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Report

Efficacy of magnesium chloride in the treatment of Hailey–Hailey disease: from serendipity to evidence of its effect on intracellular Ca^{2+} homeostasis

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Abstract

Background Hailey–Hailey disease (HHD), also known as familial benign chronic pemphigus, is a rare autosomal dominant inherited intraepidermal blistering genodermatosis. Mutations in the ATP2C1 gene encoding for the Golgi secretory pathway Ca^{2+}/Mn^{2+}-ATPasi protein 1 (SPCA1) affect the processing of desmosomal components and the epidermal suprabasal cell–cell adhesion by deregulating the keratinocyte cytosolic Ca^{2+} concentration. We report the unexpected, dramatic, and persistent clinical improvement of the skin lesions of a patient affected with longstanding HHD with daily intake of a solution containing magnesium chloride hexahydrate ($MgCl_2$).

Materials and methods We investigated the effect of $MgCl_2$ on the intracellular Ca^{2+} homeostasis and on the activity of particular Ca^{2+}-effectors in HeLa cells transfected with chimeric aequorins (cytAEQ, mtAEQ, erAEQ and GoAEQ) targeted to different subcellular compartments (cytosol, mitochondria, endoplasmic reticulum, and Golgi, respectively).

Results Experimental investigations on HeLa cells showed the effect of $MgCl_2$ on the function of Ca^{2+}-extrusor systems, resulting in increased cytosolic and mitochondrial Ca^{2+} levels, without altering the mechanisms of intraluminal Ca^{2+}-filling and Ca^{2+}-release of stores.

Conclusions Based on our clinical observation and experimental results, it can be hypothesized that $MgCl_2$ could act as an inhibitor of the Ca^{2+}-extruding activity in keratinocytes favoring intracellular Ca^{2+}-disponibility and Ca^{2+}-dependent mechanisms in desmosome assembly. This may represent the molecular basis of the good response of the HHD clinical features with $MgCl_2$ solution in the patient described.

Introduction

The genetics and pathophysiology of Hailey–Hailey disease (HHD) have been linked to about 100 mutations in the ATP2C1 gene encoding for the Golgi secretory pathway Ca^{2+}/Mn^{2+}-ATPasi protein 1 (SPCA1).[1] SPCA is a Ca^{2+} and Mn^{2+} pump that transports these ions into the lumen of Golgi from cytosolic space. The role of Ca^{2+} in disease development is well documented.[2] In fact, Ca^{2+} regulates the proliferation and the differentiation of keratinocytes as well as desmosomes assembly.[3,4] It is plausible to speculate that a defective SPCA1 might affect the processing of desmosomal components, leading to the loss of epidermal suprabasal cell–cell adhesion and consequent acantholysis.

We report the unexpected, dramatic, and persistent clinical improvement of the skin lesions of a patient affected with longstanding HHD with daily intake of a solution containing magnesium chloride hexahydrate ($MgCl_2$). In order to find a conceivable explanation of our extraordinary observation, we investigated whether the treatment with $MgCl_2$ could have any effect on the intracellular Ca^{2+} homeostasis, capable of ameliorating the clinical features of HHD.

Case report

We present the case of an otherwise healthy 72-year-old woman with a history of biopsy-confirmed HHD localized on her vulva and inguinal folds. The lesions had started at the age of 25 years, and a family history of HHD was present. Over the years, the patient had applied, without regular medical checking, a great number of topical treatments, including corticosteroids, antifungals, antibiotics, antiseptics, and moisturizers, which were only partially effective in controlling the disease. No

systemic drugs or surgical approaches had ever been attempted. The disease progressively worsened, and the patient complained of increasing chronic pruritic and burning erythematous weeping plaques with fissuring and crusting, complicated by frequent acute and painful exacerbations that needed an almost continual use of a topical corticosteroid, mainly mometasone furoate 0.1% cream or 0.1% lotion, 0.3% desametasone–1% clotrimazole fixed combination, econazole 1% cream, zinc oxide paste, and antiseptic cleansers. The patient referred with diffused joint pain during the last two years. She chose to treat her arthralgia with magnesium chloride solution, as suggested by a close friend of hers suffering from arthritis. The patient dissolved 33 g of magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) in 1 l of water and drank a coffee cup of the solution daily before breakfast, namely about 70 ml of the solution.

