Plasmas Membrane Voltage-dependent Anion Channel Mediates Antiestrogen-activated Maxi Cl− Currents in C1300 Neuroblastoma Cells*

Received for publication, March 19, 2003, and in revised form, May 16, 2003
Published, JBC Papers in Press, June 5, 2003, DOI 10.1074/jbc.M302814200

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The cell membrane large conductance voltage-dependent chloride channel (Maxi Cl− channel) has been recorded in different cell types following excision of membrane patches or stimulation by antiestrogens under whole-cell recording conditions. However, both its molecular nature and relevance to cell physiology await elucidation. Its electrophysiological properties resemble those of the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane. This observation led to the controversial hypothesis that VDAC could be the molecular correlate of the plasma membrane Maxi Cl− channel. We have investigated the cellular localization of VDAC and its relationship with the antiestrogen-activated Maxi Cl− current in C1300 neuroblastoma cells. The presence of a plasma membrane VDAC was demonstrated by immunoblotting of membrane fractions with monoclonal antibodies against VDAC and by reverse transcription-PCR using primers that hybridize to a VDAC sequence coding for an N-terminal leader peptide required for its plasma membrane sorting. Besides, VDAC colocalized with markers of plasma membrane lipid rafts (cholera toxin β subunit) but not caveolin-1. Transfection of C1300 cells with an antisense oligonucleotide directed against the specific membrane leader sequence of VDAC markedly reduced both VDAC immunostaining and antiestrogen-activated Maxi Cl− currents, suggesting that VDAC forms the plasma membrane Maxi Cl− channel or a part thereof.

Chloride channels of large conductance were first identified in excised patches from the plasma membrane of skeletal muscle 20 years ago (1). The search for their functional relevance, regulation, and molecular identity has continued, with irregular success, over all these years. Among the different roles associated with their function, the most frequently mentioned are the regulation of cell volume (2, 3) and apoptosis (4, 5). This channel has also been activated by extracellular agonists (6–9) and modulated by intracellular GTP (10).

The molecular identity of Maxi Cl− channels1 has been even more difficult to prove. The fact that Maxi Cl− channel electrophysiological properties resemble those of the mitochondrial voltage-dependent anion channel (VDAC) (11) encouraged some investigators to assume that the two channels were one and the same protein (12, 13). This hypothesis was based on early observations suggesting the presence of VDAC protein on the plasma membrane (12, 14) but was questioned by others (15). The proposal suggesting the presence of VDAC in extramitochondrial locations has received recent strong support from two independent reports: 1) the identification of a VDAC isoform (pl-VDAC) that contains a leader sequence for its trafficking to the plasma membrane (16) and 2) the presence of VDAC in caveolae (17). In the present study, we have evaluated the presence of VDAC in the plasma membrane of C1300 neuroblastoma cells and, more interestingly, its contribution to the Maxi Cl− currents activated by antiestrogens in these cells.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Chemicals—C1300 mouse neuroblastoma cells, obtained from the Imperial Cancer Research Fund Laboratories, Clare Hall, Herts, UK, were cultured as described previously (8). For experimental procedures, cells were plated onto 35-mm culture dishes (NUNC) or 13-mm glass coverslips treated with poly-β-lysine (10 μg/ml for 1 h). All chemicals were obtained from Sigma unless otherwise indicated. Toremifene was obtained from Farmos, Torku, Finland.

Preparation of Cell Lysates and Plasma Membrane Fractions—T-75 flasks containing confluent cells were washed three times with 5 ml of PBS without calcium, magnesium, and sodium bicarbonate (Invitrogen). After the final wash, 600–1,000 μl of ice-cold cell lysis buffer (50 mm Tris·HCl, pH 8.0, 120 mm NaCl, 0.5% Brij-35, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was added per T-75 flask. The cell lysate was transferred to a 2-ml Eppendorf tube, rocked for 30 min at 4 °C, and subsequently centrifuged at 10,000 × g for 10 min at 4 °C. A 50-μl aliquot was removed from the supernatant and stored at −20 °C for subsequent determination of protein concentration (Method DC protein assay, Bio-Rad). The remaining supernatant was aliquoted into cryotubes and stored at −80 °C.

Immunoblotting—Proteins were resolved by SDS-PAGE (12%) and blotted onto nitrocellulose. The primary antibodies used were: monoclonal mouse anti-perin 31 HL (Calbiochem) raised against the N-terminal leader sequence of VDAC (12) and 2) the presence of VDAC in caveolae (17). In the present study, we have evaluated the presence of VDAC in the plasma membrane of C1300 neuroblastoma cells and, more interestingly, its contribution to the Maxi Cl− currents activated by antiestrogens in these cells.

