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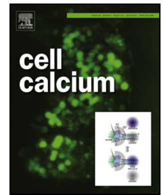

Bax Inhibitor-1-mediated Ca^{2+} leak is decreased by cytosolic acidosis

Santeri Kiviluoto[a], Tomas Luyten[a], Lars Schneider[b], Dmitrij Lisak[b], Diego Rojas-Rivera[c], Kirsten Welkenhuyzen[a], Ludwig Missaen[a], Humbert De Smedt[a], Jan B. Parys[a], Claudio Hetz[c], Axel Methner[b], Geert Bultynck[a,*]

[a] Laboratory of Molecular and Cellular Signaling, Department of Cellular and Molecular Medicine, KU Leuven, Campus Gasthuisberg O/N-I, Herestraat 49 – box 802, BE-3000 Leuven, Belgium

[b] Focus Program Translational Neuroscience (FTN), Rhine Main Neuroscience Network (rmn[2]), Johannes Gutenberg University Medical Center Mainz, Department of Neurology, Langenbeckstr. 1, D-55131 Mainz, Germany

[c] Biomedical Neuroscience Institute, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, P.O. Box 70086, Santiago, Chile

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ABSTRACT

Bax Inhibitor-1 (BI-1) is an evolutionarily conserved six-transmembrane domain endoplasmic reticulum (ER)-localized protein that protects against ER stress-induced apoptotic cell death. This function is closely connected to its ability to lower steady-state ER Ca^{2+} levels. Recently, we elucidated BI-1's Ca^{2+}-channel pore in the C-terminal part of the protein and identified the critical amino acids of its pore. Based on these insights, a Ca^{2+}-channel pore-dead mutant BI-1 (BI-1^{D213R}) was developed. We determined whether BI-1 behaves as a *bona fide* H^+/Ca^{2+} antiporter or as an ER Ca^{2+}-leak channel by investigating the effect of pH on unidirectional Ca^{2+}-efflux rates. At pH 6.8, wild-type BI-1 expression in BI-1$^{-/-}$ cells increased the ER Ca^{2+}-leak rate, correlating with its localization in the ER compartment. In contrast, BI-1^{D231R} expression in BI-1$^{-/-}$, despite its ER localization, did not increase the ER Ca^{2+}-leak rate. However, at pH < 6.8, the BI-1-mediated ER Ca^{2+} leak was blocked. Finally, a peptide representing the Ca^{2+}-channel pore of BI-1 promoting Ca^{2+} flux from the ER was used. Lowering the pH from 6.8 to 6.0 completely abolished the ability of the BI-1 peptide to mediate Ca^{2+} flux from the ER. We propose that this pH dependence is due to two aspartic acid residues critical for the function of the Ca^{2+}-channel pore and located in the ER membrane-dipping domain, which facilitates the protonation of these residues.

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1. Introduction

Intracellular Ca^{2+} signals originating from the endoplasmic reticulum (ER) critically control cell-fate decisions [1,2]. In particular, a lowering of the ER Ca^{2+}-store content has been implicated as a protective cellular mechanism against apoptosis [3,4]. Recently, a family of transmembrane Bax Inhibitor-1 (BI-1)-containing motif proteins (TMBIM) emerged as conserved and ancient cell-death suppressors by affecting ER-Ca^{2+} homeostasis and dynamics [5–13].

BI-1, the founding member of this protein family, protects against ER stress-induced apoptosis [5,14] and the underlying molecular mechanisms have been explored in more detail [6,15–17]. BI-1 contains a pH-sensing domain in its last 9 amino acids [6]. However, this domain also serves other functions [18]. Consistent with the presence of this pH-sensing domain, BI-1 was proposed to function as a H^+/Ca^{2+} antiporter, in which extraluminal protons are exchanged for intraluminal Ca^{2+} ions [19]. As a result, a reduction in pH was found to increase BI-1-mediated lowering of the ER Ca^{2+} content. This process was further influenced by cardiolipin, phosphatidylserine, the BH4 domain of Bcl-2 and chemotherapeutic drugs [20,21]. Recently, we described that the C-terminal domain of BI-1 contains a Ca^{2+}-permeable channel pore that permeates Ca^{2+} through ER membranes and interacts with IP_3R channels to sensitize them to low levels of IP_3 [10]. Both mechanisms contribute to BI-1's ability to lower the steady-state ER Ca^{2+} levels and to preserve mitochondrial bio-energetics and survival processes like autophagy [8]. However, other reports showed that BI-1 negatively regulates autophagy [22] similarly to anti-apoptotic Bcl-2 family members [23,24]. Importantly, the domain harboring the BI-1 Ca^{2+}-channel pore (a.a. 198–217) is located upstream of a proposed pH-sensing domain and dips into the ER membrane [9]. This structure is supported by a recent study of Smith and co-workers, who came to the same conclusion that the topology of BI-1 fits a six-transmembrane domain protein with a C-terminal loop domain [12]. We identified the negatively charged residues that are critical for BI-1's potency to lower the ER Ca^{2+}-store content [9]. Mutating one Asp residue in full-length BI-1 (BI-1^{D213R}) is

* Corresponding author. Tel.: +32 16 330215.
E-mail address: geert.bultynck@med.kuleuven.be (G. Bultynck).

