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Interactions of polyamines in the measurement of free magnesium concentration by mag-fura-2 and ^{31}P-NMR

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Abstract

Polyamines, particularly spermine, in physiological concentrations interact with mag-fura-2 and the mag-fura-2/Mg^{2+} complex, resulting in reduced values of free Mg^{2+} concentration. Similarly, polyamines interact with ATP and MgATP. Thus, free Mg^{2+} concentration, as measured by ^{31}P-NMR or mag-fura-2, is underestimated in the presence of polyamines, particularly of spermine.

Key words: Intracellular free magnesium; Magnesium ion, intracellular; Polyamine; Mag-fura-2; NMR, ^{31}P-

1. Introduction

The concentration of intracellular free Mg^{2+} ($[Mg^{2+}]_i$) has been measured for 25 years with different methods in various cell types. These measurements were done by means of Mg^{2+}-dependent enzymes, Mg^{2+}-sensitive electrodes, null-point method, ^{31}P-NMR, and Mg^{2+}-binding fluorescent dyes [1,2]. The two latter methods are now predominantly being used. In these methods, Mg^{2+} is bound to negatively charged mag-fura-2, changing its fluorescence, or Mg^{2+} is bound to ATP^{4-}, changing its ^{31}P-NMR spectrum. Both methods require specificity of Mg^{2+} binding to mag-fura-2 or ATP^{4-}. However, it is well known that polyamines have similar binding properties to negatively charged ligands as Mg^{2+} [3,4] and may compete with Mg^{2+} in ATP binding.

At pH 7.5 putrescine, spermidine and spermine are bound to ATP^{4-} with apparent binding constants of 290 M^{-1}, 900 M^{-1} and 9500 M^{-1}. For comparison, the MgATP complex binding constant amounted to 17 200 M^{-1} when measured by the same method [5]. Moreover, a second spermidine and spermine molecule is bound to ATP with an apparent binding constant of 280 M^{-1} and 2400 M^{-1}. With similar affinity as Mg^{2+}, polyamines are also bound to ADP [5], which was also used to measure $[Mg^{2+}]_i$ by means of ^{31}P-NMR [6].

Since intracellular concentrations of polyamines are in the millimolar range and fluctuate in response to stimuli (for references, see ref. 5), we investigated the interaction of Mg^{2+} and polyamines with mag-fura-2 and the interaction of polyamines with ATP and MgATP in the estimation of free Mg^{2+} by ^{31}P-NMR and mag-fura-2.

2. Materials and methods

2.1. Fluorescence spectra

Mag-fura-2 (0.2 μM) was dissolved in K^+ medium containing: 140 mM KCl, 10 mM NaCl, 30 mM Hepes-Tris (pH 7.4). $MgCl_2$ and polyamines were added as indicated.

Fluorescence spectra were recorded with a Perkin Elmer LS 50 Luminescence Spectrometer. Excitation: 300–400 nm, Emission: 505 nm.

2.2. Measurement of free Mg^{2+} concentration

Mag-fura-2 (0.2 μM) was dissolved in K^+ medium (see above). Every 50 s, 0.2 mM $MgCl_2$ was added in

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the presence of various concentrations of spermine as indicated.

Fluorescence ratios of the mag-fura-2/Mg^{2+} complex at excitation 335 and 378 nm and emission 505 nm were measured at 37°C with a Perkin Elmer LS 50 Luminescence Spectrometer using the 'Intracellular Biochemistry' software of the producer. For the mag-fura-2/Mg^{2+} complex a K_d of 1.5 mM [7] was used. Maximal fluorescences were measured after addition of 1.2 mM $CaCl_2$ and minimal fluorescences after addition of 5 mM EDTA.

2.3. [31]P-NMR spectra

The solutions contained (in mM): 5 Na_2ATP, or 5 Na_2ATP plus 5 $MgCl_2$ or 12.5 $MgCl_2$ without

