Mitochondrial voltage-dependent anion channel is involved in dopamine-induced apoptosis

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Abstract

Neuronal NMB cells were used to determine changes in gene expression upon treatment with dopamine. Twelve differentially expressed cDNAs were identified and cloned, one of them having 99.4% sequence homology with isoform 2 of a voltage-dependent anion channel (VDAC-2). The known role of VDAC, a mitochondrial outer-membrane protein, in transport of anions, pore formation, and release of cytochrome C prompted us to investigate the possible role of VDAC gene family in dopamine-induced apoptosis. Semi-quantitative PCR analysis indicated that expression of the three VDAC isoforms was reduced by dopamine. Immunoblotting with anti-VDAC antibodies detected two VDAC protein bands of 33 and 34 kDa. Dopamine decreased differentially the immunoreactivity of the 34 kDa protein. Whether the decrease in VDAC expression influence the mitochondrial membrane potential (ΔΨm) was determined with the dye Rhodamine-123. Dopamine indeed decreased the mitochondrial ΔΨm, but the maximum effect was observed within 3 h, prior to the decrease in VDAC mRNA or protein levels. Cyclosporin A, a blocker of the mitochondrial pore complex, prevented the decrease in ΔΨm, but did not rescue the cells from dopamine toxicity. To elucidate possible involvement of protease caspases in dopamine-induced apoptosis, the effect of the caspase inhibitor z-Val-Ala-Asp(Ome)-FMK (zVAD) was determined. zVAD decreased dopamine toxicity, yet it did not rescue the mitochondrial ΔΨm drop. Dopamine also decreased ATP levels. Finally, transfection of NMB cells with pcDNA–VDAC decreased the cytotoxic effect of dopamine. These findings are in agreement with the notion that the mitochondria, and VDAC, are important participants in dopamine-induced apoptosis.

Keywords: differential display PCR, immediate-early genes, ion channel, neurodegeneration, Parkinson’s disease.


It is now well established that dopamine triggers programmed neuronal cell death (apoptosis) in cultured cells (Ziv et al. 1994; Simantov et al. 1996; Coronas et al. 1997; Junn and Mouradian 2001). Oxidation products and reactive oxygen species (Jenner et al. 1992; Korsmeyer 1995), lipid peroxidation (Groc et al. 2001), depletion in glutathione (Riederer et al. 1989; Sofic et al. 1992; Gabbay et al. 1996), and other neurochemical changes (Olanow 1993) may be responsible for dopamine toxicity. The neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, often used as experimental models for Parkinson’s disease, interact with the mitochondria and inhibit complex I activity (Gerlach et al. 1991; Cleeter et al. 1992; Tipton and Singer 1993). As to dopamine, however, the question of direct or indirect interaction with the mitochondria, and the potential molecular targets, is still enigmatic.

Programmed cell death may be triggered by activation of mitochondria-dependent or -independent pathways, as reviewed by others (Green and Reed 1998; Li and Yuan 1999; Orth and Schapira 2001). The signaling pathway leading to apoptosis via the mitochondria is triggered by the binding of proteins of the Bcl2 family, such as Bcl2, Bax and Bad, to the mitochondrial outer membrane. This interaction forms large pores in the mitochondria membrane, through which cytochrome c is released (Desagher and Martinou 2000; Gros et al. 2000; Harris and Thompson 2000; Zamzami et al. 2000 and references therein). It has been shown that a voltage-dependent anion channel (VDAC)
located at the mitochondrial outer membrane plays a key role in the formation of these pores, and in cytochrome c release (Shimizu et al. 1999, 2000; Tsujimoto and Shimizu 2000). Microinjection of anti-VDAC antibodies significantly inhibited apoptotic cell death induced by three different agents (Shimizu et al. 2001). VDAC make up a gene family of several genes sharing high sequence homology, and three isoforms were identified in human cells (Blachly Dyson et al. 1993, 1994).

In an effort to verify further the molecular mechanisms underlying dopamine-induced apoptosis and cell differentiation (Simantov et al. 1996; Porat and Simantov 1999; Porat et al. 2001), the differential display PCR (DD–PCR) approach was used to determine changes in gene expression upon dopamine treatment. Out of 12 cDNAs differentially expressed, isolated and sequenced, the current report is focused on the voltage-dependent ion channel VDAC.