1 The abbreviations used are: Maxi Cl− channel, large conductance voltage-dependent chloride channel; Maxi K+ channels, large conductance Ca2+−activated K+ channels; CTXβ, cholera toxin β subunit; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EGFP, enhanced green fluorescent protein; VDAC, voltage-dependent anion channel; pl-VDAC, plasma membrane VDAC; FITC, fluorescein isothiocyanate; RT, reverse transcription.
corresponding complex of the mitochondrial oxidative phosphorylation system, and goat polyclonal anti-ribophorin I (1:100; Santa Cruz Biotechnology) that recognizes integral membrane glycoproteins that localize exclusively to the rough endoplasmic reticulum. Non-specific binding was avoided by incubating the nitrocellulose membranes in a blocking solution consisting of Tween 20-Tris buffer solution (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with 5% non-fat milk for 1 h at room temperature or overnight at 4 °C. Mouse and rabbit antibodies were detected either with alkaline-phosphatase-conjugated antibodies (goat anti-mouse IgG or donkey anti-rabbit IgG, both at a 1:2,000 dilution (Amersham Biosciences)). The membranes were then washed, and the bands were visualized by using the enhanced chemiluminescence substrate Super Signal (Pierce) and autoradiographed on either Amersham Biosciences Hyperfilm ECL or nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt solution for detection of alkaline phosphatase.

Confocal Microscopy—Double-label analysis was carried out on C1300 cells adhered to glass coverslips coated with poly-n-lysine. Cells were fixed in 2% paraformaldehyde, 0.15 M sucrose, and 0.1% glutaraldehyde for 10 min and permeabilized with 0.1% Triton for 10 min. Prior to antibody incubation, cells were treated with NH4Cl for 30 min to minimize the number of reactive aldehyde groups and blocked for 30–60 min (room temperature) with 5% fetal bovine serum and 1% bovine serum albumin in washing buffer. Cells were then incubated with anti-ribophorin 31 HL (20 ng/ml) or rabbit anti-caveolin-1 (1:200, Santa Cruz Biotechnology) for 2 h at room temperature. Unbound antibody was removed by washing the cells three times with 1 ml of blocking solution for 10 min each time. Staining with FITC-labeled cholera toxin β subunit (CTXβ) was performed under non-permeabilized conditions by incubating the cells with blocking solution for 30 min and then staining them with FITC-CTXβ for 30 min at room temperature. Subsequently, cells were washed, fixed, and permeabilized as described above. Following several washes in blocking solution, cells were incubated with secondary Alexa Fluor 488 goat anti-rabbit (1:500) and/or goat anti-mouse IgG coupled to the fluorochrome Cy3 (1:2,000) for 1 h at room temperature. Prior to incubation, the secondary antibody was centrifuged at 13,000 × g for 15 min at 4 °C to pellet any precipitated constituents. Negative controls were performed in which the cells were solely incubated with the secondary antibody. Digital images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Heidelberg, Germany).

Antisense Oligonucleotides—Cells were seeded in 24-well dishes (NUNC) at a concentration of 10^5 cells/well 2 days before transfection. On reaching 60% confluency, the cells were exposed to an antisense oligonucleotide hybridizing to the pl-VDAC specific leader sequence (5’-CAC GAG AAA GGA TGA ACA C-3’) and then plated onto poly-l-lysine-coated coverslips. β-Globin antisense oligonucleotide (5’-CTT CTT ACC TCA GGT ACA ATT TAT A-3’) was used as the negative control. Immediately before transfection, cells were washed and placed in 200 μl of fresh serum-free Dulbecco’s modified Eagle’s medium. Each well received 200 ng of antisense pl-VDAC plus 100 ng of pEGFP plasmid (Clontech) diluted into 25 μl of serum-free DMEM. Cells were transfected by a LipofectAMINE Plus (Invitrogen) procedure following
Fig. 3. pl-VDAC antisense treatment of C1300 cells. A, confocal VDAC immunofluorescence images of C1300 cells treated with antisense oligonucleotides against pl-VDAC. B, EGFP fluorescence signal in the same cells as in A. C, transmitted light image of the cells. D, VDAC immunofluorescence analysis of C1300 cells transiently transfected with β-globin antisense oligonucleotides. E, EGFP fluorescence signal in the same cells as in D, F, transmitted light image of the cells. G, bar chart plot of VDAC immunofluorescence intensity versus EGFP signal intensity in C1300 cells transfected with pl-VDAC antisense.

the manufacturer’s instructions. Each transfection experiment was carried out in triplicate, and the cells were used for both patch-clamp and immunofluorescence experiments.

