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The role of calcium in VDAC1 oligomerization and mitochondria-mediated apoptosis☆

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ABSTRACT

The voltage-dependent anion channel (VDAC), located at the outer mitochondria membrane (OMM), mediates interactions between mitochondria and other parts of the cell by transporting anions, cations, ATP, Ca^{2+}, and metabolites. Substantial evidence points to VDAC1 as being a key player in apoptosis, regulating the release of apoptogenic proteins from mitochondria, such as cytochrome *c*, and interacting with anti-apoptotic proteins. Recently, we demonstrated that VDAC1 oligomerization is a general mechanism common to numerous apoptogens acting via different initiating cascades and proposed that a protein-conducting channel formed within a VDAC1 homo/hetero oligomer mediates cytochrome *c* release. However, the molecular mechanism responsible for VDAC1 oligomerization remains unclear. Several studies have shown that mitochondrial Ca^{2+} is involved in apoptosis induction and that VDAC1 possesses Ca^{2+}-binding sites and mediates Ca^{2+} transport across the OMM. Here, the relationship between the cellular Ca^{2+} level, $[Ca^{2+}]_i$, VDAC1 oligomerization and apoptosis was studied. Decreasing $[Ca^{2+}]_i$ using the cell-permeable Ca^{2+} chelating reagent BAPTA-AM was found to inhibit VDAC1 oligomerization and apoptosis, while increasing $[Ca^{2+}]_i$ using Ca^{2+} ionophore resulted in VDAC1 oligomerization and apoptosis induction in the absence of apoptotic stimuli. Moreover, induction of apoptosis elevated $[Ca^{2+}]_i$, concomitantly with VDAC1 oligomerization. AzRu-mediated inhibition of mitochondrial Ca^{2+} transport decreased VDAC1 oligomerization, suggesting that mitochondrial Ca^{2+} is required for VDAC1 oligomerization. In addition, increased $[Ca^{2+}]_i$ levels up-regulate VDAC1 expression. These results suggest that Ca^{2+} promotes VDAC1 oligomerization via activation of a yet unknown signaling pathway or by increasing VDAC1 expression, leading to apoptosis. This article is part of a Special Issue entitled: 12th European Symposium on Calcium.

1. Introduction

Apart from their metabolic and apoptotic roles, mitochondria sequester Ca^{2+} at the expense of energy [1]. Mitochondria thus serve as a major hub of cellular Ca^{2+} homeostasis, fundamental for a wide range of cellular activities, such as control of oxidative phosphorylation [2,3], cell death [4–6] and secretion [7].

To regulate cytosolic and mitochondrial Ca^{2+} concentrations, mitochondria are endowed with multiple Ca^{2+} transport mechanisms located in the inner mitochondrial membrane (IMM) that mediates the uptake and release of Ca^{2+} [1]. These include the mitochondrial Ca^{2+} uniporter (MCU) [8,9], as well as its regulatory protein, the EF hand-containing protein termed MICU1 (for mitochondrial calcium uptake 1) [10]. Letm1 (leucine-zipper-EF hand-containing transmembrane region) was proposed to function in mitochondrial Ca^{2+}/H^+ exchange [11]. However, other functions, such as in mitochondrial K^+ homeostasis, have been proposed such that the contribution of Letm1 to Ca^{2+} transport has been questioned [12]. The Na^+/Ca^{2+} exchanger superfamily member NCLX serves as the major agent of Ca^{2+} efflux [13]. While all these Ca^{2+} transport systems mediate the transport of Ca^{2+} across the inner mitochondrial membrane (IMM), Ca^{2+} must first cross the outer mitochondrial membrane (OMM) before being transported across the IMM. To date, the only identified protein mediating Ca^{2+} transport in the OMM is the voltage-dependent anion channel 1 (VDAC1) [14]. Accordingly, VDAC1 is permeable to Ca^{2+} [14–16] and possesses Ca^{2+}-binding sites [14,17,18].

Located in the OMM, VDAC1 assumes a crucial position in the cell, serving as the main interface between mitochondrial and cellular metabolisms, controlling cross-talk between mitochondria and the rest of the cell [19]. VDAC1 serves as a controlled passage for anions, Ca^{2+} and other cations, adenine nucleotides and other metabolites into and out of mitochondria, thus playing a crucial role in regulating the metabolic and energetic functions of mitochondria. In addition, VDAC1 functions as an anchor point for mitochondria-interacting proteins [19] and is also recognized as a key protein in mitochondria-mediated apoptosis, participating in the release of apoptotic proteins and interacting with anti-apoptotic proteins [19].

Abbreviations: Cyto *c*, cytochrome *c*; EGS, ethylene glycol bis[succinimidylsuccinate]; OMM, outer mitochondrial membrane; PLB, planar lipid bilayer; RuR, Ruthenium red; STS, staurosporine; VDAC, voltage-dependent anion channel

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VDAC1 is highly Ca^{2+}-permeable and allows Ca^{2+} access to the mitochondrial inter-membrane space [14]. Permeability of the VDAC1 channel to Ca^{2+} was demonstrated upon reconstitution of the purified protein into a planar lipid bilayer [14,16]. In addition, La^{3+} [14], ruthenium red and AzRu [14,17,20], all of which compete with Ca^{2+} for binding sites in various proteins, are capable of inhibiting VDAC1-mediated Ca^{2+} conductivity in the lipid bilayer-reconstituted system. Finally, it was shown that Ca^{2+} regulates the uptake of Ca^{2+} into VDAC1-reconstituted liposomes [21] and that over-expression of VDAC1 in HeLa cells and skeletal myotubes enhances the transfer of Ca^{2+} into mitochondria [15].

Several lines of evidence suggest that VDAC1 possesses Ca^{2+}-binding site(s) [14,17,18]. Firstly, RuR [14], ruthenium amine binuclear complex (Ru360) [22] and AzRu, a recently-synthesized photoactivatable reagent [20], all able to specifically interact with several Ca^{2+}-binding proteins, decrease VDAC1 channel conductance in a time-dependent manner and stabilize the channel in the closed state. Such decrease in conductance can be prevented by Ca^{2+} [23], strongly suggesting that RuR and Ca^{2+} share a common binding site(s) or recognize the same VDAC1 conformation. Likewise, AzRu was also found to interact with VDAC1 and decrease channel conductance, an effect that was prevented by Ca^{2+} but not by Mg^{2+}, again suggesting interaction of AzRu with the VDAC1 Ca^{2+}-binding site(s) or a defined protein conformation [23]. RuR [17,24], like AzRu [23], had no effect on mutated E72Q- or E202Q-VDAC1 channel conductance, with [^{103}Ru]AzRu labeling native but not mutated VDAC1, suggesting that mutation of these residues stabilizes a VDAC1 conformation that doesn't bind RuR and AzRu, or alternatively, that their interaction with this conformation does not modify VDAC1 conductance.

