Concentration of intracellular free calcium measured by quin-2 fluorescence in isolated cardiomyocytes from normal and magnesium-deficient rats

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Zusammenfassung

In Kardiomyozyten, die durch Kollagenase-Behandlung isoliert wurden, wurde die Konzentration des intrazellulären freien Ca^{2+} ($[Ca^{2+}]_i$) mit Hilfe von Quin-2 gemessen. In Kardiomyozyten von normalen Ratten, die bei 0,5 mM Mg^{2+} inkubiert wurden, betrug $[Ca^{2+}]_i$ 242 nM und hing in reziproker Weise von der extrazellulären Mg^{2+}-Konzentration ab. Nach Erhöhen der extrazellulären K^+-Konzentration auf 30 mM stieg $[Ca^{2+}]_i$ auf das 2,2-fache. In Kardiomyozyten von Mg-Mangelratten waren $[Ca^{2+}]_i$ und die Zunahme von $[Ca^{2+}]_i$ durch 30 mM K^+ geringer als bei normalen Kardiomyozyten.

Summary

In cardiomyocytes, isolated by treatment with collagenase, the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured by quin-2 fluorescene. In cardiomyocytes from normal rats, incubated at 0.5 mM Mg^{2+}, $[Ca^{2+}]_i$ amounted to 242 nM and was reciprocally dependent on the extracellular Mg^{2+} concentration. Increasing the extracellular K^+ concentration to 30 mM increased $[Ca^{2+}]_i$ by the factor 2.2. In cardiomyocytes from chronic Mg-deficient rats, $[Ca^{2+}]_i$ and the increase of $[Ca^{2+}]_i$ by 30 mM K^+ were lower than in normal cardiomyocytes.

Résumé

Les auteurs ont utilisé une technique de quin-2-fluorescence pour mesurer les concentrations de calcium libre intra-cellulaire, ($[Ca^{++}]_i$), dans des cellules musculaires cardiaques isolées par un traitement par la collagénase. Dans les cellules de rats normaux, incubées dans 0,5 mM de Mg^{++}, la valeur de cette concentration a atteint 242 nM et s'est montrée inversement proportionnelle à la concentration extra-cellulaire en ions Mg^{++}. L'augmentation de la concentration extra-cellulaire en ions K^+ jusqu'à 30 mM a multiplié par 2,2 la valeur de la $[Ca^{++}]_i$. Dans les cellules musculaires cardiaques des rats ayant une carence chronique en Mg, la concentration de calcium libre intra-cellulaire et son augmentation par l'accroissement à 30 mM du taux de K^+ extra-cellulaire ont été plus faibles que dans les cellules musculaires cardiaques des rats normaux.

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The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) plays an important rọle in various cellular responses such as muscle contraction and as second messenger in the action of hormones and mitogenes, $[Ca^{2+}]_i$ being increased either by enhanced influx or enhanced release from intracellular stores.

At reduced extracellular Mg^{2+} concentration the influx and efflux of $^{22}Na^+$ and $^{42}K^+$ as well as $^{45}Ca^{2+}$ influx were found to be increased.

An increased $[Ca^{2+}]_i$ was discussed as a fundamental pathobiochemical mechanism of Mg deficiency [5]. The development of new methods offers the opportunity to measure $[Ca^{2+}]_i$ directly in isolated cells from Mg-deficient animals. In a preceding paper [17] we determined $[Ca^{2+}]_i$ in thymocytes from Mg-deficient rats and found the same $[Ca^{2+}]_i$ and the same increase of $[Ca^{2+}]_i$ induced by the mitogenic lectin concanavalin A. In the present paper we measured $[Ca^{2+}]_i$ in isolated heart muscle cells and found a somewhat lower $[Ca^{2+}]_i$ and a smaller increase of $[Ca^{2+}]_i$ by K^+ depolarization in Mg-deficient than in normal cells.

Materials and Methods

Isolation of cardiomyocytes

Normally fed female Wistar rats, weighing 200–250 g, and female Wistar rats of the same age, fed an Mg-deficient diet (Ssniff, Soest/FRG) and distil. water for 10 to 14 weeks were used for the experiments. The composition of the diet has been described in detail [7]. The rats were anesthetized with an intraperitoneal injection of Nembutal (50 mg/kg). Hearts were excised and a soft plastic tube was introduced into the aorta and fixed. According to *Cheung* et al. [1a] the spontaneously beating hearts were perfused for 15 min at a rate of 4 ml/min without recirculation with Krebs-Henseleit bicarbonate (buffer A, composition in mM: NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 15 and rat plasma levels of 20 amino acids) gassed with 95% O_2 – 5% CO_2 and prewarmed to 37 °C. During this perfusion period, the heart assumed a regular rate of contraction, recovcering from the period

