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Intracellular free magnesium in synaptosomes measured with entrapped eriochrome blue

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The free Mg^{2+} concentration within synaptosomes has been measured with an entrapped Mg^{2+} indicator, eriochrome blue. Ionophores gramicidin and A23187 slowly increased the absorbance of the entrapped dye. Calibration of the dye response in a Na^+-based medium gave a value around 0.3 mM for the internal free Mg^{2+} concentration at 1 mM external Mg^{2+}. The replacement of Na^+ by choline increased this value to around 0.65 mM. Depolarisation with a high K^+ concentration or depletion of intrasynaptosomal ATP with FCCP and iodoacetate did not affect the level of intracellular free Mg^{2+} concentration. An elevation of the external Ca^{2+} concentration significantly reduced internal Mg^{2+} to about 0.1 mM. Ca^{2+} had no significant effect when Na^+ was replaced by choline. The results indicate that the intrasynaptosomal Mg^{2+} activity is partially regulated by a Na^+-Mg^{2+} exchange mechanism which does not directly require ATP as an energy source.

Introduction

In nervous, as well as in other tissues, the importance of Mg^{2+} as a cellular regulator is well established. Mg^{2+} has presynaptic as well as postsynaptic effects on synaptic transmission. Presynaptically Mg^{2+} inhibits the uptake of $^{45}Ca^{2+}$ [1] and calcium-dependent transmitter release [2] by competitive antagonism. Mg^{2+} also has a stabilizing effect on synaptosomal plasma membranes [3]. Postsynaptically Mg^{2+}-ions block the NMDA sub-type of glutamate receptor [4]. This effect is dependent on magnitude of the membrane potential. Apart from this Mg^{2+} is required in a wealth of metabolic processes [5].

In excitable tissues the cytoplasmic, free Mg^{2+} is far below the electrochemical equilibrium due to regulatory transport systems (for a review, see Ref. 5). The mechanisms for the regulation of cytoplasmic Mg^{2+} have been studied in squid axons. $^{28}Mg^{2+}$ influx [6,7] as well as efflux [6,8] across the plasma membrane has been studied. The principal mechanism for the regulation of the internal Mg^{2+} concentration in squid axons seems to be a Na^+-Mg^{2+} exchange mechanism [6,8]. A fast influx of Mg^{2+} during depolarizing pulses has also been demonstrated [9].

In small sized nerve-endings isolated from mammalian brain, the measurement of cytoplasmic, free Mg^{2+} has proved to be difficult. Recently we have shown that it is possible to

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonate.

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measure internal ionized Mg^{2+} spectrophotometrically in synaptosomes directly with a Mg^{2+}-sensitive dye eriochrome blue by entrapping the dye during homogenization of the brain tissue [10]. A similar method has previously also been applied for measurements of intrasynaptosomal free Ca^{2+} concentration with arsenazo III [11].

The aim of the present study was to investigate a possible role of Na^+ and Ca^{2+} as well as the membrane potential in the regulation of intrasynaptosomal, free Mg^{2+}.

Methods and Materials

Preparation of synaptosomes

Synaptosomes were isolated from the cortical hemispheres of guinea pigs of either sex, aged 4–6 weeks, by the one-step sucrose gradient method described by Hajos [12] as modified by Kreuger et al. [13]. 10 mM eriochrome blue recrystallized before use [14] or 1 mM phenol red, were added to the homogenization medium in order to entrap the dyes within subsequently prepared synaptosomes. After isolation the synaptosomes were washed by centrifugation at $15000 \times g$ for 15 min in the standard Na^+-based medium (see below) and the synaptosomal pellets were kept on ice until use.

Spectrophotometric measurements

The synaptosomal pellets were resuspended into the experimental medium for spectrophotometric recordings. The protein content varied between 1.28 and 2.0 mg/ml. The absorbance of eriochrome blue was measured with an Aminco DW2 or a Shimadzu UV 3000 dual wavelength spectrophotometer using the wavelength pairs of 554–592 or 554–580 nm. The absorbance of phenol red was measured at the wavelength pair of 558–482 nm according to Scarpa [14]. Both spectrophotometers were equipped with thermostated cuvette holders. The temperature was kept at 32°C during recording.