RuR was found to protect against cell death induced by various means [24–28]. Furthermore, RuR and AzRu protected against apoptosis induced in T-REx-293 cells expressing native but not E72Q- or E202Q-mutated VDAC1 [23,24]. RuR did not interact with E72Q-VDAC1 to reduce its channel activity or protect against apoptosis in cells expressing this mutant [17]. RuR- and AzRu-mediated protection against cell death, as induced by several apoptotic stimuli [24–29], may arise from interaction with a VDAC1 Ca^{2+}-binding site or with a specific protein conformation or by inhibiting mitochondrial Ca^{2+} transport. These findings also indicate that VDAC1 functions as a Ca^{2+}-sensitive Ca^{2+} transporter in the OMM.

Mitochondrial Ca^{2+} is involved both in physiological and pathophysiological conditions [30]. Non-physiological Ca^{2+} overload depolarizes mitochondria by opening the permeability transition pore (PTP), with concomitant release of cytochrome *c* (Cyto *c*) and other IMS-located proteins, leading to both apoptotic and necrotic cell death, conditions associated with disease pathogenesis [31–33]. It is now well demonstrated that local Ca^{2+} transfer between adjacent domains of the sarco/endoplasmic reticulum (ER/SR) and mitochondria permits Ca^{2+} release from the ER/SR, leading to an enhancement of mitochondrial Ca^{2+} uptake and evoking an increase in matrix [Ca^{2+}] [34–36]. Release of Ca^{2+} from the ER via inositol-1,4,5-trisphosphate receptors (IP_3Rs) has been observed in models of apoptosis and has been directly implicated in mitochondrial Ca^{2+} overload [37]. The specific sites of physical association between the ER and mitochondria, known as mitochondria-associated membranes (MAMs), include high levels of VDAC1, among other proteins [38,39]. It has been recently established that VDAC1 (but not VDAC2 or VDAC3) selectively interacts with IP_3Rs and is preferentially involved in the transmission of low-amplitude apoptotic Ca^{2+} signals to mitochondria [40]. The involvement of VDAC1 in ER-mitochondria Ca^{2+} cross-talk places VDAC1 at a central position on the route transferring Ca^{2+} signals from the ER to the mitochondria, and thus couples ER and mitochondrial functions [40].

Although changes in mitochondrial Ca^{2+} concentration are known to trigger apoptosis, the mitochondrial target for Ca^{2+}-mediated activation of Cyto *c* release and the precise mechanism are not known. Substantial evidence, however, suggests that VDAC1 may play a role in this process.

Recently, we proposed that Cyto *c* release from the mitochondria is mediated via a central pore formed within a VDAC1 oligomeric structure, creating a pathway large enough for passage of a folded protein, such as Cyto *c*. We demonstrated that VDAC1 oligomerization is coupled to Cyto *c* release and apoptotic cell death, as induced by various stimuli [41–45]. While apoptosis inducers stimulated VDAC1 oligomerization, the apoptosis inhibitor, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), prevented STS-induced VDAC1 oligomerization and apoptosis [42,44]. In addition, VDAC1 oligomerization occurs upstream of caspase activation [42]. These results clearly indicate VDAC1 to be a component of the apoptosis machinery and support the suggestion that VDAC1 oligomerization is coupled to apoptosis induction.

In this study, the effect of alterations in cellular Ca^{2+} homeostasis on VDAC1 oligomerization and apoptosis induction was studied. We show that various apoptosis stimuli increase intracellular [Ca^{2+}] and VDAC1 oligomerization. Moreover, increasing intracellular $[Ca^{2+}]_i$ using a Ca^{2+} ionophore or thapsigargin resulted in VDAC1 oligomerization, whereas decreasing $[Ca^{2+}]_i$ using the chelator BAPTA-AM inhibited both VDAC1 oligomerization and apoptosis induction. Finally, we demonstrate that the Ca^{2+} ionophore A23187 and thapsigargin enhanced VDAC1 expression levels. The results suggest that VDAC1 oligomerization, and thus apoptosis, are regulated by cellular Ca^{2+} levels.

2. Materials and methods

2.1. Materials

Arsenic (III) oxide (As_2O_3), calcium chloride dehydrate, carboxymethyl (CM)-cellulose, *n*-decane, dimethyl sulfoxide (DMSO), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), Hepes, leupeptin, mannitol, phenylmethylsulfonyl fluoride (PMSF), propidium iodide, soybean asolectin, staurosporine (STS), sucrose, tetramethylrhodamine-methyl ester (TMRM), thapsigargin (TG) and Tris were purchased from Sigma (St. Louis, MO). Lauryl-(dimethyl)-amine oxide (LDAO) was obtained from Fluka (Buchs, Switzerland). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad (Hercules, CA) and celite comes from Merck (Darmstadt, Germany). Coelenterazine (DeepBlueC [DBC]) was obtained from Bioline (Taunton, MA). Monoclonal anti-VDAC1 antibodies produced against the N-terminal region of 31HL human porin came from Calbiochem-Novobiochem (Nottingham, UK). Ethylene glycolbis (succinimidylsuccinate) (EGS) was obtained from Pierce. Rabbit polyclonal antibodies against VDAC1 amino acids 150–250 came from Abcam (Cambridge, UK). Monoclonal antibodies against actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-mouse antibodies were obtained from Promega (Madison, WI). Annexin V (FITC) was from Enzo Life Sciences (Lausen, Switzerland), BAPTA-AM was obtained from Tocris Bioscience (Bristol, UK), Fluo-4-AM was obtained from Invitrogen (Grand Island, NY), dihydro-rhodamine-2-acetylmethyl ester (Rhod-2-AM) was from Teflabs (Austin, Texas), and siRNA was purchased from Dharmacon (Lafayette, CO). JetPRIME was from PolyPlus Transfection (Illkirch, France). Hank's balanced salts solution (HBSS) without calcium, magnesium and phenol red, Dulbecco's modified Eagle's medium (DMEM) growth media, and the supplements fetal calf serum, L-glutamine, penicillin-streptomycin, were obtained from Biological Industries (Beit Haemek, Israel).

2.2. Cell growth and transfection

T-REx-293 (transformed primary human embryonal kidney) or HeLa (human cervical adenocarcinoma) cells were maintained in DMEM supplemented with 10% fetal calf serum (1% or 0% fetal calf serum when conducting ionomycin or thapsigargin treatments, respectively), 2 mM L-glutamine, 1000 U/ml penicillin and 1 μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C with 5% CO_2.

2.3. Cellular and mitochondrial Ca^{2+} imaging and analysis

Fluo-4-AM was used to monitor changes in cytosolic Ca^{2+} levels. HeLa cells (1×10^6 cells/ml) were harvested after the appropriate treatment, collected ($1500 \times g$ for 10 min), washed with HBSS buffer (5.33 mM KCl, 0.44 mM KH_2PO_4, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.6 mM glucose, 0.03 mM phenol red) supplemented with 1.8 mM $CaCl_2$ (HBSS+) and incubated with 2.5 μM Fluo-4 in 200 μl HBSS(+) buffer in the dark for 30 min at 37 °C. After washing the remaining dye, the cells were incubated with 200 μl HBSS(+) buffer and changes in cellular free Ca^{2+}concentration were measured immediately via fluorescence-activated cell sorter (FACS) analysis. At least 10,000 events were recorded on the FL1 detector, represented as a histogram, and analyzed by the FACSCalibur flow cytometer software (BD Biosciences, Franklin Lakes, NJ). Positive cells showed a shift to an enhanced level of green fluorescence (FL1). Changes in cellular Ca^{2+} were monitored in live cells using the high content Operetta screening system (Perkin-Elmer, Hamburg, Germany). In each well, ten fields were imaged using a 20× wide field objective and excitation filter of 520–550 nm and emission filter of 560–630 nm.