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sufficient to prevent this action of BI-1, indicating a critical role for negatively charged residues in the membrane–dipping part of the C-terminus to permeate Ca^{2+}.

In our present study, we further explored the Ca^{2+}-flux properties of BI-1 in native ER membranes in response to changes in pH. We discovered that the BI-1 dependent Ca^{2+} leak from the ER is reduced by acidic pH, indicating a pore mechanism that is regulated during cytosolic acidosis.

2. Materials and methods

2.1. Generation of BI-1-deficient MEF cells expressing empty vector, wild-type BI-1 and BI-1^{D213R}

These cell lines were described previously [10]. In short, BI-1$^{-/-}$ MEF cells were used for the stable and ectopic expression of empty vector (RFP only), wild-type human BI-1 or human BI-1^{D213R} with a bicistronic N-terminal RFP-IRES sequence. The RFP was used as a probe to select and enrich transfected cells expressing these vectors using fluorescence-activated cell sorting. These cells therefore express BI-1 or BI-1^{D213R} as untagged proteins in a BI-1-deficient background.

2.2. Constructs

The pcDNA3-BI-1 construct was prepared by ligation of an mCherry into the C-terminus of a human BI-1. BI-1^{D213R}-mCherry was then created from the wild-type BI-1 by site-directed mutagenesis (Stratagene). Constructs used for labeling specific organelles: Cameleon-D1ER (a kind gift of Dr. Roger Tsien, UCSD, San Diego), pECFP-LAMP1 (a kind gift of Dr. Haoxing Xu, University of Michigan, Ann Arbor) and GFP-LC3 (a kind gift of Dr. J. Debnath, UCSF, San Fransisco).

2.3. Western blot

Five million MEF cells were lysed in RIPA buffer and 30 μg of total protein was loaded on Tris-Acetate gels (Invitrogen) in the presence of urea sample buffer [9]. The proteins were transferred on a PVDF membrane and blotted for IP_3R1 [25], IP_3R3 (BD Biosciences), SERCA2b [26] or non-muscle myosin IIA (Abcam) for loading control. Images were acquired with Bio-Rad Chemidoc MP and the quantity of each protein quantified with Image Lab software.

2.4. Confocal imaging

Hela cells were seeded on borosilicate chamber slides and cotransfected with 0.25 μg DNA for pcDNA3 (mock), wtBI-1 or BI-1^{D213R} and organelle marker constructs using Fugene 6 (Promega). The next day, confocal images of the cells were acquired with the Zeiss LSM510 confocal microscope and a 63×/1.4NA oil immersion objective.

2.5. Verification of human BI-1 or BI-1^{D213R} expression in BI-1$^{-/-}$ MEF cells

RNA was isolated from the cells using Trizol RNA isolation kit (Invitrogen), followed by cDNA synthesis (Applied Biosystems). For detecting mRNA expression in the samples, PCR was