of anoxia associated with removal from the rat. The hearts were then perfused for 5 min with an oxygenated Ca^{2+}-free bicarbonate buffer (composition in mM: NaCl 120, KCl 2.7, KH_2PO_4 1.4, $MgCl_2$ 1.4, $NaHCO_3$ 25, glucose 15, amino acids). The hearts ceased to contract within 1 min and remained in diastolic arrest throughout the remaining perfusion period. Collagenase 0.1 % (type I from Clostridium histolyticum, Sigma), bovine serum albumin (BSA) 0.1 % (analytical grade, Serva/Heidelberg) and $CaCl_2$ (final concentration: 50 µM) were added to the Ca^{2+}-free bicarbonate buffer, and the buffer was recirculated through the heart for an additional 15 min. The hearts became pale and swollen. At the end of perfusion, the hearts were removed from the cannulas and the atria and valves were trimmed. The ventricles were cut into pieces, incubated for another 10 min in the collagenase-containing buffer with 2 % BSA, and gently dissociated by pipetting. The suspension was filtered through four layers of gauze and centrifuged for 1 min at 50 g. The sediment was resuspended in 1 ml buffer A with 0.1 % BSA and layered on 8 ml buffer with 2 % BSA. The cells were sedimented for 5 min at 1 g.

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

The sedimented isolated cells were loaded with quin-2 by incubation with 50 µM quin-2/AM (Sigma, freshly dissolved in dimethyl sulfoxide) in 20 ml buffer A plus 2 % BSA for 1 h at 37 °C under gentle shaking, according to *Tsien* et al. [16]. Extracellular quin-2/AM was removed by centrifugation (1 min, 50 g) and washing the cells twice in buffer A with 0.1 % BSA. Approximately 15 min before fluorescence measurement, the cells were transferred to buffer A with various Mg^{2+} concentrations, as indicated in Tab. 1, and incubated at 37°C until measurement. Fluorescences were recorded with a Perkin-Elmer 650-10 S fluorescence spectrophotometer. Standard monochromator settings were 339 nm excitation with 4 nm slits; 492 nm emission with 10 nm slits.

Under these conditions a constant fluorescence (F) was measured for 1 – 2 min. Thereafter, 30 mM KCl (final concentration) was added in a small volume to depolarize the cells. After addition of 50µM digitonin (final concentration) maximal fluorescence (Fmax) was obtained. For the measurement of Fmin, 2 mM EGTA and 10 mM Tris (final concentration), resulting in a pH > 8.3, were added. After measurement of F, 0.5 mM $MnCl_2$ (final concentration) was added for correction of extracellular quin-2, released from quin-2-loaded cells. This value was substracted from F and Fmax. $[Ca^{2+}]_i$ was calculated according to $[Ca^{2+}]_i = K_D \cdot \frac{F\text{-}Fmin}{Fmax\text{-}F}$.

For K_D, a value of 115 nM was taken [16]. Cell viability was tested by trypan blue exclusion. For more details see Ref. [1].

Results and Discussion

Characterization of isolated cardiomyocytes

Using the applied method approximately 10^6 cells/g wet weight were obtained. More than 90 % of the cells were not stained by trypan blue. Approximately 70 % of the isolated cardiomyocytes were rod-shaped with squared corners and visible striations, the rest of the cells were rounded without distinct cross striations, as already published in detail [14], and as was observed by *Williamson* et al. [20], who used the same procedure as in the present experiments. Only a part of the rounded cells were stained by trypan blue. No cells spontaneously contracted.

$[Ca^{2+}]_i$ in cardiomyocytes

At an extracellular Ca^{2+} and Mg^{2+} concentration of 1.2 and 0.5 mM, we measured a $[Ca^{2+}]_i$ = 242 nM (Tab. 1) which is in good agreement with the results other authors obtained when using quin-2 [11], aequorin [18] or null-point titration and arsenazo III as Ca^{2+} indicator [20].

Tab. 1: Concentration of intracellular free calcium ($[Ca^{2+}]_i$) in isolated cardiomyocytes from normal and Mg-deficient rats at various extracellular Mg^{2+} concentrations ($[Mg^{2+}]_o$) and after depolarisation with 30 or 60 mM extracellular K^+ ($[K^+]_o$). Mean ± SEM. Numbers of experiments in brackets. Significant difference between normal and Mg-deficient rats by unpaired Student's t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significant difference between $[Ca^{2+}]_i$ at 3 mM and 0.5 mM$[Mg^{2+}]_o$: +, $p < 0.05$

$[Mg^{2+}]_o$ (mM)	$[Ca^{2+}]_i$ (nM)		
	control	$[K^+]_o$ = 30 mM	$[K^+]_o$ = 60 mM
Hearts from normal rats			
0.1	288 ± 57 [4]	—	—
0.5	242 ± 32 [6]	523 ± 16 [4]	—
1.2	162 ± 14 [11]	374 ± 29 [3]	575 [1]
3.0	127 ± 11 [2]+	—	—
Hearts from Mg-def.rats			
0.1	71 ± 6 [5]**	104 [1]	—
0.5	148 ± 16 [5]*	262 ± 13 [4] ***	—