Experimental solutions

The composition (in mM) of the incubation media were Na^+-medium: 137 NaCl, 5 KCl, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 20 Tes (pH 7.4) and 10 glucose. K^+-medium: 128 KCl, 0.44 KH_2PO_4, 2.5 K_2CO_3, 20 Tes (pH 7.4) and 10 glucose. In order to obtain a medium with 68 mM K^+, Na^+- and K^+ medium were used in combination (1 : 1, v/v). In the choline-medium NaCl was replaced by choline chloride.

Materials

Digitonin, EDTA and choline chloride were obtained from Merck AG, (Darmstadt, F.R.G.). Eriochrome blue, phenol red, tetrodotoxin, gramicidin and A23187 wre purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Verapamil was a gift from Knoll AG (Ludwigshaven, F.R.G.). All the other reagents were commercial products of the highest available purity.

Results

Effect of gramicidin and A23187 on the absorbance of entrapped eriochrome blue

When the cortex of guinea pigs was homogenized in the presence of eriochrome blue and synaptosomes were subsequently isolated the dye was entrapped within the material as judged from the bright red colour (not shown). When these

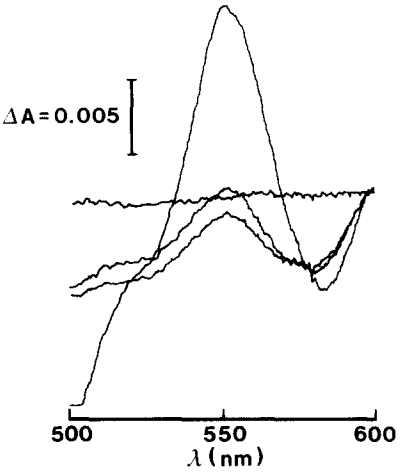

Fig. 1. Difference spectra on the effects of gramicidin and A23187 on the absorbance of entrapped eriochrome blue. The extracellular Mg^{2+} was 5 mM. The horizontal line is the baseline obtained after 10 min incubation in the Na^+-based medium. After preincubation for 10 min, 2 μM gramicidin was added to the synaptosomal suspension. The lowermost spectrum is obtained 5 min and the middle recording 10 min after the addition of gramicidin. The uppermost spectrum is obtained 5 min after the addition of 10 μM A23137. The synaptosomal protein was 1.34 mg/ml and temperature 32°C.

synaptosomes were resuspended in the Na^+-based medium containing 5 mM $MgCl_2$ at 32°C and gramicidin, a cation-selective ionophore, or A23187, a bivalent cation selective ionophore, were added a difference spectrum typical of the eriochrome blue-Mg^{2+} complex was observed [14] consisting of an increase in the absorbance at 551 nm. This suggests that the ionophores caused an increase in the intrasynaptosomal free Mg^{2+} concentration (Fig. 1).

In order to find out whether the gramicidin-induced increase in the absorbance of intrasynaptosomal eriochrome blue was due to depolarisation induced flow of Mg^{2+} through voltage-dependent Na^+- or Ca^{2+}-channels the effects of tetrodotoxin (0.2 μM) and verapamil (0.1 mM) was studied. The absorbance of the entrapped dye was recorded at the wavelength pair 554 nm–580 nm. Gramicidin caused a slow increase in the absorbance of the dye (Fig. 2). A subsequent addition of ionophore A23187 caused a further response. Tetrodotoxin or verapamil had no significant effect on the absorbance changes induced by the ionophores.