**Materials and methods**

**Materials**

Dopamine, rotenone, MPP⁺, ethidium bromide, propidium iodide, agaroase RNase A, ethidium bromide, cyclosporin A, and protease inhibitors cocktail were purchased from Sigma (St Louis, MO, USA). Tri-reagent was from Molecular Research Center (Cincinnati, OH, USA), Luciferin-luciferase kit (ENLITEN) and pGEM-T cloning vector were from Promega (Madison, WI, USA), and MTT Cell Proliferation Kit 1 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)-2-(4-nitrophenyl) was from Roche Diagnostic (Indianapolis, IN, USA). Rhodamine-123 (Rh123) was from Mowiol bromid))-2-(4-nitrophenyl) was from Roche Diagnostic (Indianapolis, IN, USA). Rhodamine-123 (Rh123) was from Molecular Probes (Eugene, OR, USA), Taq Zol DNA polymerase from Biological Industries (Bef Haemek, Israel), z-Val-Ala-Asp(Ome)-FMK (zVAD) from Alexis Biochemicals (Lausen, Switzerland), and iron-supplemented calf serum from HyClone (Logan, UT, USA). Gene Shuttle transfection reagent was from Qbiogene Inc. (Illkirch, France), anti-VDAC antibody from Affinity Bioreagents Inc. (Golden, CO, USA), and α-32P-dATP and horseradish–peroxidase-conjugated anti-rabbit IgG from Amersham (Piscataway, NJ, USA). All other compounds used were of analytical grade.

**Cell cultures and viability assay**

NMB cells were kindly provided by Drs M. N. Goldstein, University of St Louis, MO, USA and Y. Wollman, Department of Nephrology, Tel-Aviv Medical Center, Tel-Aviv, Israel, adapted to Dulbecco’s modified Eagle medium (DMEM) with 5% HyClone calf serum and cultured as reported (Simantov et al. 1996). Cytotoxicity was determined by the MTT Cell Proliferation Kit I, and statistical significance was determined by ANOVA and the Student’s t-test.

**RNA isolation, DD–PCR analysis, and identifying cDNAs induced by dopamine**

Total RNA was extracted with Tri Reagent. For DD–PCR, mRNA was isolated from total RNA using the Oligotex kit (Qiagen, Valencia, CA, USA) and reverse transcribed with oligo dT anchored primers. Each reaction containing 0.2 μg RNA template and 0.2 μM oligo dT primer was incubated at 65°C for 10 min, then cooled on ice for 2 min. cDNA synthesis was initiated by addition of 1 x first-stand buffer (Life Technologies, Rockville, MD, USA), 25 μM dNTPs, 10 μM diethiothiol (DTT), and 2 units SuperScript II RT RNase H reverse transcriptase in a 20-μL reaction volume, and incubated at 42°C for 50 min. DD–PCR were carried out by mixing 0.1 volume of the first-strand cDNA, 1 x PCR buffer [made of 50 mM Tris–HCl (pH 9.1), 16 mM ammonium sulfate, 3.5 mM MgCl₂ and 150 μM/μL bovine serum albumin (BSA)], 20 μM dNTPs, 2.5 μM primer, and 1 unit Taq Zol DNA polymerase. The conditions for PCR were as follows: 5 min at 95°C, 35 cycles of 1 min at 92°C and 50 s at 46°C, and finally 2 min of an extension period at 72°C. Samples were then mixed with an equal volume of the loading buffer, denatured for 2 min at 95°C and analyzed on a 6% denaturing polyacrylamide gel (4 h at 500 V). The gels were dried, exposed to an X-ray film, differentially expressed bands were excised, transferred to 100 μL sterile water, boiled for 15 min, and centrifuged for 10 min at 10 000 g. The supernatant (10 μL) was used as a template for reamplification (40 cycles) at the same conditions with the same primers, and the products cloned into a pGEM-T. Cloned DNA fragments were sequenced and compared with genome databank using blast search.

**Semi-quantitative RT–PCR**

The semi quantitative analysis of vdac mRNA levels was carried out by RT–PCR. Two micrograms of DNase-digested total RNA were reverse transcribed using superscript reverse transcriptase, by following the recommendations of the manufacturer. The following primers were used to amplify the corresponding VDAC isoforms.

VDAC1: F– 5¢-GCCAAGTACAGATTGCCACC-3¢, R– 5¢-CTTGGAAATTTGACGTCTAAGGGC-3¢;
VDAC2: F– 5¢-CTACCCTCCTACCCAAACACAG-3¢, R– 5¢-CCAGGACAGAGAGTGAAGG-3¢;

The DNA products of the three VDAC isoforms provided the expected product size (180 bp, 480 bp, and 175 bp, respectively). A 317-bp 18S rRNA was amplified in parallel in separate reactions for normalization of the results by using the primers F– 5¢-TCAAGCGGTCGATTCC-3¢ and R– 5¢-AAATACCCAGACATGGCTCC-3¢. Each PCR reaction contained mRNA template, 1 x Taq DNA polymerase buffer, 0.2 μM primer, 200 μM dNTPs and 2 units of Taq DNA polymerase. The PCR amplification consisted of 19–21 cycles of 1 min at 94°C, 40 s at 58°C and 1 min at 72°C. Relative levels of VDAC gene expressions were calculated by determining the ratio between the vdac products and the internal 18S ribosomal RNA.