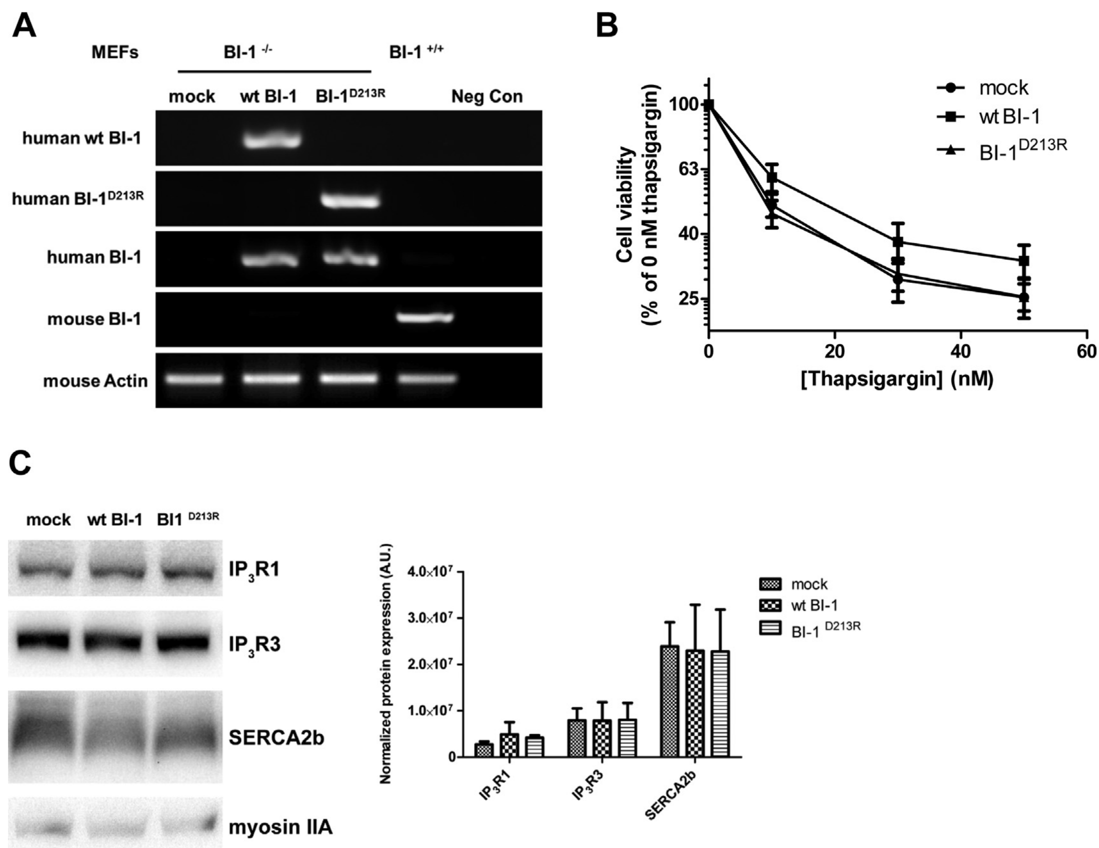

Fig. 1. Validation of the BI-1$^{-/-}$ MEF cells overexpressing empty vector (mock), wild-type BI-1 (wt BI-1) or BI-1^{D213R}. (A) RT-PCR validation of BI-1$^{-/-}$ MEF cells overexpressing empty vector (mock), wild-type BI-1 (wt BI-1) or BI-1^{D213R}. (B) Assessment of the survival of empty vector (mock), wt BI-1- and BI-1^{D213R}-expressing BI-1$^{-/-}$ MEF cells in the presence of different [thapsigargin]. Cell death was quantitated by the cell-titer blue assay. (C) Protein levels of IP_3R1, IP_3R3 and SERCA2b in BI-1 MEF cells normalized to non-muscle myosin IIA with mean ±SD of 3 independent experiments.