RNA Extraction and RT-PCR—RNA extraction and RT-PCR were performed as described previously (19). In brief, RNA was isolated from C1300 cells using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse-transcribed to cDNA. We used the following primer pair for PCR amplification of pl-VDAC (16): forward, 5’-TCA TGT GTC TCT TTC TCG TGC-3’ and reverse, 5’-CCA GTC TTC GGC GAG AAT GAC-3’. PCR products were analyzed on a 2% agarose gel containing a final concentration of 0.5 μg/ml ethidium bromide.

Electrophysiology and Statistics—C1300-transfected cells were plated on 15-mm poly-D-lysine-coated glass coverslips and mounted on the stage of an inverted Olympus IX70 microscope. Ion currents were recorded 48–72 h after transfection using the inside-out or whole-cell patch clamp modes as described previously (8). The pClamp8 software (Axon Instruments, Foster City, CA) was used for pulse generation, data acquisition through an Axon Digidata A/D interface, and subsequent analysis. Borosilicate glass patch pipettes had 3–5 megohms resistance and were filled with a solution containing 140 mM N-methyl-D-glucamine chloride, 1.2 mM MgCl₂, 1 mM EGTA, and 10 mM HEPES (295 mosmol/liter, pH 7.3). ATP (4 mM) and GTP (0.1 mM) were added to the pipette solution for whole-cell experiments. Cells were bathed in a solution containing 140 mM N-methyl-D-glucamine chloride, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES (305 mosmol/liter, pH 7.4), which was also the solution bathing the cytoplasmic face of the patch membrane in inside-out experiments. Data were collected using a List Medical n-6100 Darmstadt or Axopatch 200A amplifier. Inside-out currents were acquired at 1 kHz and low pass-filtered at 1 kHz. Membrane patches were clamped at 0 mV and pulsed for 5 s to both −80 mV and +80 mV. Whole-cell currents were acquired at 5 kHz and low pass-filtered at 1 kHz. Cells were clamped at 0 mV and pulsed for 500 ms from −80 to +80 mV in 40-mV steps before and after addition of toremifene (10 μM) to the bath solution. All experiments were performed at room temperature (22–26 °C). Data are expressed as means ± S.E. Differences between the three different groups were compared by one-way analysis of variance followed by the Bonferroni post-test for multiple comparisons. Data are taken as significant at a probability of 0.05.

RESULTS AND DISCUSSION

The presence of VDAC protein on C1300 cells was tested by Western blot (Fig. 1). The monoclonal anti-VDAC antibody used (anti-31HL, in particular Ab-2) was raised against an N-terminal synthetic peptide (21). Fig. 1A shows a Western blot obtained from a membrane fraction or whole lysate of C1300 cells. A single band of 32 kDa, which is the expected size for VDAC, was obtained from both preparations, indicating the absence of cross-reactivity with other cellular proteins. Contamination of the membrane fraction with organelle membranes was tested with antibodies against the cytochrome oxidase IV (a mitochondrial marker) or ribophorin I (endoplasmic reticulum marker). Fig. 1B shows that our membrane preparation is negative for the mitochondrial marker but positive for the marker of the endoplasmic reticulum, an organelle where the presence of VDAC has already been reported (22).

The experiments shown in Fig. 1, A and B, are consistent with the targeting of VDAC through two different pathways: the plasma membrane (via the endoplasmic reticulum/golgi pathway) and the mitochondria. The lack of a mechanism to explain how this protein is selected for its targeting to the plasma membrane, instead of the outer mitochondrial membrane, has fuelled the reticence to accept its extramitochondrial localization (15). However, the recent identification of an alternative exon in the murine vdac-1 gene evidenced a plasmalemmal form of VDAC (pl-VDAC) with an N-terminal leader sequence for its targeting to the plasma membrane (via the endoplasmic reticulum/golgi pathway) and the mitochondria. The presence of a pl-VDAC form in C1300 cells was confirmed by RT-PCR using primers specific to the pl-VDAC, including the leader sequence (Fig. 1C). A band of the expected size (350 bp) was identified and confirmed to be pl-VDAC by sequencing.

