{2+} concentration in vertebrate cardiac and skeletal muscle is a highly controversial subject, and values reported range from 0·2

to 6 mM depending on technique and investigator. For instance in frog skeletal muscle, Maughan (1983) deduced a value of 0·2 mM for $[Mg^{2+}]_i$ from measurements of total diffusible Mg^{2+} with a novel liquid sampling and X-ray spectroscopic method; using nuclear magnetic resonance (n.m.r.) methods, Gupta & Moore (1980) calculated a value of 0·6 mM. Wu, Pieper, Salhany & Eliot (1981), measuring a different MgATP dissociation constant, argue that the results of Gupta & Moore are an underestimate and that the right value is 2·5 mM, which is close to the 3 mM reported earlier by Cohen & Burt (1977) using similar techniques. More recently, Baylor, Chandler & Marshall (1982) using three metallochromic indicator dyes obtained three different estimates of $[Mg^{2+}]_i$, depending on the dye used: Arsenazo I yielded values of 3–6 mM, Arsenazo III, 0·5–1·2 mM and Dichlorophosphonazo III, 0·2–0·3 mM. The explanation they gave for this discrepancy was that the dyes behave differently inside muscle fibres and in calibrating solutions.

The reported values of $[Mg^{2+}]_i$ in squid axoplasm are several-fold higher than those in snail neurones, ranging from 2 to 4 mM (Baker & Crawford, 1972; Brinley & Scarpa, 1975; De Weer, 1976). This may reflect a genuine difference between marine and terrestrial molluscs. However, none of the methods used in squid axons gave a direct signal of $[Mg^{2+}]_i$ in intact cells. A critical discussion is provided by De Weer (1976).

The main conclusion from our results is that the values found in the present study are in line with the values reported for a variety of vertebrate cells, although with techniques less direct than ISE (e.g. Flatman & Lew, 1977; Flatman, 1980; Rink *et al.* 1982; Tsien, 1983; Maughan, 1983). The mean value of 0·76 mM for $[Mg^{2+}]_i$ in snail neurones, in the presence of 5 mM-external Mg^{2+} and with a membrane potential of -50 mV implies that Mg^{2+} is far from electrochemical equilibrium. It can readily be calculated that if Mg^{2+} was in electrochemical equilibrium the ratio $[Mg^{2+}]_i/[Mg^{2+}]_o$ would be about 55, and so there must be an outwardly directed extrusion mechanism to keep $[Mg^{2+}]_i$ low and constant.

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