Changes in mitochondrial Ca^{2+} were monitored in live cells using Rhod-2-AM, a compound that undergoes enhancement of fluorescence upon binding to Ca^{2+} and accumulates in the mitochondria [46], with mitochondrial membrane potential serving as the driving force. It should be noted that Rhod-2 staining is not exclusive to mitochondria and also appears in the cytosol. HeLa cells were seeded in a 96-well plate, treated with TG or As_2O_3, incubated at 37 °C for 30 min with or without medium containing 20 μM FCCP. The cells were incubated at 37 °C for 30 min in HBSS(+) containing 1 μM Rhod-2-AM and washed twice with HBSS(+). Finally Rhod-2 fluorescence was analyzed using a plate reader. Rhod-2 fluorescence sensitivity to FCCP reflects mitochondrial Ca^{2+} accumulation. FCCP is a proton ionophore that dissipates the mitochondrial membrane potential ($\Delta\Psi$), the driving force for Rhod-2 uptake by the mitochondria. Thus, in cells not treated with FCCP, the fluorescence signal reflects both cytosolic and mitochondrial [Ca2+] levels, while in cells treated with FCCP, the fluorescence signal reflects the Rhod-2 background or/and cytosolic [Ca^{2+}].

2.4. Chemical cross-linking

T-REx-293 or HeLa cells (1.5 to 3 mg/ml in PBS, pH 8.2) were harvested after the appropriate treatment and incubated with the cross-linking reagent EGS (150–300 μM) for 15 min at 30 °C. Samples (60 to 90 μg) were subjected to SDS-PAGE and immunoblotting using N-terminal-directed anti-VDAC1 antibodies, as described below. Quantitative analysis of immune-reactive VDAC1 dimer bands was performed using Image Gauge software (version 4.0; Science Lab 2001) provided by Fujifilm.

2.5. Mitochondrial membrane potential determination

Mitochondrial membrane potential ($\Delta\Psi$) was determined using TMRM, a potential-sensitive dye, and a plate reader. HeLa cells were treated with ionomycin and subsequently incubated with TMRM (1 μM, 20 min). Cells were then washed twice with PBS and examined in the plate reader. FCCP-mediated $\Delta\Psi$ dissipation served as a control.

2.6. Apoptosis analysis

T-REx-293 or HeLa cells (2×10^5), untreated or treated with BAPTA-AM, ionomycin or other reagents, were analyzed for apoptotic cell death using propidium iodide (PI), annexin V-fluorescein isothiocyanate (FITC) and flow cytometer analysis. Cells were collected ($1500 \times g$ for 10 min), washed, and resuspended in 200 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Annexin V–FITC was added according to the recommended protocol, and the cells were incubated in the dark for 15 min. Cells were then washed once with binding buffer and resuspended in 200 μl binding buffer, to which PI was added immediately before flow cytometry analysis. At least 10,000 events were collected, recorded on a dot plot, and analyzed by the FACSCalibur flow cytometer software (BD Biosciences).

2.7. Gel electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli [47]. Gels were stained with Commasie Brilliant Blue or electro-transferred onto nitrocellulose membranes for immunostaining. Membranes containing the transferred proteins were blocked with 5% non-fat dry milk and 0.1% Tween-20 in Tris-buffered saline and incubated with monoclonal anti-VDAC1 antibodies (1:10,000), followed by incubation with HRP-conjugated anti-mouse IgG secondary antibodies (1:10,000). After treatment with the appropriate primary and secondary antibodies, enhanced chemiluminescence (Pierce, Rockford, IL) was performed.

2.8. VDAC1 channel conductance measurement

VDAC1 was purified from sheep liver mitochondria following LDAO solubilization and chromatography using hydroxyapatite resin and CM-cellulose [18]. Single and multiple channel current recording and data analysis were carried out as described previously [14]. Briefly, a planar lipid bilayer (PLB) was prepared from soybean asolectin dissolved in *n*-decane (30 mg/ml). Purified VDAC1 was added to the *cis* chamber containing 1 M NaCl or 150 mM $CaCl_2$ and 10 mM Hepes, pH 7.4. After one or several channels were inserted into the PLB, excess protein was removed by perfusing the *cis* chamber with ~20 volumes of solution to prevent further channel incorporation. Recordings were made under voltage clamp using a Bilayer Clamp BC-535B amplifier (Warner Instruments, Hamden, CT). Currents were measured with respect to the *trans* side of the membrane (ground). The currents were low pass-filtered at 1 kHz and digitized on-line using a Digidata 1440-interface board and pClampex 10.2 software (Axon Instruments, Union City, CA). The effects of RuR and AzRu, added to the *cis* chamber, on channel conductance were carried out as above in 1 M NaCl, with recordings taken before and after RuR and AzRu addition.

3. Results

We have shown that apoptosis inducers, such as STS, selenite and cisplatin, acting via different mechanisms, all induced VDAC1 oligomerization [42]. Since these agents are also known to disrupt Ca^{2+} homeostasis, we investigated here whether Ca^{2+} is the common signaling molecule leading to VDAC1 oligomerization.

3.1. Ca^{2+} is required for VDAC1 oligomerization and apoptosis

To assess the requirement of Ca^{2+} for VDAC1 oligomerization, HeLa or T-REx-293 cells were pre-incubated with the cell-permeable Ca^{2+} chelating reagent, BAPTA-AM (acetoxymethyl ester form of bis(aminophenoxy) ethan -N,N,N′,N′-tetraacetic acid), which is cleaved by intracellular esterases to release the free acid form that subsequently decreases the intracellular Ca^{2+} concentration. Pre-incubation of cells with BAPTA-AM resulted in a significant inhibition of both selenite-induced VDAC1 oligomerization (Fig. 1A, B) and apoptotic cell death (Fig. 1C).