Unexpectedly, one week after the beginning of the treatment with magnesium chloride hexahydrate, a significant improvement in the skin lesions was noted, and an almost complete remission of signs and symptoms was obtained after four weeks. On examination after three months of daily intake of magnesium chloride, we observed the complete re-epithelialization of the previously affected areas that were hitherto scarcely responsive to treatments. The patient denied her consent to take pictures. However, the visual analog scale (0–10) of itching, burning, and pain fell from 8, 7, and 4 to 1, 0, 0, respectively. The objective assessment of erythema, essudation, and vessiculobullous using a four-point scale (0 = absence; 1 = mild; 2 = moderate; 3 = severe) fell from 3, 3, 3 before treatment to 1, 0, 0, respectively. This clinical improvement continued to persist after 12 months of ongoing treatment, including during the hot and humid summer period. No relevant relapses occurred, except for two exacerbations of HHD in situations of great stress; however, these were minor for severity and duration compared with previous recurrences. These minor relapses were treated with topical corticosteroid, and remission was obtained in a few days, with persistent recovery. No side effects have been reported by the patient, though the magnesium chloride solution has been referred to have an unpleasant taste. After 12 months of magnesium chloride intake, laboratory findings were normal, including magnesiemia and the other electrolytes. Pre-treatment serum levels of electrolytes, in particular calcium, magnesium, and zinc, were unknown. The patient's quality of life was significantly improved.

Materials and methods

Preparation of $MgCl_2$ solution

Magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$; 33 g) was dissolved in 1 l of water, obtaining a sterilized solution of 0.16 M $MgCl_2$. The cells were incubated with this solution before performing Ca^{2+}-measurement.

Cell culture and transfection

Human cervical adenocarcinoma cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), in 75-cm^2 Falcon flasks. For the aequorin experiments, the cells were seeded onto 13-mm glass coverslips and allowed to grow to 75% confluence. At this stage, transfection with 4 μg aequorin chimera cDNA was carried out as previously described.[5]

Aequorin measurements

The probes employed (cytAEQ, mtAEQ, erAEQ and GoAEQ) are chimeric aequorins targeted to the cytosol, mitochondria, endoplasmic reticulum (ER), and Golgi, respectively. Reconstitution protocols for cytAEQ and mtAEQ are different from those for erAEQ and GoAEQ. For the experiments with cytAEQ and mtAEQ, cells were incubated with 5 μM coelenterazine for 1–2 hours in DMEM supplemented with 1% FCS. Next, the coverslip with transfected cells was placed in a perfused chamber with thermostat, located in close proximity to a low-noise photomultiplier, with a built-in amplifier-discriminator. To reconstitute the erAEQ and GoAEQ with high efficiency, the luminal [Ca^{2+}] of the stores first had to be reduced. This was achieved by incubating the cells for 1 hour at 4 °C in Krebs–Ringer buffer (KRB), supplemented with 5 μM coelenterazine, Ca^{2+} ionophore ionomycin, and 600 μM EGTA. After 45 minutes of incubation, the cells were extensively washed with KRB, supplemented with 2% bovine serum albumin, and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB supplemented with either 1 mM $CaCl_2$ (cytAEQ and mtAEQ) or 100 μM EGTA (erAEQ and GoAEQ). Agonist and other drugs were added to the same medium, as specified in the figure legends. The experiments were terminated by lysing the cells with 100 μM Triton in a hypotonic Ca^{2+}-containing solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The output of the discriminator was captured by a Thorn-EMI photon-counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated offline into [Ca^{2+}] values, using a computer algorithm based on the Ca^{2+}-response curve of wt aequorin. Statistical data are presented as mean ± SE, and statistical significance was determined using the *t*-test.