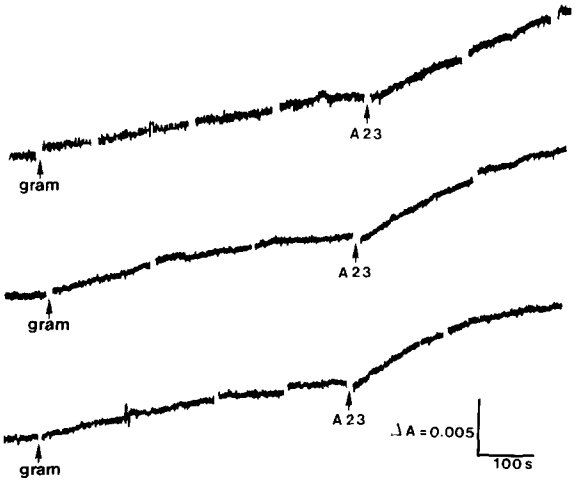

Fig. 2. The lack of effect of TTX and verapamil on the gramicidin-induced increase in the absorbance (554–580 nm) of the entrapped eriochrome blue. The recordings were performed in the Na^+-based incubation medium with 5 mM external Mg^{2+}. The uppermost trace is a control recording. In the middle trace 0.2 μM TTX and in the lowermost trace 100 μM verapamil was added before the recording. The additions of 2 μM gramicidin (gram) and 10 μM A23187 (A 23) are indicated by arrows. The protein concentration was 1.55 mg/ml and the temperature was 32°C.

Determination of the cytosolic free Mg^{2+} concentration

In order to be able to convert absorbance changes into values of free Mg^{2+} the synaptosomes were suspended into the different experimental media, the entrapped dye was released by the addition of 2 mg/ml digitonin, which disrupts the plasma membrane [10] and the response of the dye to increasing Mg^{2+} concentrations was monitored. The results were corrected for the contamination of eriochrome blue in the external medium by centrifugation of the synaptosomes at 15 000 × g for 15 min in the absence of digitonin. The absorbance values obtained upon additions of Mg^{2+} to the supernatant in this case were reduced from those obtained after the digitonin treatment. The Mg^{2+} contamination in the nominally Mg^{2+} free external medium did not affect the results because an addition of EDTA (0.5 mM) did not significantly affect the absorbance of the released

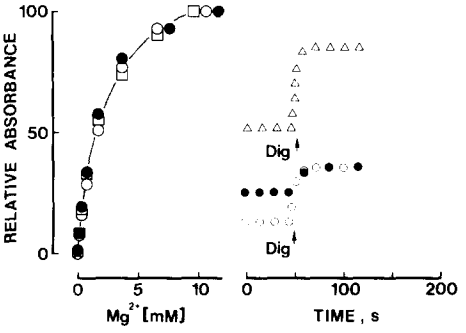

Fig. 3. The calibration of the Mg^{2+}-sensitive absorbance of the intrasynaptosomally entrapped eriochrome blue (on the left) and replotted recordings of the absorbance of eriochrome blue at 1 and 5 mM external Mg^{2+} (on the right). The calibration plot at increasing Mg^{2+} concentrations was obtained from the difference in absorbance of eriochrome blue in digitonin-treated synaptosomes and the supernatant obtained after centrifugation of synaptosomes at 15000 × g for 15 min. The values are expressed as % of the maximal response of the dye at saturating Mg^{2+} concentrations. (○) in the standard Na^+-based medium (mean value, $n = 6$) (●) the choline-based medium ($n = 4$), or (□) a medium containing 68 mM K^+ ($n = 4$). The temperature was 32°C. On the right typical recordings similar to those in Fig. 2 of entrapped eriochrome blue have been replotted. Where indicated an addition of digitonin was made (Dig) in (○) the standard Na^+-based medium, (●) the choline medium at 1 mM external Mg^{2+}, and (△) in the standard Na^+-based medium, at 5 mM external Mg^{2+}. The value on the abscissa of the calibration plot gives the corresponding Mg^{2+} concentrations.