Next, to test the effect of elevating $[Ca^{2+}]_i$ on VDAC1 oligomerization, the Ca^{2+} ionophore ionomycin was used to increase cellular Ca^{2+} levels and to allow for re-equilibration of Ca^{2+} gradients between mitochondria, cytosol and the extracellular space [48,49]. Ionomycin treatment is frequently used as a model of Ca^{2+}-dependent cell death, as this ionophore can induce both necrotic and apoptotic cell death [50,51]. Incubation of cells with ionomycin resulted in a marked increase in VDAC1 oligomerization (Fig. 2A), as well as in apoptotic cell death (Fig. 2B). In addition, an anti-VDAC1 antibody-labeled protein band that migrated

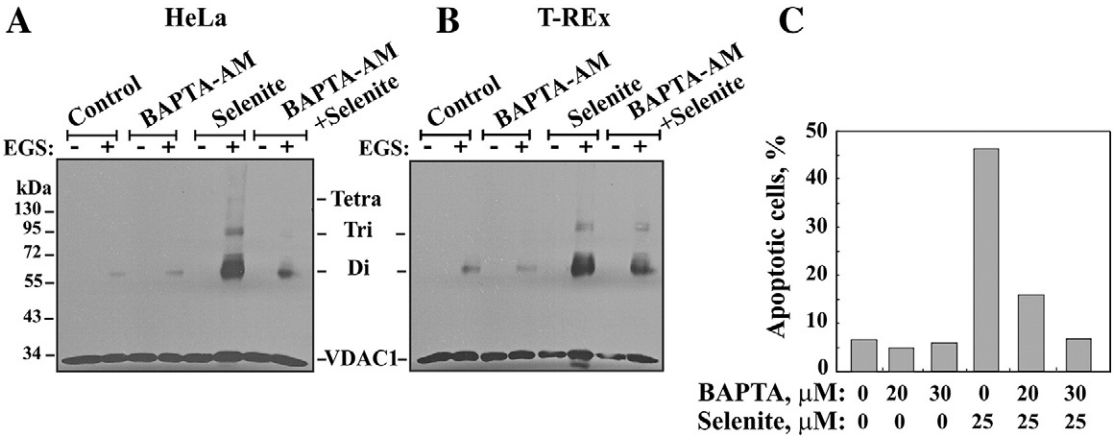

Fig. 1. BAPTA-AM, a Ca^{2+}-chelating reagent, prevents VDAC1 oligomerization. HeLa (A) or T-REx-293 (B) cells were incubated with BAPTA-AM (20 μM, 1.5 h) and then with or without selenite (25 μM, 2.5 h), harvested, cross-linked with EGS (250 μM, 15 min), and analyzed by immunoblotting using anti-VDAC antibodies. (C) Apoptotic cell death induced by the different treatments was assayed using annexin V–FITC, PI staining and FACS analysis. Results are representative of two similar experiments.

faster than the position of monomeric VDAC1 was obtained upon apoptosis induction and EGS treatment (asterisk in Fig. 2A). This band appeared only when apoptosis was induced and most likely represents a monomer that underwent conformational changes that were fixed upon intermolecular cross-linking, thereby modifying its mobility in SDS-PAGE.

As expected, ionomycin increased Ca^{2+} intracellular levels, as monitored using Fluo-4 and FACS analysis (Fig. 2C).

Increase in intracellular Ca^{2+} was also induced using thapsigargin, an inhibitor of the sarcoplasmic reticulum/ER Ca^{2+}-ATPase that disrupts the homeostatic balance of $[Ca^{2+}]_i$ and induces ER stress [52]. Thus, the effects of thapsigargin on VDAC1 oligomerization and apoptotic cell death were also evaluated. Incubation with thapsigargin resulted in an increase in $[Ca^{2+}]_i$, apoptosis and induced an increase in VDAC1 oligomerization (Fig. 3).

3.2. Apoptotic stimuli elevate intracellular Ca^{2+} levels and encourage VDAC1 oligomerization

Next, we examined whether induction of apoptosis by agents that are known to affect intracellular Ca^{2+} is associated with VDAC1 oligomerization. Thapsigargin, STS, selenite and other apoptosis inducers were shown to induce apoptotic cell death and disrupt Ca^{2+} homeostasis [53]. Incubation of HeLa cells with STS, selenite or As_2O_3 resulted in elevated intracellular Ca^{2+} levels, as monitored using Fluo-4 and FACS analysis (Fig. 4A and B) or by cell imaging using the Operetta system (Fig. 4C). Some images show highly intense fluorescent punctuations, which may represent mitochondria. As shown previously [42], these apoptosis stimuli induced apoptotic cell death (Fig. 4D) and VDAC1 oligomerization, as revealed using chemical cross-linking followed by Western blotting with anti-VDAC1 antibodies (Fig. 4E). Several anti-

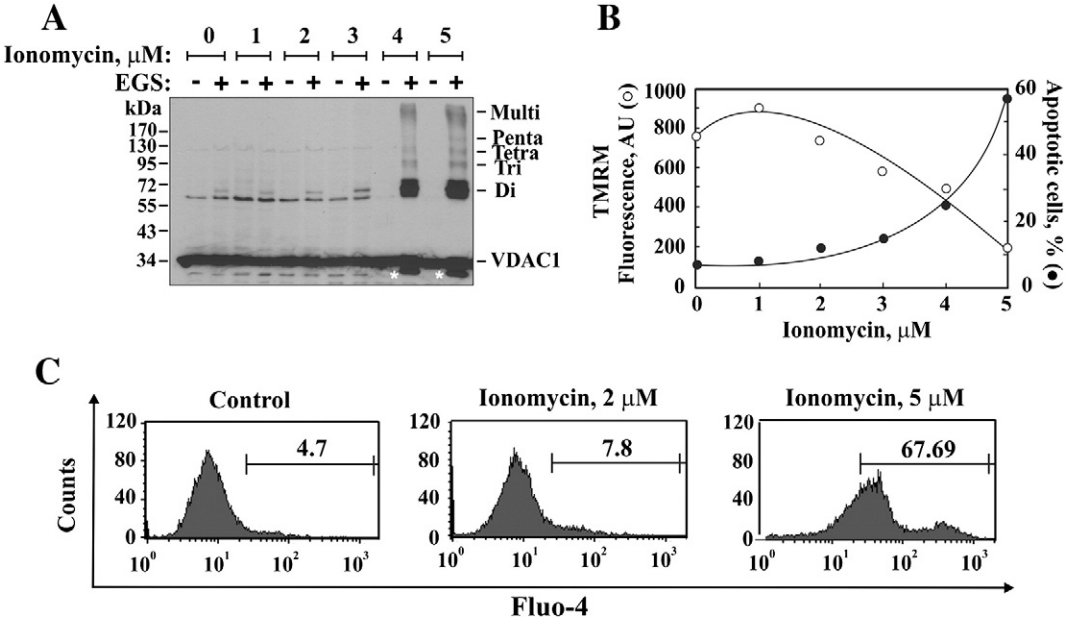

Fig. 2. VDAC1 oligomerization and apoptosis are enhanced by ionomycin. HeLa cells were incubated with the indicated concentrations of ionomycin (1–5 μM, 45 min), followed by analysis of VDAC1 oligomerization (A). Apoptotic cell death and TMRM fluorescence (ΔΨ) were analyzed as a function of ionomycin concentration (B). $[Ca^{2+}]_i$ levels (C). Representative FACS analysis of $[Ca^{2+}]_i$ levels in control cells and in cells treated with ionomycin, as monitored using Fluo-4 and FACS analysis. The values shown indicate the increase in $[Ca^{2+}]_i$.