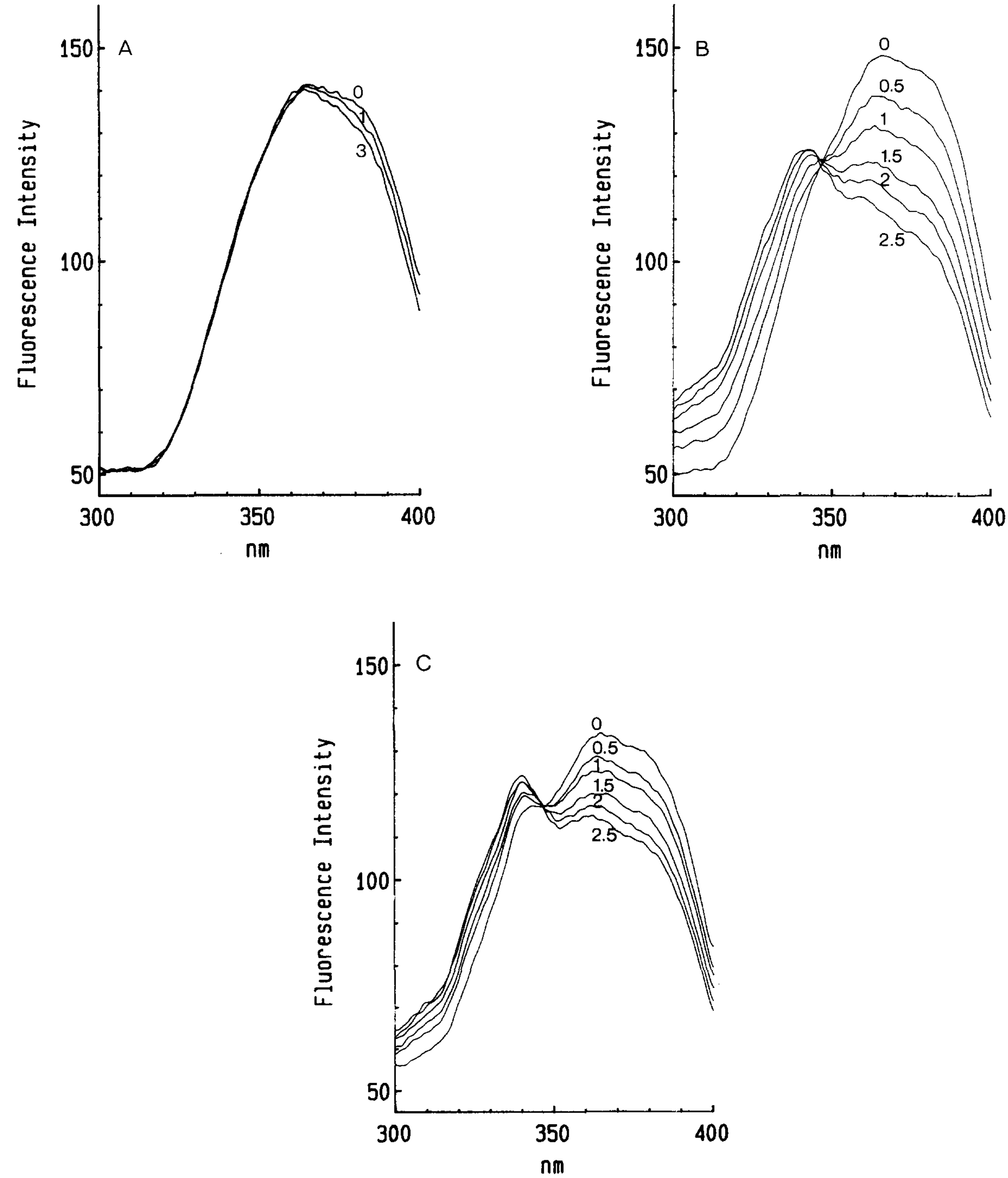

Fig. 1. Fluorescence spectra of mag-fura-2. Mag-fura-2 (0.2 μM) was dissolved in dimethyl sulfoxide and diluted in K^+ medium. (A) Mag-fura-2 plus various spermine concentrations (mM) as indicated; (B) mag-fura-2 plus various $MgCl_2$ concentrations (mM) as indicated; (C) mag-fura-2 with 3 mM spermine plus various $MgCl_2$ concentrations (mM) as indicated.

polyamines or with 5 putrescine · 2 HCl, 5 spermidine · 3 HCl or 5 spermine · 4 HCl as indicated, dissolved in 140 KCl, 20 TrisCl (pH 7.2). Na_2ATP was neutralized with Tris. To 2.0 ml of the solutions 0.7 ml D_2O was added to permit heteronuclear field frequency locking on deuterium.

^{31}P-NMR measurements were done on a Bruker AM500 spectrometer with a ^{31}P-resonance of 202 MHz. All measurements used a one-pulse sequence (90° pulse) without proton decoupling. The probe head temperature was held constant at 310 K and 128 acquisitions were recorded with a pulse repetition delay of 1 s and a sweep width of 20 kHz. Prior to the fast Fourier transformation an exponential multiplication (line broadening factor = 1.2 Hz) of the 64 Kbyte time domain data points was carried out and yielded a digital resolution of 0.31 Hz/point. The resonances were referenced to external H_3PO_4.