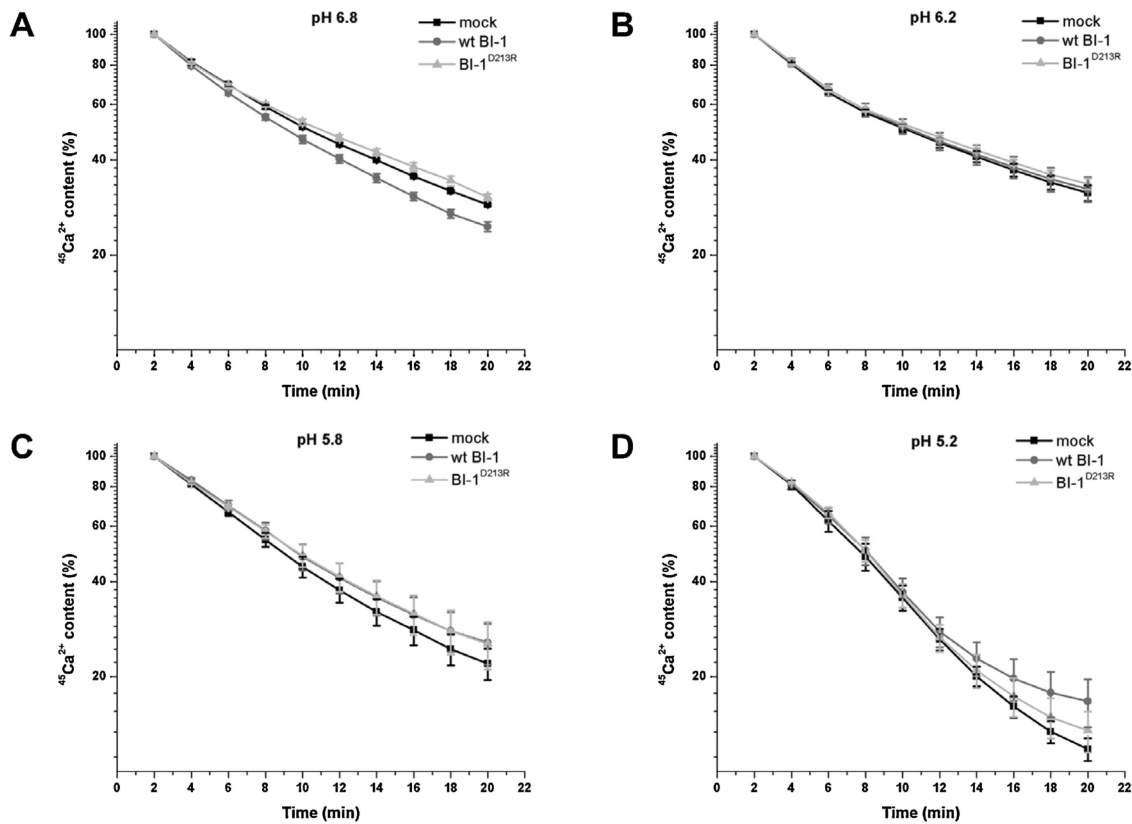

Fig. 2. Overexpression of BI-1, but not BI-1^{D213R}, enhances the Ca^{2+}-leak from the ER of BI-1$^{-/-}$ MEF cells at pH 6.8, but not below pH 6.2. After loading the ER Ca^{2+} stores to steady-state levels with $^{45}Ca^{2+}$ at pH 6.8, unidirectional $^{45}Ca^{2+}$ leak was triggered by washing the cells in a Ca^{2+}-free efflux medium in the presence of 2 μM thapsigargin at pH 6.8 (A), pH 6.2 (B), pH 5.8 (C) or pH 5.2 (D). The data were plotted as the $^{45}Ca^{2+}$-store content as a function of time. The mean ±SEM of 3–5 independent experiments is shown.

performed using primers specific for wild-type human BI-1 mRNA (5′-ATTAGCAATGATCTTTACCTGCTTCA-3′ and 5′-ATTAGAGTTTTCT GAAGACAGTAATGAAATC-3′), human BI-1^{D213R} mRNA (5′-ATTAGCAATGATCTTTACCTGCTTCA-3′ and 5′-ATTATTCTGAAGACAGTAATGAACCG-3′), or both wild-type human BI-1 and BI-1^{D213R} mRNAs (5′-ATTAGCAATGATCTTTACCTGCTTCA-3′ and 5′-ATTACATGGCCAGGATCATCATG-3′). Endogenous mouse BI-1 mRNA (5′-AAGGCCAGGATGAACATGAG-3′ and 5′-GACCGAGCAAAAGAGACTGG-3′) and beta-actin mRNA (5′-TACCACCATGTACCCAGGCA-3′ and 5′-CTCAGGAGGAGCAATGATCTTGAT-3′) were also assayed. The amplicons were detected in a 2% agarose/Tris-acetate-EDTA (TAE) gel.

2.6. Cell-death assays

Cell-death assays were performed exactly as described before [10]. In short, cell death was determined using Sytox green (Invitrogen) in the presence of different thapsigargin concentrations. After incubation with thapsigargin for 48 h, the cells were analyzed by high-content imaging using a BD Pathway 855 microscope. Data were obtained from 4 independent experiments using 3 replicates per experiment. The data were normalized to the untreated cells, which were set as 100%. The corresponding values were represented as mean ± S.E.M. and * indicates $p < 0.05$.

2.7. $^{45}Ca^{2+}$-flux assays

$^{45}Ca^{2+}$-flux assays were performed in plasma membrane-permeabilized MEF cells grown for 6 days to confluent monolayers in gelatin-coated 12-well plates. Only cell layers grown to nearly the same density were analyzed for their ER Ca^{2+}-leak rate. The assays were performed as previously described [25,27], except that different buffers were used for proper pH control in the efflux medium (imidazole for pH 6.8 and 6.2; MES for pH 5.8 and acetate for pH 5.2). In the experiments in which a peptide corresponding to the C-terminal Ca^{2+}-channel pore of BI-1 was used, the peptide (CTP1) was added acutely at a concentration of 40 μM, well above the EC_{50} for provoking a Ca^{2+} flux (32 μM, [9]). Purity (>80%) was verified by mass spectrometry (ThermoFisher, Germany).

3. Results

3.1. BI-1, but not BI-1^{D213R}, overexpression in BI-1$^{-/-}$ MEFs leads to increased ER Ca^{2+} leak and protection against apoptosis

To obtain a suitable model system to study the Ca^{2+}-leak properties of BI-1, we reconstituted BI-1$^{-/-}$ MEFs with empty vector, wild-type BI-1 or BI–1^{D213R}. The lack of mouse BI-1 expression and re-expression of either human wild-type BI-1 or human BI-1^{D213R} was confirmed using RT-PCR (Fig. 1A). In addition, we validated that BI-1 overexpression protects against thapsigargin-induced apoptosis. Elaborating our previous findings [10], we found that BI-1 overexpression in BI-1$^{-/-}$ MEFs enhanced cell survival upon prolonged exposure to a range of [thapsigargin], while this was not the case for the cells overexpressing BI-1^{D213R} (Fig. 1B).

To exclude that BI-1 indirectly accelerates the ER Ca^{2+}-leak rate by altering the expression level of other ER-Ca^{2+}-transport mechanisms, we assessed the protein levels of IP_3R1, IP_3R3 and the sarco/endoplasmic-reticulum Ca^{2+} ATPase SERCA2b, the predominant isoform in MEF cells using a quantitative immunoblot analysis. This demonstrated that the expression of IP_3Rs and SERCA2b was

similar in all cell lines used in this study and is therefore not involved in the observed Ca^{2+} leak (Fig. 1C).