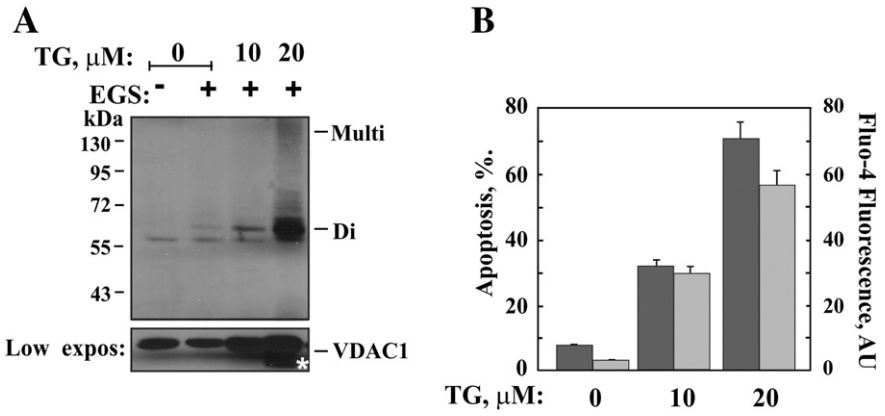

Fig. 3. Thapsigargin induces VDAC1 oligomerization. HeLa cells were incubated with the indicated concentrations of thapsigargin (TG) for 18 h, followed by analysis of VDAC1 oligomerization (A), apoptotic cell death (dark gray column) and intracellular Ca^{2+} levels (light gray column) (B), as described in the legend to Fig. 2. Note the increase in VDAC1 levels upon TG treatment. Low expo indicates a short time of exposure to the film.

VDAC1 antibody-labeled protein bands were obtained upon exposure to the apoptosis stimuli and found to correspond to VDAC1 dimers, trimers, tetramers and multimers. The appearance of the intramolecular cross-linked monomeric VDAC1 species was also obtained upon apoptosis induction and EGS treatment (asterisk in Fig. 4E).

These results thus clearly demonstrate that strong association exists between the level of apoptosis, the extent of $[Ca^{2+}]_i$ increase and the degree of VDAC1 oligomerization.

3.3. VDAC1 Ca^{2+} permeability and Ca^{2+}-binding sites function in the oligomerization of the protein

The effect of Ca^{2+} on VDAC1 oligomerization can be mediated via the transport or binding of Ca^{2+} to VDAC1. Since VDAC1 allows passage of both cations and anions, its permeability to Ca^{2+} can be estimated from the reversal potential determined upon encountering different $CaC1_2$ concentration gradients [14]. As such, VDAC1 channel conductance was analyzed in a gradient of 150–500 mM $CaCl_2$ as a function of voltage, generating a profile in the form of ramp (Fig. 5A). The reverse potential of 10.3 mV obtained under these conditions was used to estimate the permeability ratio of PCa^{2+}/PCl^- (Fig. 5A) [14]. The obtained ratio of 0.36 indicates that VDAC1 permeability to Ca^{2+} is only 2.8-times less than that to Cl^-. This value is similar to a previously reported value [14] and indicates that VDAC1 is highly permeable to Ca^{2+}. The channel conductance in the presence of $CaCl_2$, as with NaCl, is voltage-dependent, decreasing at highly negative or positive voltages (Fig. 5B).

RuR and AzRu decreased VDAC1 channel conductance at all tested voltages (Fig. 5C) in a manner that can be prevented by Ca^{2+} [14,20]. RuR and AzRu were shown to inhibit the channel activity of recombinant wild type but not of mutated VDAC1 [17], suggesting that VDAC1 possesses Ca^{2+}-binding sites. In addition, these reagents inhibit mitochondrial Ca^{2+} transport [14,20] and protect against apoptosis in T-REx-293 cells expressing native but not mutated VDAC1 [17,20,23,24]. These findings suggest that RuR and AzRu interact with VDAC1 to mediate their anti-apoptotic activities. Thus, to evaluate the effects of AzRu on VDAC1 oligomerization induced by STS, cells were pre-incubated with AzRu, followed by incubation with STS (Fig. 5D). AzRu inhibition of VDAC1 oligomerization points to the involvement of mitochondrial Ca^{2+} in VDAC1 oligomerization.

3.4. Mitochondrial Ca^{2+} and VDAC1 oligomerization

Mitochondrial Ca^{2+} overload has been shown to lead to a dissipation of the mitochondrial potential ($m\Delta\Psi$), the opening of the permeability transition pore and the release of cytochrome *c* [21,31]. Therefore, to verify whether ionomycin also increased mitochondrial Ca^{2+} levels, $m\Delta\Psi$ was measured using TMRM. Such analysis revealed that ionomycin indeed decreased $m\Delta\Psi$ and concomitantly enhanced apoptotic cell death (Fig. 2B).

To further demonstrate that VDAC1 oligomerization involves mitochondrial $[Ca^{2+}]$, we used the fluorescent dye, Rhod-2-AM. Rhod-2 fluorescence was analyzed in cells subjected to apoptosis induced by As_2O_3 or thapsigargin, using a plate reader (Fig. 6A). To distinguish between mitochondrial and cytosol-derived signals, before loading with Rhod-2-AM, the cells were treated with FCCP to dissipate $m\Delta\Psi$ and thus, the driving force for mitochondrial Ca^{2+} accumulation. Cells treated with thapsigargin or As_2O_3 showed an increased FCCP-sensitive Rhod-2 fluorescent signal (Fig. 6A), reflecting an increase in $[Ca^{2+}]_m$.

Next, to further evaluate the function of mitochondrial Ca^{2+} in VDAC1 oligomerization, we tested the effect of $m\Delta\Psi$ dissipation by FCCP on VDAC1 oligomerization as induced by ionomycin and thapsigargin. We found that even in the presence of FCCP, ionomycin induced VDAC1 oligomerization (data not shown). This is not surprising as decreasing the $m\Delta\Psi$ by FCCP would not prevent Ca^{2+} entry into the mitochondria via ionomycin, since ionomycin allows for equilibration of cytosolic and the mitochondrial Ca^{2+} levels. Thapsigargin, on the other hand, would increase cytosolic but not mitochondrial Ca^{2+} in the presence of FCCP. VDAC1 oligomerization, as induced by thapsigargin, is reduced in the presence of FCCP (Fig. 6B), which decreased similarly the $m\Delta\Psi$ (measured with TMPM) in the absence and the presence of thapsigargin (data not shown). These results suggest that mitochondrial Ca^{2+} is important for induction of VDAC1 oligomerization.

3.5. Increased intracellular Ca^{2+} levels induce VDAC1 over-expression

To examine whether intracellular Ca^{2+} also affects VDAC1 protein levels, we followed VDAC1 expression in cells treated with the Ca^{2+} ionophore A23187. It was found that incubation of HeLa cells with A23187 results in increased VDAC1 protein levels (Fig. 7A and B). An increase in VDAC1 expression was also obtained when $[Ca^{2+}]_i$ was enhanced with thapsigargin (see Fig. 3), increasing VDAC1 level by 2.7- and 4.1-fold upon exposure to 10 and 20 μM of thapsigargin, respectively (Fig. 7C).