Free Mg^{2+} concentration ($[Mg^{2+}]_{free}$) in the solutions was calculated according to Eq. (1) [6,8]:

$$[Mg^{2+}]_{free} = K_d^{MgATP}(\Phi^{-1} - 1) \quad (1)$$

For K_d^{MgATP} (dissociation constant of MgATP) 50 μM was taken [8].

Φ was calculated from the chemical shift differences between the α-and β-phosphoryl group resonances of ATP ($\delta_{\alpha\beta}$), MgATP ($\delta_{\alpha\beta}^{MgATP}$, Mg^{2+} excess) and shift difference in the presence and absence of polyamines (($\delta_{\alpha\beta}^{x}$) according to Eq. (2):

$$\Phi = \left(\delta_{\alpha\beta}^{x} - \delta_{\alpha\beta}^{MgATP}\right)/\left(\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}\right) \quad (2)$$

Additionally, $[Mg^{2+}]_{free}$ was calculated from the $\alpha\beta$ and $\beta\gamma$ shift difference according to Eq. (3) [9]:

$$\Phi = \frac{1}{2}\left\{\frac{\left(\delta_{\alpha\beta}^{x} - \delta_{\alpha\beta}^{MgATP}\right)}{\left(\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}\right)} + \frac{\left(\alpha_{\beta\gamma}^{x} - \delta_{\beta\gamma}^{MgATP}\right)}{\left(\delta_{\beta\gamma}^{ATP} - \delta_{\beta\gamma}^{MgATP}\right)}\right\} \quad (3)$$

For the calculation of the shift differences, the arithmetic means of the α (α_1, α_2) and γ (γ_1, γ_2) peaks and the β_2 and β peak respectively (in ppm) were used.

Furthermore, $[Mg^{2+}]_{free}$ of the equimolar MgATP solution was calculated according to Eq. (4):

$$[Mg^{2+}]_{free} = -\tfrac{1}{2}\left([ATP]_{tot} - [Mg^{2+}]_{tot} + K_D^{MgATP}\right) + \left(\tfrac{1}{4}\left([ATP]_{tot} - [Mg^{2+}]_{tot} + K_D^{MgATP}\right)^2 + [Mg^{2+}]_{tot} \cdot K_D^{MgATP}\right)^{1/2} \quad (4)$$

($[ATP]_{tot}$ = total ATP concentration, $[Mg^{2+}]_{tot}$ = total Mg^{2+} concentration).

3. Results

3.1. Interaction of polyamines with mag-fura-2 and Mg^{2+}/mag-fura-2

As can be seen from Fig. 1A, spermine alone had only a small effect on the fluorescence of mag-fura-2 in the range of 378 nm and 335 nm, which are used to determine $[Mg^{2+}]_{free}$ [6,7]. Putrescine and spermidine did not have any significant effect on mag-fura-2 fluorescence either alone or in the presence of Mg^{2+} (not shown). However, the fluorescence of the Mg^{2+}/mag-fura-2 complex (Fig. 1B) was considerably changed in the presence of spermine (Fig. 1C). The interaction of spermine in the fluorescence of mag-fura-2 and Mg^{2+}/mag-fura-2 was caused by reaction of spermine with mag-fura-2, since spermine itself did not fluoresce or absorb at the wavelengths where measurements were done (data not shown).

To ascertain the interaction of spermine with the Mg^{2+}/mag-fura-2 complex quantitatively, every 50 s 0.2 mM $MgCl_2$ was added to a solution of mag-fura-2 in K^+ medium (pH 7.4) and the concentration of free Mg^{2+} was measured. As shown in Fig. 2 (curve A), in the absence of spermine correct values of $[Mg^{2+}]_{free}$ were obtained as expected. However, in the presence of 0.5 mM spermine (curve B), 30% and in the presence of 3 mM spermine (curve C) 50% lower values of $[Mg^{2+}]_{free}$ were determined.

3.2. Interaction of polyamines with ATP and MgATP in ^{31}P-NMR

As can be seen from Table 1A, there was only a slight interaction of the polyamines with ATP, according to the series putrescine < spermidine < spermine.