We then compared the Ca^{2+}-leak rate from the ER of these cells using unidirectional $^{45}Ca^{2+}$-flux assays in permeabilized cells (Fig. 2A). This approach allows a very accurate analysis of the Ca^{2+}-leak rate of the ER in the absence of ER Ca^{2+} uptake by SERCAs, mitochondrial Ca^{2+} fluxes and plasma-membrane Ca^{2+} fluxes. We also chose to overexpress human BI-1 in a BI-1-knockout background to avoid confounding Ca^{2+} fluxes through endogenous BI-1. Thus, this system provided a unique manner to study Ca^{2+}-flux properties of ectopic BI-1.

Direct comparison of the unidirectional Ca^{2+} leak from the ER was assayed for vector-, BI-1 and BI-1^{D213R}-expressing BI-$1^{-/-}$ MEF cells at pH 6.8, which resembles the physiological intracellular pH [28] (Fig. 2A). Overexpression of BI-1 enhanced the unidirectional ER Ca^{2+}-leak rate in comparison to empty vector-expressing cells. Overexpression of BI-1^{D213R} did not stimulate the unidirectional ER Ca^{2+}-leak rate and did not protect against cell death triggered by ER Ca^{2+}-store depletion using thapsigargin [9], suggesting that the two observations are correlated.

Collectively, these data indicate that BI-1 promotes ER Ca^{2+} leak by functioning as an ER Ca^{2+}-leak channel, which is critical for its anti-apoptotic function.

3.2. *Lowering the pH decreases the BI-1-mediated Ca^{2+} leak from the ER*

To assess whether the Ca^{2+}-flux property of BI-1 changes with pH, we lowered the pH of the efflux medium and compared the BI-1-dependent ER Ca^{2+} leak in the BI-$1^{-/-}$ MEF cells expressing either empty vector, wild-type BI-1 or BI-1^{D213R} (Fig. 2A–D). Interestingly, the ER Ca^{2+} leak specifically mediated by wild-type BI-1 was decreased by lowering the pH of the efflux medium. Strikingly, while at pH 6.8 wild-type BI-1, but not BI-1^{D213R}, expression significantly enhanced Ca^{2+} leak from the ER (Fig. 2A), there were no significant differences between the three cell lines at pH 6.2 (Fig. 2B). Similar findings were observed at pH 5.8 and pH 5.2 (Fig. 2C and D). However, the Ca^{2+} flux from the ER was increased in BI-$1^{-/-}$ MEF cells by cytosolic acidosis, indicating the presence of a BI-1-independent Ca^{2+}-leak pathway that is enhanced at low pH.

Previous reports have shown that BI-1 is able to act as a stimulator of V-ATPase, suggesting that BI-1 is expressed at lysosomal membranes. To investigate the subcellular localization of BI-1 further, we tested seven commercially available antibodies (shown in a supplemental figure) and two custom-made antisera (not shown) for their ability to stain over-expressed and native BI-1 using immunoblots and immunohistochemistry. In our hands, none of these antibodies were able to recognize BI-1. We therefore chose to clone a mCherry-BI1 expression vector and to cotransfect Hela cells with fluorescently tagged markers for ER (D1ER), autophagosomes (GFP-LC3) and lysosomes (GFP-LAMP1) and mCherry-BI-1. The next day, expression patterns of the proteins were imaged using confocal microscopy. Results show that BI-1 was abundantly coexpressed with D1ER, a marker for ER, but not with GFP-LC3 (autophagosomes) or GFP-LAMP (lysosomes) (Fig. 3). Together, this indicates that the effect of pH on BI-1 dependent Ca^{2+} leak is exclusively from BI-1 localized at the ER.

3.3. *Ca^{2+} flux through the BI-1 Ca^{2+}-channel pore decreases by lowering the pH*

We recently identified a 20-amino acid peptide corresponding to residues 198–217 of human BI-1 (CTP1) as being responsible and sufficient to cause ER Ca^{2+} release, indicating that the C-terminus of BI-1 harbors the Ca^{2+}-channel pore [9]. Adding this peptide to ER