Results

It was investigated whether the treatment with $MgCl_2$ had any effect on the intracellular Ca^{2+} homeostasis and whether it affected the activity of particular Ca^{2+}-effectors. To these aims, recombinant aequorins targeted to different subcellular compartments were transfected in

HeLa cells. HeLa cells were preferred because they are considered a good cellular model for the study of Ca^{2+} signaling. This kind of cell presents elevated intracellular Ca^{2+} content, high Ca^{2+}-dependent agonist-responsivity, very low Ca^{2+}-response variability; indeed they are easy to manage and to transfect. Thirty-six hours after transfection, the cells were treated with 0.16 M $MgCl_2$.

Figure 1 shows the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) monitored with the cytosolic aequorin chimera (cytAEQ). The cells were challenged with histamine (100 μM) in a medium containing 1 mM $CaCl_2$, causing a rapid rise in $[Ca^{2+}]_c$ in both sets of cells. However, the $[Ca^{2+}]_c$ increase evoked by agonist in $MgCl_2$-treated cells was significantly higher than in untreated cells ($[Ca^{2+}]_c$ untreated cells 1.45 ± 0.06 μM vs. $MgCl_2$-treated 2.05 ± 0.07 μM, $P < 0.01$; Fig. 1a). HeLa cells transfected with mitochondrial aequorin chimera (mtAEQ) showed a peak of mitochondrial response that was markedly increased (almost 15%) with respect to untreated cells, when stimulated (3.76 ± 0.32 μM in untreated cells vs. 4.43 ± 0.53 μM in $MgCl_2$-treated cells, $P < 0.01$; Fig. 1b). A possible explanation for the increased cytosolic and mitochondrial $[Ca^{2+}]$ response in cells treated with $MgCl_2$ could be the increased amount of Ca^{2+} released by Ca^{2+} stores (ER and Golgi apparatus) as a consequence of a higher level of Ca^{2+}-filling of the lumen, favored by $MgCl_2$ treatment. This was tested by directly monitoring the intralumenal $[Ca^{2+}]$ with an aequorin chimera targeted to the ER and Golgi (erAEQ, GoAEQ). Under the experimental conditions, the $[Ca^{2+}]_{ER}$ was <10 μM in Ca^{2+}-depleted cells, and it gradually increased upon switching the perfusion medium to a buffer supplemented with 1 mM Ca^{2+}, reaching plateau levels that in the control cells were about 300 μM. In $MgCl_2$-treated cells, $[Ca^{2+}]_{ER}$ reached a similar steady-state level (314.17 ± 24.21 μM in untreated cells vs. 316.00 ± 14.44 μM in $MgCl_2$-treated cells, $P > 0.05$; Fig. 2a). Also in Golgi apparatus, no differences in $[Ca^{2+}]_{Go}$ and inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} release were measured (281.33 ± 15.68 μM in untreated cells vs. 288.00 ± 14.09 μM in $MgCl_2$-treated cells, $P > 0.05$; Fig. 2b).

All these data confirm the ability of $MgCl_2$ treatment to perturb the intracellular Ca^{2+} homeostasis, without altering the mechanisms of intraluminal Ca^{2+}-filling and Ca^{2+} release of stores. This aspect was confirmed by experiments in which the cytosolic Ca^{2+}-response, evoked by perfusing cells with the SERCA pump inhibitor thapsigargin, was performed. In $MgCl_2$ treatment and in untreated cells, the inhibitor promoted a small transient increase in $[Ca^{2+}]_c$, due to release of Ca^{2+} from the ER, similar in both conditions (1.04 ± 0.03 μM in untreated cells vs. 1.01 ± 0.04 μM in $MgCl_2$-treated cells, $P > 0.05$). However, the $[Ca^{2+}]_c$ increase evoked by the inhibitor in $MgCl_2$-treated cells was followed by a slower declining phase (Fig. 3a). This suggests that increased cytosolic and mitochondrial Ca^{2+}-responses were sustained by an inhibitory effect of $MgCl_2$ on Ca^{2+}-extrusor system, resulting in increased height of the $[Ca^{2+}]$ transient, after agonist stimulation.