4. Discussion

4.1. Ca^{2+} overload signals VDAC1 oligomerization and apoptosis

Anti-cancer drugs and cytotoxic agents, such as thapsigargin, STS, As_2O_3 and selenite and, reagents that induce apoptotic cell death, were also shown to disrupt cell Ca^{2+} homeostasis [53], all shown here

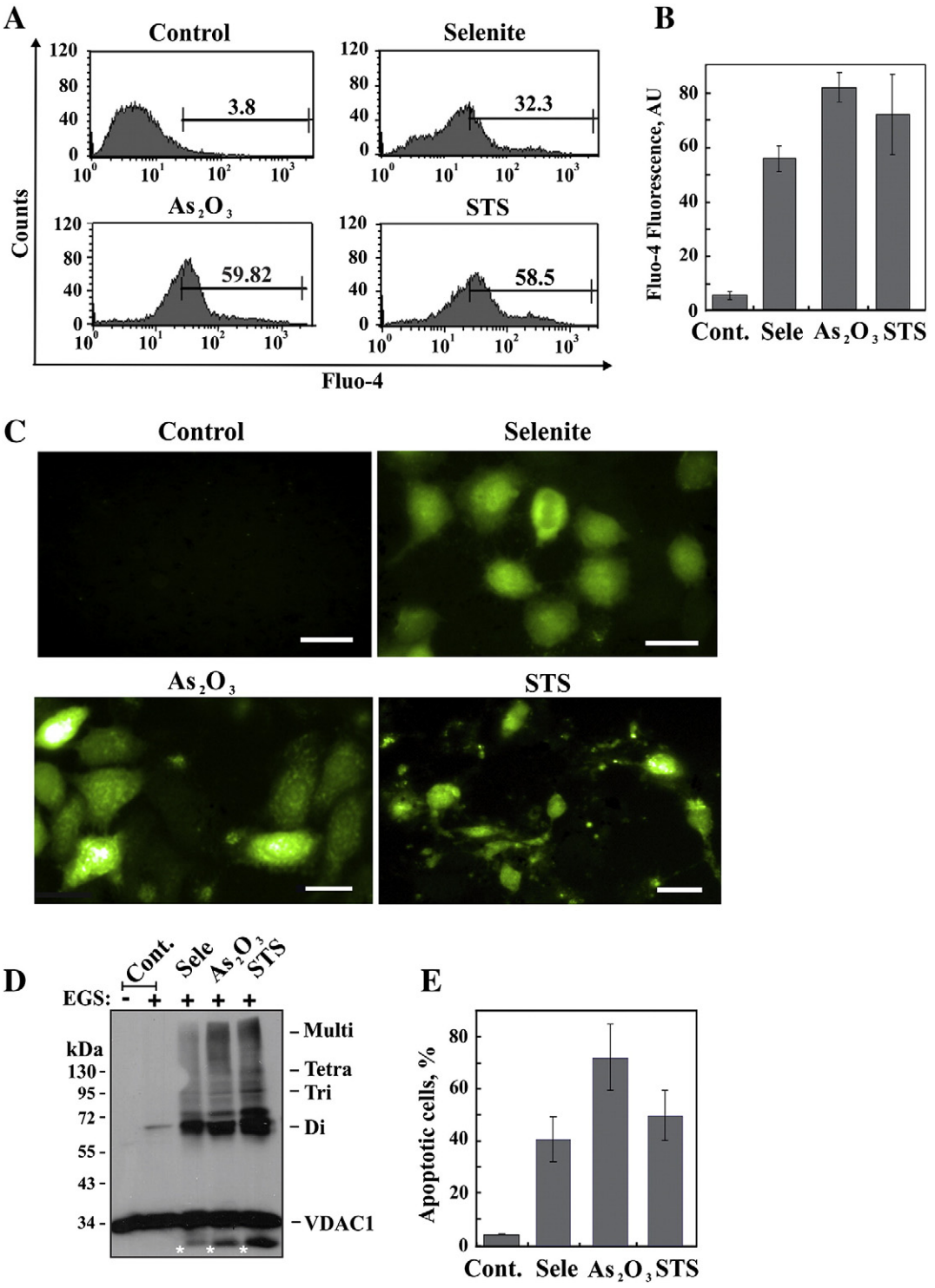

Fig. 4. Apoptosis inducers elevate intracellular Ca^{2+} levels and induce VDAC1 oligomerization. HeLa cells were incubated for 22 h with selenite (8 μM), As_2O_3 (20 μM) or STS (0.5 μM), harvested and intracellular Ca^{2+} levels (A, B, C) apoptotic cell death (D) and VDAC1 oligomerization (E) were assessed. A. Representative FACS analysis of $[Ca^{2+}]_i$ levels, determined using Fluo-4 and FACS. B. Quantitative analysis of $[Ca^{2+}]_i$, as assessed by FACS (data from panel A) (n=3). C. HeLa cells were plated on black CellCarrier 96-wells plates overnight, and then treated for 22 h with selenite (6 μM), As_2O_3 (10 μM) and STS (0.5 μM), followed by incubation with Fluo-4. Cell fluorescence was recorded using the Operetta and representative images are shown. D. Quantitative analysis of apoptosis, as measured by FACS analysis (using annexin V–FITC and PI staining) (n=3). E. VDAC1 oligomerization revealed using EGS-based cross-linking. Cells (2.5 mg/ml) were washed with PBS, pH 8.3 and incubated with EGS (250 μM) at 30 °C for 15 min and then subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies. The positions of VDAC1 monomers to multimers are indicated. The asterisks indicate an anti-VDAC1 antibody-labeled protein band that migrates below the position of monomeric VDAC1.

to increase $[Ca^{2+}]_i$ (Fig. 4). The mode of action of drugs that interfere with $[Ca^{2+}]_i$ signaling includes an elevation of $[Ca^{2+}]_i$ by increasing Ca^{2+} uptake from the extracellular space in some tumor cells, as in the case of cisplatin, or triggering Ca^{2+} release from stores that consequently encourages apoptosis, as in the case of As_2O_3 [53]. However, As_2O_3 toxicity is not limited to $[Ca^{2+}]_i$ disturbances, as it can also elicit