eriochrome blue. A typical calibration plot in different conditions is shown in Fig. 3. Fig. 3 also shows a typical replotted recording of the absorbance of entrapped eriochrome blue (corrected for the absorbance of external dye) in the Na^+-based and choline-based media in the presence of 1 mM external $MgCl_2$. An addition of digitonin increased the absorbance of the dye denoting the absorbance at the level of external free Mg^{2+}. The intrasynaptosomal free Mg^{2+} concentration before digitonin addition was then obtained from the values on the abscissa. As can be seen this basal absorbance is higher in the choline-based medium as compared to the Na^+-based medium suggesting that the intrasynaptosomal free Mg^{2+} is higher in the absence of external Na^+. There is also a significant increase in the basal absorbance when the external Mg^{2+} concentration is increased from 1 mM to 5 mM (Fig. 3). Table I summarizes data from experiments similar to those in Fig. 3 performed in different conditions. None of the experimental media by itself had any significant effect on the response of eriochrome blue to changes in the free Mg^{2+} concentration. The relation of eriochrome blue absorbance to the Mg^{2+} concentration was also not affected by Ca^{2+} concentrations between 0 and 5 mM (data not shown).

The absorbance of eriochrome blue is sensitive to changes in pH which might limit its usefulness for quantitative determination of internal free Mg^{2+} concentration. This might especially be the case with ionophore A23187 which causes an increase in intrasynaptosomal pH [15]. in order to exclude possible effects through changes in pH the effect of nigericin, which is expected to decrease the pH gradient due to Na^+-H^+ exchange, on the absorbance of entrapped eriochrome blue and phenol red was studied. Nigericin had no significant effect on the absorbance of eriochrome blue (Fig. 4A) but slowly increased the absorbance of phenol red (Fig. 4B). The calibration of the nigericin-induced signal gave an internal pH of 7.2 at an external pH of 7.4. A similar value is obtained if internal pH is measured by [^{14}C]methylamine distribution as described earlier [16]. The intrasynaptosomal pH as measured with this method was 7.18 ± 0.01 ($\pm$S.D., $n = 3$) in the absence and 7.43 ± 0.02 ($\pm$S.D., $n = 3$) in the presence of 0.1 μM nigericin, respectively. Thus changes in internal pH do not seem to affect the calibration of the internal eriochrome blue signal.

TABLE I

EFFECTS OF EXTRACELLULAR Na^+, Ca^{2+} AND INTRASYNAPTOSOMAL ATP DEPLETION ON CYTOPLASMIC, FREE Mg^{2+}

The incubation time prior to calibration was 10 min. See Methods and Results for experimental details. Values are given as means $\pm$S.D., n is number of observations. Concentrations of FCCP and iodoacetate were 10 μM and 1 mM respectively.

Condition	$Ca^{2+}_{ext.}$ (mM)	$Mg^{2+}_{ext.}$ (mM)	$Mg^{2+}_{int.}$ (μM)	$\pm$S.D.	n
Na^+		1	342	26	6
Na^+		5	2150	329	4
Na^+	2	1	130	10	3
Na^+/68 mM K^+		1	375	35	3
Choline		1	625	82	6
Choline	2	1	675	139	3
Choline/ FCCP+IAA		1	715	55	5
Choline/ FCCP+IAA	2	1	833	76	3
Na^+/FCCP+ IAA		1	350	35	3

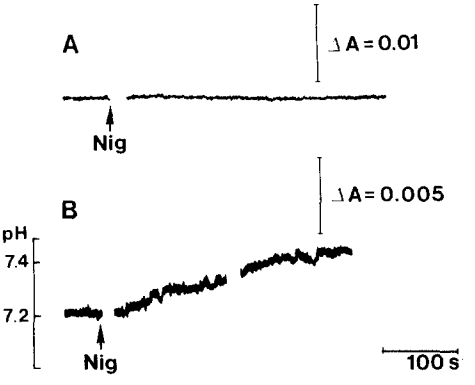

Fig. 4. Effects of nigericin on the absorbance of entrapped eriochrome blue (A) and phenol red (B). In (A) conditions were exactly as in Fig. 2. In (B) synaptosomes were loaded with phenol red as described in Methods and the absorbance was recorded at the wavelength pair 558–482 nm. The dye response was calibrated in a similar way as that of eriochrome blue by addition of pulses of HCl to digitonin-treated synaptosomes and the supernatant after centrifugation of intact material. 0.1 μM nigericin was added where indicated (Nig). The synaptosomal protein concentration was 1.34 mg/ml.