To reinforce the suggestion that $MgCl_2$ treatment acted as an inhibitor of the Ca^{2+}-extruding activity, we investigated whether $MgCl_2$ had any effect on the Ca^{2+}-influx and Ca^{2+}-efflux responses in HeLa. However, two different sets of observations militate against Ca^{2+}-influx perturbation. The first was the responses of erAEQ and GoAEQ measurements (Fig. 2a, b). During the first phase of ER and Golgi Ca^{2+}-refilling, the Ca^{2+}-uptake rates were the same in both experimental conditions.

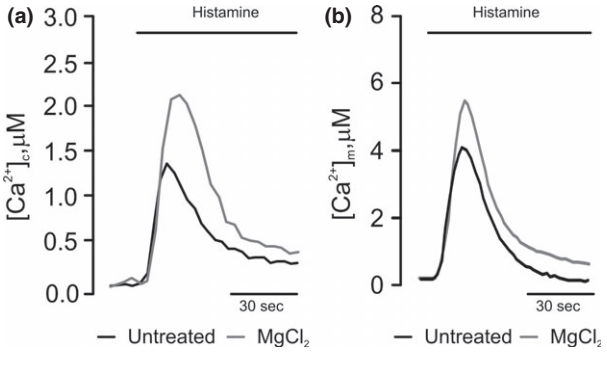

Figure 1 Intracellular Ca^{2+} in $MgCl_2$-treated HeLa cells. (a) Cytosolic, (b) mitochondrial representative Ca^{2+}-measurements performed in HeLa cells treated with $MgCl_2$. Where indicated, the cells were challenged with 100 μM histamine to induce IP_3-dependent store Ca^{2+} release

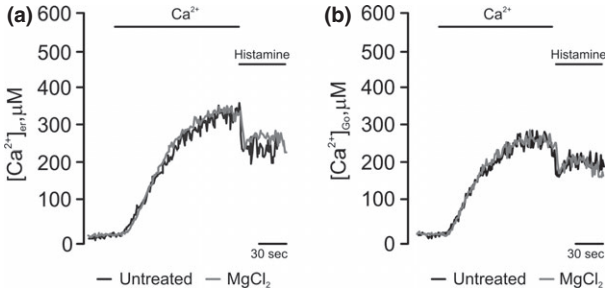

Figure 2 No effects on store Ca^{2+}-filling in $MgCl_2$-treated HeLa cells. (a) ER and (b) Golgi Ca^{2+}-measurements performed in HeLa cells treated with $MgCl_2$. Under the experimental conditions, the $[Ca^{2+}]_{ER}$ was <10 μM in Ca^{2+}-depleted cells, and it gradually increased upon switching the perfusion medium to a buffer supplemented with 1 mM Ca^{2+}. Where indicated the cells were challenged with 100 μM histamine to induce IP_3-dependent Ca^{2+} release

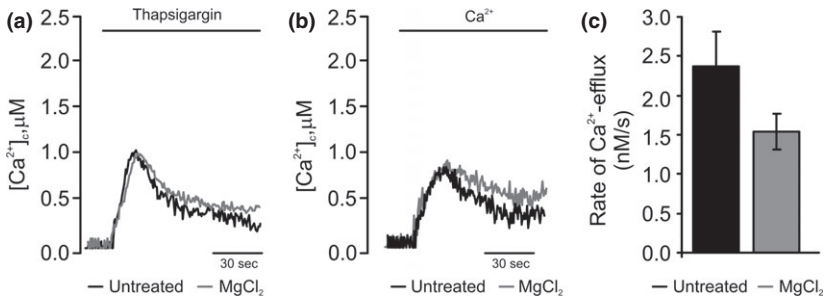

Figure 3 Inhibitory effect of $MgCl_2$ on the Ca^{2+}-extrusion system in HeLa cells. (a) Cytosolic Ca^{2+}-transients evoked by perfusion of the SERCA blocker thapsigargin in $MgCl_2$-treated and -untreated cells. (b) Effect of Ca^{2+}-filling (2 mM) on cells transfected with cytAEQ. This allows the estimation of the cytosolic Ca^{2+} concentration under condition of active Ca^{2+}-influx through plasma membrane channels before the cells have been pre-incubated with SERCA blocker thapsigargin to exclude the contribution of stores. (c) Rate of intracellular Ca^{2+}-extrusion of reduction in $[Ca^{2+}]_c$ in mock and $MgCl_2$-treated cells, expressed as ratio nM/sec