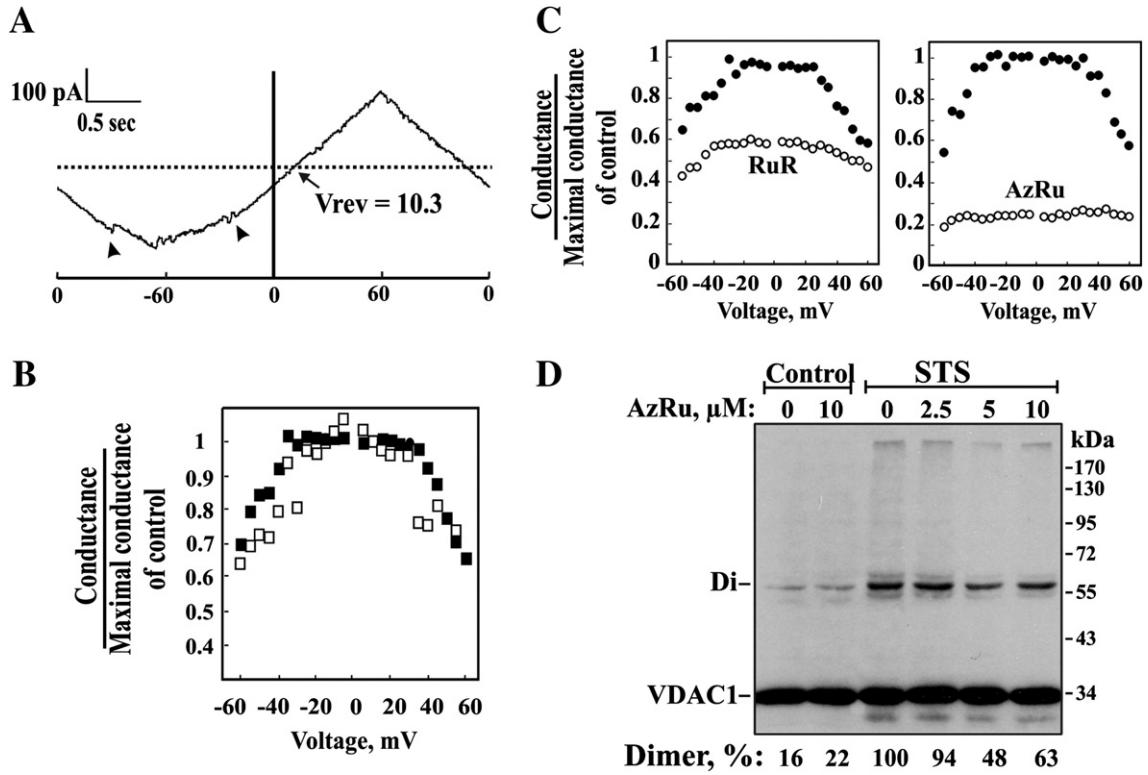

Fig. 5. VDAC1 is a Ca^{2+}-permeable channel and its conductance is inhibited by RuR and AzRu. Sheep liver mitochondria-purified VDAC1 was reconstituted into a PLB and recording of channel conductance was carried out as described previously [14]. A. Currents through VDAC1 obtained in response to voltage ramps (−60 to +60 mV, at 1 mV/ms) in the presence of a $CaCl_2$ concentration gradient (150–500 mM) were recorded. The broken line indicates the zero current level, arrowheads indicate the channel in a sub-conducting states and the arrow indicates the reversal potential (V_{rev}). B. Multi-channel recordings of the average steady-state conductance of VDAC1 in the presence of a symmetric $CaCl_2$ concentration (150 mM) (□) or NaCl (0.5 M) (■), as a function of voltage. Relative conductance was determined as the ratio of conductance at a given voltage to maximal conductance. C. The effects of RuR and AzRu on VDAC1 conductance were measured in symmetric 1 M NaCl solution on both sides of the bilayer as a function of voltage, ranging from 60 mV to −60 mV. The average steady-state conductance at a given voltage was normalized to the conductance at 10 mV. The recordings were taken before (•) and 10 min after the addition of RuR (50 μM) (○) or 30 min after the addition AzRu (50 μM) (o). D. AzRu-mediated inhibition of VDAC1 oligomerization. HeLa cells were incubated with the indicated concentrations of AzRu (16 h), followed by incubation with or without STS (4 μM for 3.5 h). The cells were then harvested, cross-linked with EGS (300 μM, 15 min), and analyzed by immunoblotting using anti-VDAC1 antibodies. Di indicates VDAC1 dimers, while the numbers at the bottom indicate the percentage of dimers formed. The intensities of the dimer bands are given as dimer percentage relative to the maximal dimer intensity obtained in STS treatment.

numerous intracellular processes that may lead to apoptosis. These include protein kinase C (PKC) activation, mitochondrial depolarization, depletion of intracellular glutathione, up-regulation of caspase-3, down regulation of Bcl-2, and Bax/Bak-dependent Cyto *c* release [54–57]. Thapsigargin decreases the ER Ca^{2+} pool by inhibiting SERCA pumps, causing a transient increase in $[Ca^{2+}]$, resulting in ER stress and apoptosis [58]. It was reported that thapsigargin induced apoptosis in several tumor cell lines [59,60], with its effect being attributed to a reduction of the mitochondrial transmembrane potential and a concomitant increase in ROS generation [61]. STS, an ATP-competitive inhibitor of PKC, PKA, PKG, p60v-src tyrosine protein kinase and CaM kinase II [62], directly provokes Ca^{2+} leak from the ER by activating caspase-3-mediated cleavage of IP_3R1 [63]. STS was shown to significantly increase $[Ca^{2+}]_i$ [64] and promote the activation of the mitochondrial Ca^{2+}-dependent protease, calpain [65]. Selenite treatment induced apoptosis, as well as causing a rapid increase in cytosolic $[Ca^{2+}]$ and accumulation of ROS [66,67].

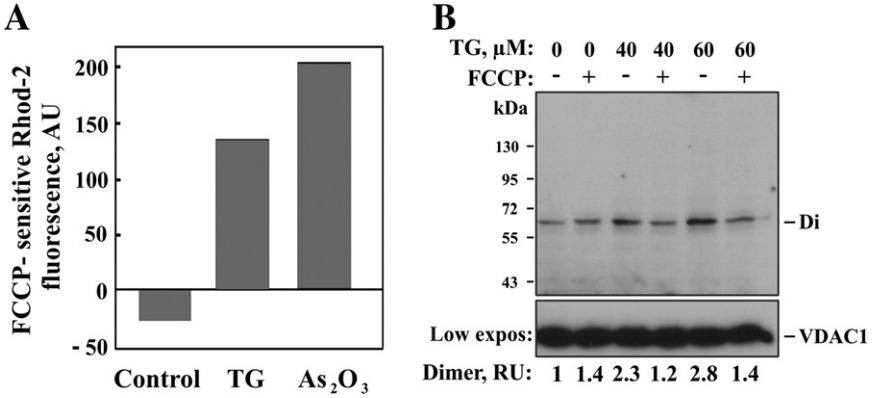

Fig. 6. Mitochondrial Ca^{2+} is increased upon apoptosis induction. HeLa cells were challenged with thapsigargin (TG, 10 μM, 30 min) or As_2O_3 (50 μM, 3 h), incubated with or without FCCP (20 μM, 30 min) and treated with Rhod-2-AM (1 μM, 30 min). Cellular fluorescence was analyzed using a plate reader. Results are representative of two similar experiments, with the FCCP-sensitive signal being presented. B. HeLa cells were incubated with FCCP (5 μM, 30 min) and then challenged with TG (40 or 60 μM, 90 min) and VDAC oligomerization was analyzed by EGS (60 μM, 1 mg protein/ml) cross-linking as described in Fig. 1. The intensities of the dimer bands are given relative (RU) to dimer intensity in a sample not treated with FCCP or TG.