A value around 0.3 mM was obtained for intracellular free Mg^{2+} in synaptosomes incubated in the Na^+-based medium at 1 mM external Mg^{2+}. When the external Mg^{2+} concentration was increased to 5 mM there was a 5–6-fold increase in the internal free Mg^{2+} concentration (Table I). When the synaptosomes were depolarized for 10 min by increasing te K^+ concentration to 68 mM with the K^+-based medium there was no significant increase in the intracellular free Mg^{2+} concentration. Incubation of the synaptosomes for 10 min in the presence of an uncoupler of oxidative phosphorylation, FCCP, in combination with an inhibitor of glycolysis, iodoacetate, also caused no significant change in the intracellular free Mg^{2+} (Table I). The latter treatment has previously been shown to reduce the intrasynaptosomal ATP concentration to one twentieth of its initial value in less than 5 min [16].

In order to find out whether Na^+ might influence intracellular Mg^{2+} experiments similar to those above were performed in the choline-based medium. Replacement of Na^+ by choline chloride caused a doubling of the free Mg^{2+} concentration. FCCP in combination with iodoacetate caused no further change (Table I).

The addition of Ca^{2+} to the Na^+-based medium caused a significant decrease in the internal free Mg^{2+} concentration (Fig. 5) from 0.32 mM to around 0.1 mM at 2 mM Ca^{2+}. A half-maximal Ca^{2+}-induced change was observed around 1 mM Ca^{2+} and the effect was maximal above 2 mM. No significant effect of Ca^{2+} was observed in the choline-based medium (Table I). The addition of EGTA (1 mM) to the nominally Ca^{2+} fre medium did not significantly affect the free intracellular Mg^{2+} concentration. A value of 0.35 mM was obtained. Ca^{2+} had no significant effect on the intrasynaptosomal free Mg^{2+} concentration in the choline-based medium in the presence or absence of FCCP in combination with iodoacetate.

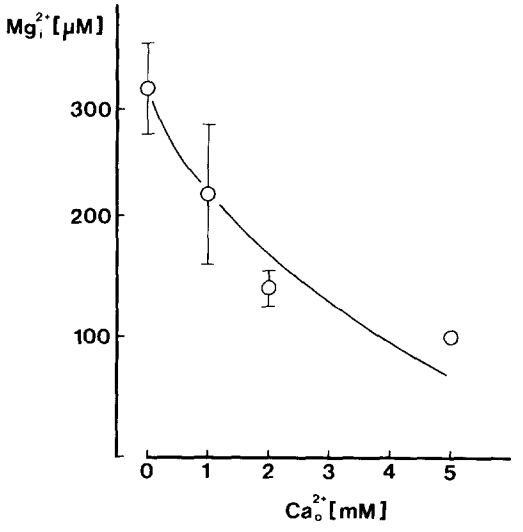

Fig. 5. The effect of the extracellular Ca^{2+} concentration on the cytosolic, free Mg^{2+}. The measurements of Mg^{2+} were performed in the Na^+-based medium with 1 mM external Mg^{2+}. The values of the internal, free Mg^{2+} at each Ca^{2+} concentration are means ± S.D. from three measurements. 0 Ca^{2+} indicates a nominally Ca^{2+}-free medium. If not indicated, the S.D. values do not exceed the diameter of the symbol. The temperature was 32°C.