The second observation was the result of a series of experiments in which the kinetics of the cytosolic Ca^{2+}-response evoked by 2 mM EGTA-buffered $[Ca^{2+}]$ was analyzed, in pre-incubated thapsigargin (to exclude the store's contributions through the release of Ca^{2+} from the ER and Golgi), $MgCl_2$-treated and untreated HeLa cells (Fig. 3b). If $MgCl_2$ had potentiated the Ca^{2+}-influx activity, a higher effect on cytosolic Ca^{2+}-transient amplitude would have appeared after Ca^{2+}-perfusion. By contrast, if the target of the $MgCl_2$ was the Ca^{2+}-extrusion system, a more sustained cytosolic Ca^{2+}-transient has been observed in the cells treated. Figure 3b shows that this was indeed the case: the amplitude of the responses was the same in untreated and in $MgCl_2$-treated cells (1.03 ± 0.08 μM in untreated cells vs. 0.97 ± 0.02 μM in $MgCl_2$-treated cells, $P > 0.05$), but a significantly slower Ca^{2+}-extrusion rate was observed in cells treated with $MgCl_2$ (Fig. 3c; rate of Ca^{2+}-efflux: untreated 2.37 ± 0.44 nM/sec vs. $MgCl_2$-treated cells 1.54 ± 0.23 nM/sec).

Discussion

Hailey–Hailey disease, also known as familial benign chronic pemphigus, is a rare autosomal dominant inherited intraepidermal blistering genodermatosis, characterized by pruritic vesicles on an erythematous base that evolve into erosion-macerate, vegetating malodorous plaques in intertriginous regions, mainly the axillary and inguinal folds. Fingernail alterations when present are highly typical, while mucosal surface involvement, including the vulvo-vaginal area, is rare but possible. The disease, which typically occurs between the second and fourth decades of life, usually around puberty, has a chronic fluctuating course with remissions and relapses triggered by friction, stress, sweating, heat, moisture, superinfection, ultraviolet radiation, or tissue damage. Current treatments are not particularly effective.

The genetic abnormality of HHD lies in the ATP2C1 gene that is localized to chromosome regions 3q21–q24,[3,6–8] while the underlying pathological process is the loss of epidermal suprabasal cell–cell adhesion due to desmosomal dysfunction and consequent acantholysis.[9–13] ATP2C1 encodes the Golgi SPCA1; human keratinocytes use almost only this pump for loading the Golgi stores with Ca^{2+}. SPCA1 also plays a crucial role in setting up the cytosolic Ca^{2+} oscillations known to control many vital cell functions, through cycles of release and re-uptake of internal Ca^{2+} stores.[14,15]

It is well known that desmosome assembly and adhesion are Ca^{2+}-dependent processes.[4,16,17] The assembly of desmosomes has been studied *in vitro* in the presence of both high and low levels of extracellular Ca^{2+}. When mouse and human keratinocytes are grown in medium containing Ca^{2+} concentrations <0.1 mM, they do not form desmosomes.[18,19] In low Ca^{2+} conditions, desmosomes are not assembled, even if constituent proteins continue to be synthesized and stored in the ER, where they are folded and processed prior to transport to the cell membrane.[20,21] On the other hand, increasing Ca^{2+} concentration in the medium to >0.1 mM leads to characteristic morphological changes, such as cell–cell contacts within five minutes and desmosome formation within two hours.[18,19] Therefore, desmosomal proteins are gradually targeted to the cell membrane, where they form mature desmosomes.[20]