These investigators measured 137 nM [11], 270 nM (20) and 300 nM [18] for isolated resting cardiomyocytes. Using fura-2, $[Ca^{2+}]_i$ was measured in single isolated mechanically quiescent cells to amount to 134 nM, in spontaneously contracting cells to amount to 270 nM and in hypercontracted (rounded up) cells to amount to 955 nM [19]. Thus, the differences in $[Ca^{2+}]_i$ in the experiments with isolated cardiomyocytes, cited above, may be explained, at last in part, to be caused by a variation in the cell population after isolating the cells.

With microelectrodes values of 120 nM [8], 210 nM [15], 251 nM [12], 260 nM [9] and 360 nM [2] were obtained for Purkinje fibres or resting ventricle muscle cells. For review see Ref. [1]. The differences can be explained by experimental artefacts because of impaling the cells with the microelectrodes and from different values for the stability constant of Ca EGTA which is used in the calibration of Ca-microelectrodes [9]. All the values represent "concentration" and not ion activity.

These values for resting cells measured by dyes or microelectrodes are in agreement with the pCa-tension relationship. Contraction only starts at pCa < 6.5 (> 300 nM). For literature see Ref. [9].

Isolated quiescent cardiomyocytes do not respond to adrenalin, noradrenalin or isoproterenol by increasing $^{45}Ca^{2+}$ exchange [13]. In agreement with this result, in isolated resting cardiomyocytes isoproterenol (0.1 mM) did not increase $[Ca^{2+}]_i$, as was also found by *Powell* et al. [11]. Thus, our techniques in cell preparation and $[Ca^{2+}]_i$ measurement with normal cardiomyocytes under identical conditions of incubation gave results identical with those reported in the literature.

Moreover, we measured $[Ca^{2+}]_i$ of normal cardiomyocytes after incubation in hypomagnesemic and hypermagnesemic media. At reduced extracellular free Mg^{2+} concentration of 0.1 mM, $[Ca^{2+}]_i$ was somewhat, not significantly increased, whereas at elevated concentrations of extracellular free Mg^{2+}, $[Ca^{2+}]_i$ was significantly reduced (Tab. 1), indicating the Ca^{2+} antagonistic effect of Mg^{2+} at drastically increased extracellular Mg^{2+} concentrations. Interestingly, at 1.2 mM extracellular Mg^{2+}, 30 mM extracellular K^+ increased $[Ca^{2+}]_i$ by the same factor of 2 as at 0.5 mM Mg^{2+}.

The most significant result of our experiments was that $[Ca^{2+}]_i$ in isolated cardiomyocytes from chronic Mg-deficient rats was lower than $[Ca^{2+}]_i$ in control cells, particularly after incubation at 0.1 mM extracellular Mg^{2+} (Tab. 1). After incubation with 30 mM extracellular K^+, the increase of $[Ca^{2+}]_i$ in the cells from Mg-deficient rats was somewhat lower than in the cells from normal rats. These results indicate that the capacity of Ca^{2+} pumping out of the cell and/or the capacity of Ca^{2+} storage in cardiomyocytes from chronic Mg-deficient rats is stronger than in those of normal rats. The increased intracellular Ca^{2+} content in hearts from Mg-deficient rats [5] is caused by increased intracellular Ca^{2+} storage. Thus, the results from cardiomyoctes agree with those from thymyocytes of Mg-deficient rats [17].

The measured values of $[Ca^+]_i$ are steady state concentrations of intracellular Ca^{2+} distribution representing the capacity of Ca^{2+} influx, Ca^{2+} pumping and Ca^{2+} storage systems because the quin-2 method needs a high loading concentration with the Ca-chelator quin-2 resulting in additional intracellular Ca^{2+} uptake. Therefore, it is possible that in the cardiomyocytes of Mg-deficient animals, particularly under the action of decreased serum Mg^{2+} concentrations, and thus increased serum catecholamine and prostaglandins [10], there may be a transiently increased Ca^{2+} influx and a transiently increased $[Ca^{2+}]_i$, followed by increased Ca^{2+} pumping and/or Ca^{2+} storage, which can not be measured under our experimental conditions. Experiments with isolated perfused hearts in a Langendorff preparation did not reveal any differences between hearts from normal and Mg-deficient rats when the hearts were perfused under identical conditions [3]. Therefore, it can be concluded that the cardiac effects of Mg deficiency are induced by the reduced serum Mg^{2+} concentration and/or in consequence by the increased concentrations of catecholamines, biogenic amines and prostaglandins which may act in concert. For a detailed discussion see Ref. [6].

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