Another piece of evidence suggesting the presence of VDAC in the plasma membrane has been its isolation from liquid-ordered membrane microdomains called lipid rafts and functional reconstitution in artificial bilayers (17). To test whether VDAC was also present in such specialized membrane microdomains in C1300 cells, we carried out colocalization experiments using confocal microscopy. Two markers for lipid raft microdomains were used: the β₁ subunit of cholera toxin, which binds to GM₁ ganglioside, with which lipid rafts are enriched, and caveolin-1, a protein present in more structured lipid rafts with flask-shaped membrane invaginations termed caveolae (23, 24). Fig. 2 shows confocal images of C1300 cells probed with anti-VDAC antibody (Fig. 2, A and D, in red) and shows anti-caveolin 1 antibody (Fig. 2B, in green) or cholera toxin β₁.
subunit (CTX-β₁) conjugated to FITC (Fig. 2E, in green). Merged images (Fig. 2, C and F) show an almost complete overlap of CTX-β₁ and VDAC at the plasma membrane level, whereas no clear VDAC overlapping was observed with caveolin-1. These results reinforced previous observations suggesting the presence of VDAC at the plasma membrane, and similar to other cell types (17), VDAC in C1300 cells segregates to GM₄-containing lipid rafts. Caveolin-1 has been detected in cells from neuronal origin such as pheochromocytoma PC12 cells (25), although its localization varies with the degree of cell differentiation, mainly appearing in the soma of undifferentiated PC12 cells, similar to the pattern observed in our study with C1300 neuroblastoma cells. The apparent lack of colocalization of VDAC with caveolin-1 in C1300 cells might be related to the undifferentiated state of the cells and, therefore, the low presence of the caveolin-1 at the plasma membrane. However, experiments using differentiated C1300 cells, confirmed by the appearance of well developed neurites, did not modify the localization pattern of either caveolin-1 or VDAC.² Further immunocolocalization experiments using antibodies against other caveolin proteins expressed in neuronal cells will be necessary to elucidate the presence of VDAC in caveolae of C1300 cells.

The large conductance chloride channel, Maxi Cl⁻ channel, has been recorded under both cell-attached and whole-cell patch clamp conditions from C1300 cells exposed to triphenylethylene antiestrogens such as tamoxifen and toremifene (8). The activation of Maxi Cl⁻ channels appears to involve the interaction of antiestrogens with an external plasma membrane binding site and the generation of intracellular signals (8). The plasma membrane Maxi Cl⁻ channel shares many of the electrophysiological and pharmacological characteristics of the mitochondrial VDAC (11), implying that VDAC might be the molecular correlate of Maxi Cl⁻ channels (13). This hypothesis received additional experimental support from excised-patch recording studies of Maxi Cl⁻ channels from cells expressing heterologous VDAC protein (16, 26) and their functional inhibition with VDAC antibodies (26). However, these approaches can be criticized based on the fact that control cells used for transfection already express the channel of interest and, secondly, the absence of a correlation between immunodetection of VDAC protein and Maxi Cl⁻ channel activity in VDAC-transfected cells. Therefore, we set out to investigate whether endogenous pl-VDAC might underlie the antiestrogen-activated Maxi Cl⁻ current in C1300 cells. For that purpose, we transfected C1300 cells with antisense oligonucleotides against the 5' end of the murine vdac-1 gene. The oligonucleotide hybridized with the leader sequence required for targeting VDAC to the plasma membrane (16). The control antisense oligonucleotide was directed against an unrelated protein, β-globin. The use of an EGFP-expressing plasmid served as a reporter gene for the identification of transfected cells. Fig. 3 shows images of cells transfected with pl-VDAC antisense/EGFP (Fig. 3, A–C) or β-globin antisense/EGFP (Fig. 3, D–F). VDAC levels in transfected cells were determined by immunofluorescence confocal microscopy with the anti-VDAC antibody (Fig. 3, A and D) and the efficiency of the transfection by the production of EGFP protein (shown as the fluorescence signal in Fig. 3, B and E). From the images shown in Fig. 3 and the graph presented in Fig. 3G, a clear inverse correlation can be seen between the EGFP and VDAC signals in those cells transfected with pl-VDAC antisense and EGFP but not in those cells transfected with β-globin antisense/EGFP. Once we demonstrated that the pl-VDAC antisense oligonucleotide reduced VDAC levels, we proceeded to analyze the activity of Maxi Cl⁻ channels.

Whole-cell Maxi Cl⁻ currents were recorded under control conditions and following the addition of 10 μM toremifene to the bathing solution. A, whole-cell Cl⁻ currents recorded from a C1300 cell (8 picofarads) transfected with β-globin antisense/EGFP-expressing plasmid before and 5 min after the addition of 10 μM toremifene to the bathing solution. B, whole-cell Cl⁻ currents recorded from a C1300 cell (9 picofarads) transfected with pl-VDAC antisense/EGFP-expressing plasmid before and 5 min after the addition of 10 μM toremifene to the bathing solution. The cells selected for measurement of ion currents were those showing the highest EGFP signal. C, summary of Maxi-Cl peak current densities obtained at +80 mV under the conditions shown. Data are presented as mean ± S.E. (n = 7–11), p < 0.05 for untreated versus β-globin antisense-transfected cells, p = 0.02 for untransfected versus pl-VDAC antisense-transfected cells, and p = 0.01 for β-globin antisense-transfected versus pl-VDAC antisense-transfected cells.