The mutations in SPCA1 found in HHD suggest that intracellular Ca^{2+} regulation is also important to desmosome assembly. A previous study revealed that the Ca^{2+} concentration in the basal layer of HHD lesions is lower than in the normal control skin, suggesting that the impaired intracellular Ca^{2+} metabolism plays an

important role in the formation of skin lesions in HHD.[22] The study of Ca^{2+} signaling in keratinocytes shows that an increase in Ca^{2+} in the culture medium results in the production of both diacylglycerol and IP3, which then trigger the release of Ca^{2+} from the intracellular stores into the cytoplasm and induce an increase in intracellular Ca^{2+} levels.[19,23] This finally results in the expression of several target genes, including those required for a proper assembly of desmosomal components and the arrangement of mature desmosomes. Because of the deficient Golgi Ca^{2+} pump, the restoration of the Golgi Ca^{2+} stores in HHD is slower than in normal keratinocytes[23,24]; regulation of cytoplasmic Ca^{2+} is impaired, and the adhesion of cultured keratinocytes obtained from patients affected with HHD is abnormal.

The results of this work showed that intracellular Ca^{2+} homeostasis changes after treatment with $MgCl_2$, and this could be associated with remission of clinical features of HHD in the patient observed. We have also demonstrated that $MgCl_2$ has an inhibitory effect on intracellular Ca^{2+}-extrusion machinery, favoring cytosolic and mitochondrial Ca^{2+} accumulation, as reported in Figure 1.

The Ca^{2+}-ATPases are key actors in the regulation of Ca^{2+} in eukaryotic cells, comprising three families of phosphorylation-type (P-type) that contribute to the correct functioning of cell machinery[25]: plasma-membrane Ca^{2+}-ATPases (PMCAs) that extrude Ca^{2+} out of the cell; and sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs) and SPCA that pump Ca^{2+} into the lumen of intracellular stores. The three pumps share the basic features of the catalytic mechanism but differ in a number of properties related to tissue distribution, regulation, and role in the cellular homeostasis of Ca^{2+}. All Ca^{2+} pumps are classified as P-type, because the energy required for generating ion gradients across the cellular membranes is derived from ATP by catalyzing hydrolysis in two Mg^{2+}-dependent steps.[26] Mg^{2+} promotes both the rate of phosphorylation and the conversion of E2 into E1.[27,28]

An important difference between PMCA and other P-type pumps is that PMCA is regulated by multiple cellular mechanisms. The Mg^{2+} ion regulates the activity of PMCA, and activity drops to <5% of maximum in the absence of Mg^{2+}, while at high intracellular Mg^{2+} concentration (>2 mM) it is inhibitory in human red blood cells.[29] The last consideration could justify our results, confirming that the elevated concentration of $MgCl_2$ (0.16 M) acts as an inhibitor of the Ca^{2+}-extruding activity of PMCA in HeLa cells.

As shown in Figure 3, we can conclude that the matured benefices could be attributed to a repair on Ca^{2+} homeostasis, notably to a major intracellular Ca^{2+}-disponibility, due to the reduced Ca^{2+}-extrusion rate. This favors the Ca^{2+}-dependent mechanism of desmosome assembly. Indeed, our data excluded the possibility that the effect of $MgCl_2$ was aimed at SPCA activity, as reported in Figure 2, where no perturbations in Golgi Ca^{2+}-filling were measured in both experimental conditions. This aspect is interesting; we could speculate on the versatility of therapeutic treatment, in this mode different genetic forms of HHD could be treated, regardless of mutations on the ATP2C1 gene. Based on our findings, $MgCl_2$ could be expected to be effective also in Darier disease, in which keratinocyte Ca^{2+} signaling is altered by genetic defects of SERCA2 pump.

To our knowledge, no previous cases of patients with HHD successfully treated with $MgCl_2$ have been reported. Wider studies are needed in order to better assess efficacy, safety, and prescription modalities of $MgCl_2$ in HHD; molecular analyses on affected keratinocytes should also be carried out to confirm our experimental results.

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