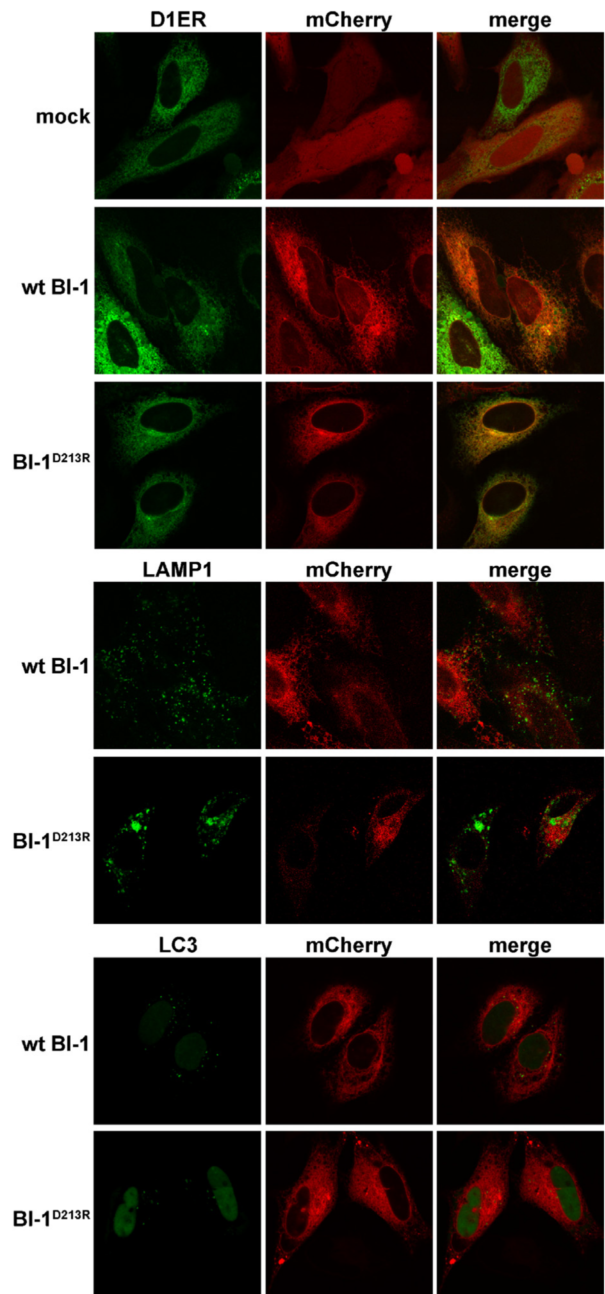

Fig. 3. BI-1 predominantly localizes to the ER. Wild-type (wt) BI-1 or BI-1^{D213R}-mCherry constructs were co-expressed for 24 h together with markers for ER (Cameleon-D1ER), lysosomes (LAMP1-CFP) or autophagosomes (GFP-LC3). Representative confocal images demonstrate that both wtBI-1 and BI-1^{D213R} localize to the ER.

membranes causes a direct release of Ca^{2+} [9]. We therefore aimed to examine whether the Ca^{2+} flux mediated by the Ca^{2+}-channel pore domain of BI-1 is directly affected by pH (Fig. 4A and B). Interestingly, we found that lowering the pH below 6.8 decreased the Ca^{2+} flux through the isolated Ca^{2+}-channel pore of BI-1. At pH 6.0, Ca^{2+} flux through the BI-1 Ca^{2+}-channel pore was completely blocked (Fig. 4B). In addition, we observed a decreased Ca^{2+}-flux in alkaline conditions, suggesting that the CTP1 peptide function is optimal in physiological steady-state pH (Fig. 4C and D). These data correlate with the results obtained using full-length BI-1, which show that lowering pH from 6.8 to a value around pH 6.0 blocks the difference between ER Ca^{2+} leak mediated by wild-type BI-1 and BI-1^{D213R} (Fig. 2A and B).