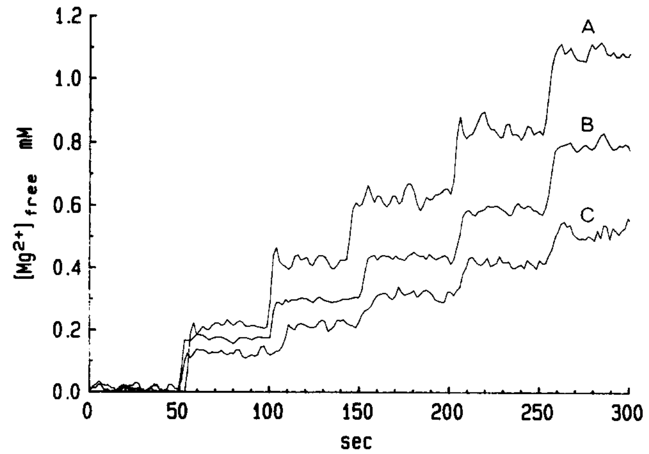

Fig. 2. Effect of spermine on $[Mg^{2+}]_{free}$. Mag-fura-2 (0.2 μM) was dissolved in dimethyl sulfoxide and diluted in K^+ medium. Every 50 s 0.2 mM $MgCl_2$ were added. (A) In the absence of spermine; (B) in the presence of 0.5 mM spermine; (C) in the presence of 3 mM spermine.

Table 1A
Effect of polyamines (3.7 mM) on ^{31}P-resonances of ATP (3.7 mM)

^{31}P-ATP resonance	ATP	MgATP *	ATP+ putrescine	ATP+ spermidine	ATP+ spermine
γ_1 **	−6.59	−5.25	−6.59	−6.58	−6.52
γ_2	−6.68	−5.33	−6.68	−6.68	−6.62
α_1	−10.80	−10.40	−10.81	−10.82	−10.84
α_2	−10.90	−10.48	−10.91	−10.92	−10.94
β_1	−21.63	−18.67	−21.64	−21.67	−21.66
β_2	−21.73	−18.75	−21.73	−21.75	−21.75
β_3	−21.83	−18.83	−21.83	−21.84	−21.85

Values in ppm. Mean of two experiments.
* MgATP, 9.25 mM Mg^{2+} plus 3.7 mM ATP.
** γ_1, γ_2, α_1, α_2, β_1, β_2, β_3 mean α and γ doublets and β triplets of ^{31}P-ATP resonances.

Table 1B
Effect of polyamines (3.7 mM) on ^{31}P-resonances of MgATP ($[Mg^{2+}]_{tot}$ = 3.7 mM; $[ATP]_{tot}$ = 3.7 mM)

^{31}P-ATP resonance	MgATP	MgATP+ putrescine	MgATP+ spermidine	MgATP+ spermine
γ_1	−5.24	−5.26	−5.34	−5.44
γ_2	−5.32	−5.34	−5.42	−5.52
α_1	−10.34	−10.35	−10.40	−10.46
α_2	−10.42	−10.43	−10.47	−10.54
β	−18.92	−18.96	−19.07	−19.33

Values in ppm. Mean of two experiments

The interaction of Mg^{2+} with ATP was particularly seen from the shift of the β peak (Table 1B). In the presence of equimolar Mg^{2+} and ATP concentrations the β peak is broadened which is due to Mg^{2+} exchange between MgATP and ATP [10]. Hence β_1, β_2 and β_3 could not be differentiated. When Mg^{2+} was in excess to ATP, again 3 β peaks occurred (Table 1A).

The polyamines interacted with MgATP. The interaction between polyamines and MgATP again corresponded to the series putrescine < spermidine < spermine (Table 1B) which is in agreement with their ATP complex binding constants [5].

For a quantitative analysis of the interaction with MgATP in ^{31}P-NMR, the concentrations of free Mg^{2+} were calculated according to the above given Eq. (1).

Table 2
Concentration of free Mg^{2+} ($[Mg^{2+}]_{free}$) in solutions with 3.7 mM $MgCl_2$ and 3.7 mM ATP in the absence and presence of 3.7 mM polyamines measured by ^{31}P-NMR

Polyamine	$[Mg^{2+}]_{free}$ (mM) (Eqs. (1), (2))	$[Mg^{2+}]_{free}$ (mM) (Eqs. (1), (3))
–	0.509	0.455
Putrescine	0.444	0.395
Spermidine	0.345	0.325
Spermine	0.197	0.177

$[Mg^{2+}]_i$ was calculated according to Eqs. (1), (2) and (3) (see Section 2).

Table 3
Concentration of free Mg^{2+} ($[Mg^{2+}]_{free}$) in solutions with 3.7 mM $MgCl_2$ and 3.7 mM ATP in the absence and presence of 3.7 mM polyamines measured at 37°C by mag-fura−2 (see Section 2)

Polyamine	$[Mg^{2+}]_{free}$ (mM)
–	0.51 ± 0.01
Putrescine	0.44 ± 0.01
Spermidine	0.35 ± 0.02
Spermine	0.24 ± 0.01

Mean ± S.E.M. of three experiments.