² M. I. Bahamonde, unpublished observations.

Fig. 4. pl-VDAC antisense inhibition of toremifene-activated Maxi Cl⁻ currents. A, whole-cell Cl⁻ currents recorded from a C1300 cell (8 picofarads) transfected with β-globin antisense/EGFP-expressing plasmid before and 5 min after the addition of 10 μM toremifene to the bathing solution. B, whole-cell Cl⁻ currents recorded from a C1300 cell (9 picofarads) transfected with pl-VDAC antisense/EGFP-expressing plasmid before and 5 min after the addition of 10 μM toremifene to the bathing solution. The cells selected for measurement of ion currents were those showing the highest EGFP signal. C, summary of Maxi-Cl peak current densities obtained at +80 mV under the conditions shown. Data are presented as mean ± S.E. (n = 7–11), p < 0.05 for untreated versus β-globin antisense-transfected cells, p = 0.02 for untransfected versus pl-VDAC antisense-transfected cells, and p = 0.01 for β-globin antisense-transfected versus pl-VDAC antisense-transfected cells.
Maxi Cl\(^-\) channels can also be activated following the excision of the membrane patch containing the channel (1, 8). This stimulus was also used to evaluate the Maxi Cl\(^-\) channel activity in cells exposed to pl-VDAC antisense. Fig. 5 shows representative excised inside-out single channel recordings obtained from control cells (Fig. 4A) or pl-VDAC antisense-treated cells (Fig. 4B). In the control cells, typical Maxi Cl\(^-\) channel transitions of about 300 picosiemens were present. The voltage dependence was identical to the whole-cell currents described above with rapid inactivation at negative potentials. On the traces obtained from pl-VDAC antisense-treated cells, no such channel activity was observed. The probability of finding Maxi Cl\(^-\) channels in the excised patches should decrease with reduction in the amount of VDAC protein. Accordingly, Maxi Cl\(^-\) channel activity was detected in 100% (8/8) of patches obtained from control cells but also in 60% (10/16) of patches obtained from pl-VDAC-treated cells, without changes in the single channel conductance (280 ± 15 picosiemens in control cells, n = 8, and 270 ± 10 picosiemens in pl-VDAC antisense-treated cells, n = 10; p = 0.5) or the open probability (results not shown). However, we acknowledge that these experiments are less representative than the whole-cell experiments described above, where the activity of all channels present in the plasma membrane was studied. Nevertheless, the Maxi Cl\(^-\) channel activity was reduced in pl-VDAC antisense-treated cells, regardless of the stimuli used (activation by toremifene or membrane excision). Mitochondrial VDAC is inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Therefore, we carried out experiments to check whether the plasma membrane Maxi Cl\(^-\) channel was also inhibited by DIDS. Fig. 5C shows current-voltage relationships obtained from control cells treated with toremifene in the presence or absence of 100 μM DIDS. Addition of DIDS completely abolished the toremifene-activated Maxi Cl\(^-\) currents of C1300 cells.

Voltage-dependent anion channels (VDAC), also known as porins, are integral membrane proteins that form a pore slightly more permeable to anions than cations and also permeable to small solutes. They have been identified in the mitochondrial outer membrane, where they provide a major pathway for the transport of metabolites, e.g. ATP (27) and cholesterol (28), among others. They have also been involved in the mitochondrial events leading to apoptosis (4, 5). Besides their mitochondrial location, several reports claim the presence of VDAC in the plasma membrane of different cell types (13, 14, 16, 17). In the present study, in addition to demonstrating the association of VDAC with membrane lipid rafts of C1300 neuroblastoma cells, we have provided the first molecular evidence relating VDAC with plasma membrane Maxi Cl\(^-\) channels activated by antiestrogens. The identification of the molecular nature of the Maxi Cl\(^-\) channel will allow us to study whether its regulation by estrogen and antiestrogens presents a double pathway, involving the generation of intracellular signals and a direct interaction of the hormones with the channel protein, similar to the modulation of Maxi K\(^+\) channels by estrogens (29, 30).

Acknowledgment—We thank A. Currid for proofreading the manuscript.

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