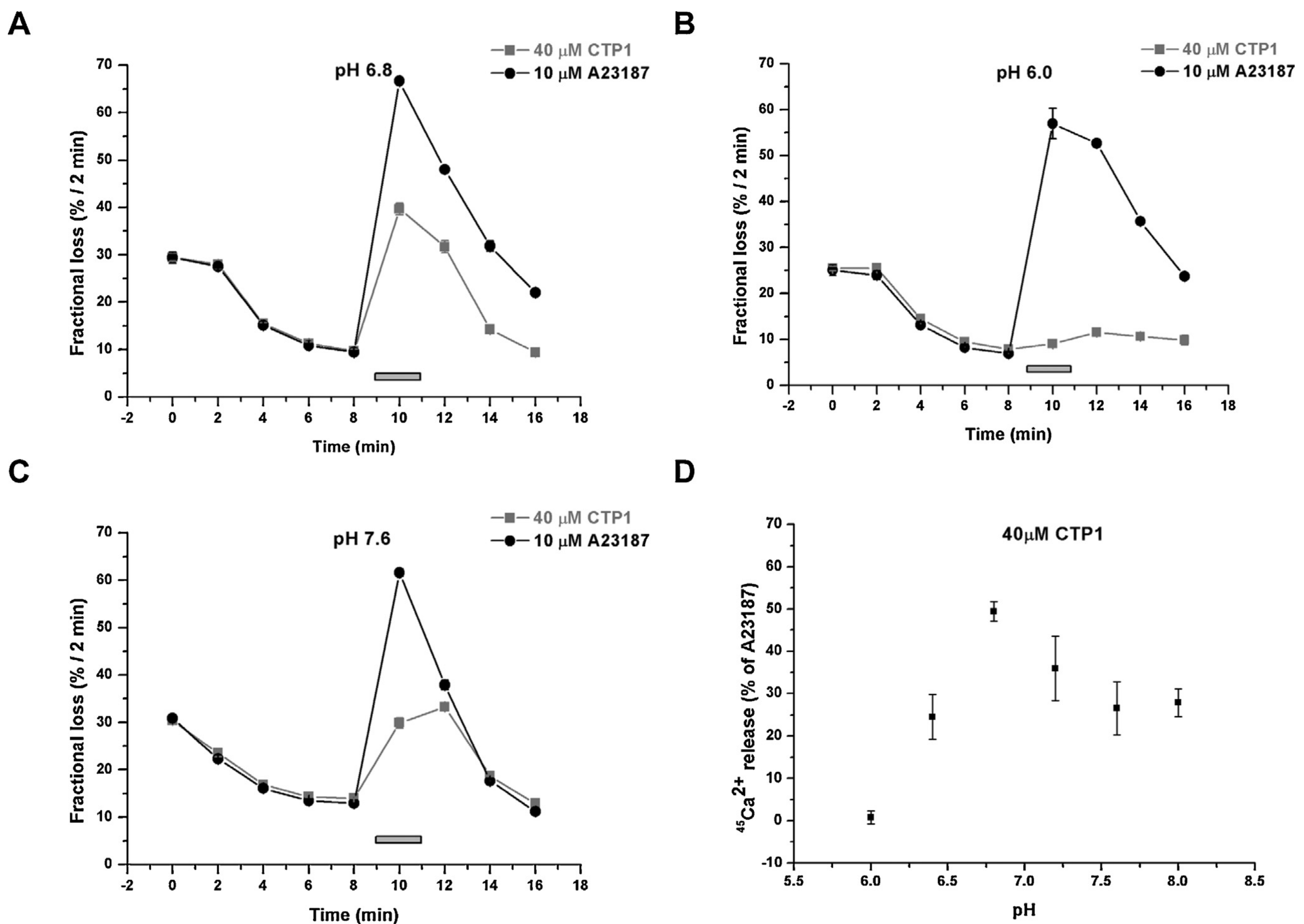

Fig. 4. A peptide corresponding to BI-1's Ca^{2+}-channel pore is less efficient to provoke ER Ca^{2+}-release at pH values lower than 6.8. (A–C) ER Ca^{2+} release provoked by 40 μM of a peptide corresponding to the C-terminal Ca^{2+}-channel pore of BI-1 (a.a. 198–217; CTP1) at different pH. The data were plotted as fractional loss, which is the amount of Ca^{2+}-release as a function of time, divided by the store content at that time point. A23187 (10 μM) was used to estimate the total amount of releasable $^{45}Ca^{2+}$ from the ER. The gray bar indicates the addition of the peptide or A23187 (A–C). A typical experiment obtained from duplicate replicates is shown as mean ±SD. *D*, CTP1-mediated Ca^{2+} release calculated as % of the A23187-releasable $^{45}Ca^{2+}$, was plotted as function of pH. The graph represents the mean values ±SEM of 3 independent experiments.

4. Discussion

The findings of this study indicate that (i) ectopic BI-1 expression enhances Ca^{2+} flux from the ER into the cytosol at pH 6.8, (ii) Ca^{2+} flux through the full-length BI-1 channel or through the isolated Ca^{2+}-channel pore of BI-1 is efficient at the physiological pH of 6.8 and decreases by mild cytosolic acidosis. Finally, (iii) the Ca^{2+} flux from the ER is increased in BI-$1^{-/-}$ MEF cells by cytosolic acidosis, indicating the presence of a BI-1-independent Ca^{2+}-leak pathway that is enhanced at low pH, predominantly at pH values below 6.0.

The dynamics of intracellular Ca^{2+} during steady-state and cellular stress are finely tuned. During unstressed conditions, BI-1 regulates the slow release of ER-Ca^{2+} and promotes cell survival by lowering steady-state Ca^{2+} levels via its pore domain [9] and by sensitizing IP_3Rs [10]. Conversely, strong and prolonged ER Ca^{2+} efflux results in ER stress and cell death, a condition associated with many pathological conditions [29,30]. In the case of BI-1, it is specifically the Ca^{2+} pore property of BI-1 which is able to prevent mitochondrial Ca^{2+}-overload and cell death (Fig. 1B and [10]). In pathological conditions such as ischemia-reperfusion injury, intracellular acidification can lead to increased ER-Ca^{2+} leak and subsequent induction of ER stress response [29,31]. Cells then respond by blocking ER-Ca^{2+} leak via BI-1 to maintain the oxidation state of the ER and to avoid potentially damaging, excessive Ca^{2+}-release to the cytosol. Results presented here follow the same reasoning as for chaperone GRP78, which blocks Ca^{2+}-leak through the translocon complex, another leak pathway of the ER [32,33]. Similarly, during the early phase of ER stress, GRP78 is displaced by ERp44 to reduce IP_3-induced Ca^{2+} release by IP_3R1s [30,34,35].

Previous reports have shown that cytosolic acidification increases a BI-1 mediated Ca^{2+} leak, presumably due to increased oligomerization of BI-1 [6,20]. We do not exclude that the pH may also affect other properties of BI-1, such as oligomerization, which has been observed under conditions of long exposure to low pH [6]. Yet, it is important to note that a recent report showed that Ca^{2+} flux through viral Golgi anti–apoptotic protein (vGAAP), another BI-1-family member, was independent of its oligomerization state [13]. Although we could not detect BI-1 expression outside the ER (Fig. 3), we do acknowledge the possibility of BI-1 expression in lysosomes and the Golgi apparatus where it can elicit other functions unrelated to the CTP1 domain of BI-1. Furthermore, we also observed an increased Ca^{2+}-leak from the ER in acid conditions, but this leak was independent of BI-1, suggesting that there are other pH-dependent elements of the ER membrane that increase its permeability to Ca^{2+}.