$[Mg^{2+}]_{free}$ calculated according to Eq. (4) amounted to 0.41 mM. Table 2 shows that the values of $[Mg^{2+}]_{free}$ determined by ^{31}P-NMR are in agreement with the value of $[Mg^{2+}]_{free}$ calculated according to Eq. (4). The values of $[Mg^{2+}]_{free}$ were reduced by the polyamines according to the series putrescine < spermidine < spermine.

3.3. Effect of polyamines on $[Mg^{2+}]_{free}$ of MgATP solutions measured by mag-fura-2

In order to compare the effects of polyamines on the measurement of $[Mg^{2+}]_{free}$ by ^{31}P-NMR, in analogous experiments $[Mg^{2+}]_{free}$ of MgATP solutions was measured by mag-fura-2. As shown in Table 3, measurement of $[Mg^{2+}]_{free}$ by mag-fura-2 yielded the same values of $[Mg^{2+}]_{free}$ as measured by ^{31}P-NMR. $[Mg^{2+}]_{free}$ measured by mag-fura-2 was similarly reduced as when measured by ^{31}P-NMR.

4. Discussion

During the last few years mag-fura-2 and ^{31}P-NMR were the most frequently used methods to measure $[Mg^{2+}]_i$. The interaction of Ca^{2+} and H^+ were considered in both methods [6,8]. The interaction of polyamines, particularly of spermine, has not yet been taken into account in these measurements.

The intracellular content of polyamines amounts to 1–5 mmol spermine/kg wet weight for liver, pancreas and prostate and to 8–9 mmol spermidine/kg wet weight for prostate and pancreas [3,4,11,12] and correspond to total intracellular Mg^{2+} content. Cellular polyamines are compartmentalized, and a part of the polyamines within the various compartments is bound to negatively charged ligands [3,4,13,14]. As yet there is no method available to determine the concentration of free polyamines besides bound polyamines. The complex binding constants of spermine are about half of the Mg^{2+} complex binding constants [5]. Therefore, in analogy to intracellular Mg^{2+}, it may be supposed that approximately 20% of total spermine is free. For putrescine and spermidine the rate of free polyamine may be higher than for spermine because of their lower complex binding constants [5]. In Figs. 1 and 2 the used spermine concentrations varied between 0.5 and 3 mM and in Tables 2 and 3 the used polyamine concentrations amounted to 3.7 mM and may be within or near the physiological range in some cell types. Thus, $[Mg^{2+}]_i$ measured by these two methods is underestimated in the presence of polyamines, particularly in the presence of spermine. This effect must be considered when values for $[Mg^{2+}]_i$ measured by mag-fura-2 and ^{31}P-NMR are compared with those measured by the null-point method or Mg^{2+}-sensitive electrodes.

The mechanism by which polyamines reduce the measured values of $[Mg^{2+}]_{free}$ can be attributed to competition of polyamines with Mg^{2+} for common binding sites of mag-fura-2 and ATP^{4-}, and to lower responses in fluorescence or ^{31}P-NMR of the polyamine-mag-fura-2 and polyamine-ATP complex than the corresponding Mg^{2+} complexes. The alternative mechanism may be binding of Mg^{2+} to polyamines and thus reducing $[Mg^{2+}]_{free}$. When Mg^{2+} binding to spermine was investigated by proton magnetic resonance, it was found that most of the spermine existed in a free form [15]. This result implies that there is no significant binding of Mg^{2+} to spermine. Hence, reduction of measured $[Mg^{2+}]_{free}$ by binding of Mg^{2+} to polyamines can be neglected. Also, an association constant for Mg^{2+} binding to spermine is not available.

Using mag-fura-2, it was found that some cell types showed an increase in $[Mg^{2+}]_i$ after stimulation with various effectors, such as epidermal growth factor [16,17], insulin [16], vasopressin [18], endothelin [18], carbachol [19], cGMP [20] and atrial natriuretic peptide [20]. An increase in $[Mg^{2+}]_i$ was also found by isoproterenol in heart muscle cells as measured by ^{31}P-NMR [21]. A decrease of $[Mg^{2+}]_i$ was induced by cAMP, PTH and calcitonin [20]. From these results it was concluded that $[Mg^{2+}]_i$ may play a role in the intracellular mechanisms of these effectors. In these experiments the alterations of $[Mg^{2+}]_i$ were very small and amounted to 0.1 to 0.2 mM. However, the concentration of polyamines (besides Ca^{2+} or H^+) is also changed by some of these effectors [3,13,14,22].

Hence, the interaction of polyamines in the measurement of $[Mg^{2+}]_i$ cannot be defined quantitatively and the real alterations in $[Mg^{2+}]_i$ by effectors remain uncertain.

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