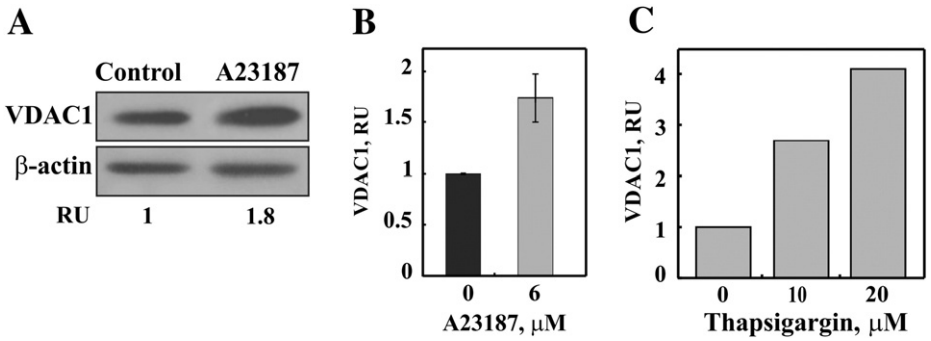

Fig. 7. Ca^{2+} overload induces up-regulation of VDAC1 expression. HeLa cells were incubated with the indicated concentration of A23187 for 24 h (A, B) or with thapsigargin (TG) for 18 h (C, D). Thereafter, VDAC1 expression levels were analyzed by immunoblotting (A, C). Quantitative analysis of VDAC1 expression is shown in (B, D).

The previously shown increase in $[Ca^{2+}]_i$ mediated by the apoptosis inducers discussed above was also demonstrated in the current study (Fig. 4). These apoptosis stimuli also induced VDAC1 oligomerization [41–44] (Fig. 4), suggesting a link between an increase in $[Ca^{2+}]_i$, VDAC1 oligomerization and apoptosis induction. This is further supported by our demonstration that an increase in $[Ca^{2+}]_i$, induced by Ca^{2+} ionophores (Fig. 2) or thapsigargin (Fig. 3), leads to VDAC1 oligomerization and apoptosis induction. Conversely, BAPTA-AM-mediated decrease in $[Ca^{2+}]_i$ inhibits VDAC1 oligomerization and prevents apoptosis induction (Fig. 1). Thus, it is possible that the common mechanism shared by all agents inducing apoptosis and enhancing VDAC1 oligomerization involves disrupting $[Ca^{2+}]_i$ homeostasis by increasing $[Ca^{2+}]_i$.

The dynamic equilibrium between VDAC1 monomers and oligomers can be influenced by $[Ca^{2+}]_i$. However, it is not clear whether cytosolic or mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) mediates VDAC1 oligomerization, as increased $[Ca^{2+}]_i$ would lead to an increase in $[Ca^{2+}]_m$. In this respect, we have shown that an increase $[Ca^{2+}]_i$, mediated by Ca^{2+} ionophore (Fig. 2) was accompanied by a decrease in $m\Delta\Psi$ produced. This is in accord with the findings that $[Ca^{2+}]_m$ overload leads to a dissipation of $m\Delta\Psi$ [21,31], although this decrease could also be a consequence of the apoptotic cell death process. In addition, thapsigargin and As_2O_3 were found to increase the FCCP-sensitive Rhod-2 fluorescent signal, reflecting an increase in $[Ca^{2+}]_m$ (Fig. 6A). Thus, Ca^{2+} ionophore treatment increases both $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, suggesting that VDAC1 oligomerization may be induced by an increase in $[Ca^{2+}]_m$. This is supported by the finding that VDAC1 oligomerization, as induced by thapsigargin, was reduced in the presence of FCCP (Fig. 6B), suggesting that mitochondrial Ca^{2+} is required. The inhibition of VDAC1 oligomerization by AzRu, an inhibitor of mitochondrial Ca^{2+} transport [20] that directly interacts with VDAC1 and inhibits its channel conductance (Fig. 5C) and oligomerization (Fig. 5D), further points to $[Ca^{2+}]_m$ as being the regulator of VDAC1 oligomerization and apoptosis.

Ca^{2+} could mediate apoptosis via directly interacting with VDAC1, able to transport Ca^{2+} across the OMM and also to bind Ca^{2+} [14,23]. Binding of Ca^{2+} to VDAC1, either from the cytosolic face or in the mitochondrial inter-membrane space, can lead to an increase in VDAC1 oligomerization. However, $[Ca^{2+}]_m$ may potentially affect VDAC1 oligomerization via a different mechanism that involves Cyto *c*. Not all Cyto *c* is free, with a substantial portion instead being associated with the anionic membrane lipid cardiolipin (CL). Thus, Cyto *c* dissociation from CL is a prerequisite step for Cyto *c* release. This could be induced by cardiolipin oxidation [68], by Ca^{2+} interaction with cardiolipin [69], or by tBid [70]. It was also demonstrated that Cyto *c* encapsulated in liposomes containing VDAC1 induced VDAC oligomerization [45]. Thus, Ca^{2+}-mediated dissociation of Cyto *c* from CL could lead to unbound Cyto *c* activating VDAC1 oligomerization. Exploring the interplay between mitochondrial Ca^{2+}, VDAC1 oligomerization and apoptosis induction will require further study.

4.2. Ca^{2+} overload encodes information that up-regulates VDAC1 expression

In this study, we observed an increase in VDAC1 expression levels upon increasing $[Ca^{2+}]_i$. Cells exposed to thapsigargin or to the Ca^{2+} ionophore A23187 (Fig. 7) resulted in up-regulation of VDAC1 expression. This suggests that elevated cellular Ca^{2+} leads to increased VDAC1 expression. A23187 was shown to induce growth arrest and expression of the DNA damage-inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153 [71].

Various studies demonstrated that Ca^{2+} activates signaling pathways that regulate gene transcription [72]. However, the exact signal transduction mechanisms involved in transforming Ca^{2+} signals into specific, finely controlled changes in gene expression have yet to be fully delineated. Several reviews have addressed the variety of regulatory mechanisms that participate in Ca^{2+}-dependent gene expression in neurons [73–75] and other cells [76,77]. There are several potential Ca^{2+}-dependent steps that could contribute to the process of gene expression. Indeed, mRNA transcription, elongation, splicing, stability, and translation have all been suggested as being regulated by Ca^{2+} [72].

The increase in VDAC1 expression level, as induced by an elevation of $[Ca^{2+}]_i$, may result from inhibition of VDAC1 degradation or an increase in the level of VDAC1 mRNA. The mechanism(s) involved in increased VDAC1 expression was not addressed here but will be the subject of further research.

We propose that the increase in VDAC1 levels in the cell is associated with apoptosis induction. VDAC1 over-expression was found to lead to its oligomerization and apoptosis in the absence of any apoptosis stimuli [24,78–80]. In addition, several apoptosis inducers, such as arbutin, prednisolone, cisplatin, viral proteins, or UV irradiation, were found to increase VDAC1 expression levels [81–87]. Thus, up-regulation of VDAC1 expression may comprise a major mechanism by which these treatments lead to cancer cell death. We further propose that the increase in $[Ca^{2+}]$ induced by the various apoptotic signals (Figs. 2–4) [53] leads to increased VDAC1 expression (Fig. 7), encouraging VDAC1 oligomerization and apoptosis induction.

To summarize, Ca^{2+} regulation of VDAC1 oligomerization and apoptosis is a complex process and may involve several possible mechanisms, from Ca^{2+}-mediated dissociation of Cyto *c* from CL leading to unbound Cyto *c* activating VDAC oligomerization, to up-regulation of VDAC1 expression.

Acknowledgements

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