In the present study, we opted to assay the Ca^{2+} release property of BI-1 in a null background (BI-$1^{-/-}$), comparing untagged, reconstituted, full-length wild-type BI-1 or BI-1^{D213R} mutant. Given that the C-terminus of BI-1 acts as a hot spot for many interacting proteins [17,18,36] and harbors the proposed pH sensor [6], our approach allowed us to isolate and study the function of the Ca^{2+} pore domain of the native BI–1 protein in detail. Finally, our assay

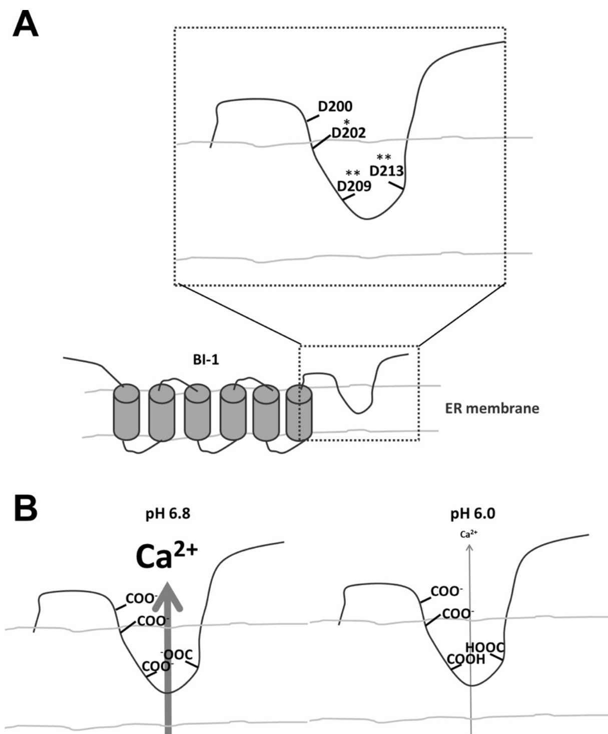

Fig. 5. A schematic model of BI-1, showing the 6 transmembrane regions and the C-terminal ER-membrane-dipping domain forming the Ca^{2+}-channel pore. (A) The aspartic acid residues (Asp202, Asp209 and Asp213) involved in establishing the Ca^{2+}-channel pore are indicated. We propose a Ca^{2+}-recruiting function for Asp202 (*) at the mouth of the pore. Asp209 and Asp213 (**) are presumably part of the Ca^{2+}-channel pore, where they might be highly susceptible to alterations in pH, allowing their protonation and the loss of negatively charged side chains, which are essential for Ca^{2+} flux through the pore. For clarity reasons, the one-letter amino-acid code was used in the figure. (B) At pH 6.8, the acidic side chains of the aspartic-acid residues in the Ca^{2+}-channel pore of BI-1 are negatively charged, allowing the ligation and permeation of Ca^{2+}. At pH 6.0, the acidic side chains of the aspartic acid residues of the Ca^{2+}-channel pore of BI-1 present in the ER membrane-dipping domain will become protonated, which might prevent proper binding and permeation of Ca^{2+}.

is designed to measure acute effect of pH (0–20 min after acidification) specifically in intact ER of permeabilized cells, whereas other studies have focused on pH-dependent effects 30 min after altering pH in microsomal preparations or proteoliposomes [6,19,20].

We propose a model where BI-1 elicits ER-Ca^{2+}-leak which is acutely blocked by a decrease in cytosolic pH. This causes the protonation of Asp209 and Asp213 in the membrane-dipping part of the Ca^{2+} pore channel of BI-1 (Fig. 5). The most likely reason for this is that the dielectric constant for Asp209 and Asp213 is considerably smaller than that for the two Asp residues present in the cytosolic aqueous part of the protein (Asp200 and Asp202). As a consequence, the weakly acidic carboxylate groups in the Asp side chain will be more easily protonated in the lipid environment. Relatively mild cytosolic acidosis (pH 6.2) is able to decrease the Ca^{2+} -leak properties of BI-1, suggesting that protonation of Asp209 and Asp213 in the membrane fraction of the pore is sufficient to block Ca^{2+}permeation. This is in line with findings described for the pH-dependence of L-type voltage-operated Ca^{2+} channels [37].

To conclude, we have shown that the effect of BI-1 as a regulator of ER Ca^{2+} homeostasis is regulated by intracellular pH by removing negative charges in the proposed Ca^{2+}-channel pore essential for Ca^{2+} flux. The relevance of the pH-dependent shutdown of BI-1-mediated Ca^{2+} flux for the regulation of cellular stress responses ought to be further scrutinized.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2013.06.002.

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