Discussion

The results of the present study demonstrate that a metallochromic Mg^{2+} indicator, eriochrome blue, is entrapped within synaptosomes, when present in the homogenisation medium, in a similar way as the Ca^{2+} indicator arsenazo III [11]. The advantage of using eriochrome blue for the determination of Mg^{2+} activity is the insensitivity of the indicator of changes in Ca^{2+} concentration as well as the low K_d (around 1.5 mM for Mg^{2+} determined as described in Ref. 17). The cytosolic free Mg^{2+} concentration around 0.35 mM at 1 mM external Mg^{2+} obtained in this study is in good agreement with the value of 0.37 mM in rat hepatocytes reported by Corkey et al. [18]. They used a null point titration method with antipyrilazo II in digitonin-treated hepatocytes in the absence of Ca^{2+}. Our values are also in general good agreement with various other indirectly determinations of the cytosolic free Mg^{2+} concentration in mammalian cells ranging from 0.1 to 1 mM [19–21]

The mechanism by which the cells maintain a low cytosolic Mg^{2+} concentration is unknown at present. In agreement with data on squid axons [6,8] our results indicate that cytosolic free Mg^{2+} is at least partially regulated by a Na^+-Mg^{2+} exchange mechanism, which extrudes Mg^{2+} ions coupled to the Na^+ electrochemical potential.

Thus replacement of extracellular Na^+ by choline increased the internal free Mg^{2+}. Gramicidin, which depolarises synaptosomes completely by increasing Na^+ influx [22] and thus is expected to equilibrate the Na^+ gradient had a similar effect. The increase in internal free Mg^{2+} in the presence of of gramicidin is probably not due to influx of Mg^{2+} through Na^+ or Ca^{2+} channels since neither tetrodotoxin nor verapamil has any effect on the rise in free Mg^{2+} induced by the ionophore. Furthermore high K^+ depolarisation had no effect on the internal free Mg^{2+} suggesting that depolarisation per se does not induce Mg^{2+} influx.

There still appears to be a Mg^{2+} gradient across the plasma membrane after Na^+ removal or in the presence of gramicidin. This would suggest that mechanisms additional to apparent Na^+/Mg^{2+} exchange exist for the regulation of internal free Mg^{2+} in synaptosomes. No evidence for direct ATP-linked mechanisms could be obtained in this study since depletion of synaptosomal ATP with FCCP and iodoacetate had no short term effects on internal free Mg^{2+} in contrast to what has been reported with respect to Ca^{2+} [16] the concentration of which increases significantly upon ATP depletion. The lack of a direct effect of ATP on Mg^{2+} regulation is in agreement with the results of Mullins et al. [23].

Physiological concentrations of Ca^{2+} (around 1 mM) cause a significant reduction in the intrasynaptosomal free Mg^{2+} concentration. This might be an indirect effect through effects upon the Na^+ permeability. If the external Ca^{2+} concentration is reduced significantly there is also a reduction in the Na^+ gradient and the membrane potential [24]. This is probably not due to non-specific permeability changes [25] since it is partially blocked by tetrodotoxin and fully reversible when the Ca^{2+} concentration is increased. Thus it is most likely that also the effect of Ca^{2+} is due to effects through the Na^+ gradient across the plasma membrane. The lack of effect of Ca^{2+} when Na^+ was replaced by choline is in agreement with this and excludes the possibility of the existence of a simple Ca^{2+}-Mg^{2+} exchange mechanism. Thus in physiological conditions (in the presence of millimolar free external Ca^{2+} concentrations) it appears that there is a significant Mg^{2+} gradient across the plasma membrane and that intrasynaptosomal free Mg^{2+} is nearly one order of magnitude lower than the extracellular concentration of this cation.

It is concluded that entrapment of eriochrome blue within synaptosomes is a useful method for studying the regulation of the internal free Mg^{2+} concentration in isolated nerve endings. The mechanism for maintaining a Mg^{2+} gradient across the plasma membrane is at least partially due to a Na^+-linked mechanism.

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