**VDAC immunoblotting**

Cells were washed once with phosphate-buffered saline (PBS), lysed with buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate and 10 μL/mL protease inhibitors cocktail. Protein concentrations were determined using a BCA kit. Protein samples of 40 μg were separated by 10% SDS polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) using miniblot transfer apparatus. After blocking for 1 h in PBS containing 5% non-fat milk and 0.5% Tween-20 (PBST) followed by three washes in PBST, membranes were incubated for 2 h with

Dopamine-induced apoptosis and VDAC

1:1000 rabbit polyclonal anti-VDAC antibody in the blocking solution. Membranes were then washed and incubated with an HRP-conjugated second antibody for 1 h and washed. Immuno-reactivity was detected using ECL kit according to the manufacturer’s instructions.

Determination of mitochondrial membrane potential and ATP

NMB cells were incubated for 20 min with Rh123 at 10 μM, washed twice with PBS, resuspended in PBS, and analyzed in FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a single 488 Argon laser. The filter in front of the fluorescence 1 (FL1) photomultiplier transmits at 530 nm and the filter used in the FL2 channel transmits at 617 nm. To eliminate dead cells, propidium iodide (2 μg/mL) was added 20 min before the FACS analysis. It was verified that propidium iodide did not interfere with Rh123 reading. At least 2 × 10^4 cells per sample were analyzed. As a positive control, the protonophore cccp (carbonyl cyanide p-chlorophenyl hydrazone, at 50 μM) was used to fully collapse the mitochondrial membrane potential. In another set of experiments, 2 μM Rh123 was used (without washing), with practically identical results.

Cellular ATP content was determined with the luciferin-luciferase assay. In brief, cells were washed once with PBS, resuspended in PBS, sonicated on ice, and centrifuged at 10 000 g for 10 min at 4°C. Twenty microliters of the supernatant were added with luciferin-luciferase reagent and luminescence read immediately using a luminometer (Turnet TD-20e). A standard curve of known ATP concentrations was used to calculate ATP concentration in the samples.

Expression vectors and transfection

The full-length hVDAC or hVDAC2 cDNA (kindly provided by Dr M. Forte, Vollum Institute, Portland, OR, USA) or GFP were inserted into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA was purified using Qiagen Maxiprep columns, and sequenced to confirm identity. For transfection of NMB cells, 2 μg of plasmid DNA were mixed with 6 μL Gene shuttle reagent in 1 mL of DMEM without antibiotics and serum, added to the cells and incubated for 10 h. Then, the transfection medium was replaced with fresh DMEM containing serum and antibiotics, and the cells were further cultured for 24 h before testing.

Results

Identifying cDNAs and genes triggered by dopamine; down-regulation of VDAC

DD–PCR analysis of RNA extracted from control and dopamine-treated NMB cells showed several cDNAs that were differentially expressed upon treatment with dopamine (20–100 μM). Twelve of such cDNAs, ranging from 220 to 576 bp were extracted from the gels, cloned into pGEM-T cloning vector (see Materials and methods), and sequenced. Table 1 shows that six of the isolated cDNAs show high homology with previously isolated genes, including Tis 11D, stathmin, VDAC-2 and elongation factor 1, two are homologs of ESTs, and four are novel sequences. Dopamine had a characteristic effect on the expression of these cDNAs, either an increase or decrease, as determined with the DD–PCR (Table 1). Up- or down-regulation of the known genes (Tis 11D, VDAC-2, Stathmin, AFIQ, VPS4B and Elongation Factor 1) was confirmed with specific primers designed according to the reported sequence of each gene (data not shown). The significance of these changes in the process of dopamine-induced apoptosis or cell differentiation need further studies. Herein we focus on the 319-bp cloned cDNA, which had 99.4% sequence homology with isoform 2 of hVDAC (Blachly Dyson et al. 1993).

To verify the effect of dopamine on expression of the VDAC gene family, specific primers for the three human isoforms were designed according to published sequences (Blachly Dyson et al. 1993, 1994), and semi-quantitative PCR was carried out with RNA isolated from dopamine-treated and untreated cells. Figure 1(a) shows a time-dependent decrease in expression of the three VDAC isoforms upon treatment with 100 μM dopamine, with 30–44% and 65–75% reduction after 12 or 24 h treatment, respectively. Dose-dependent analysis of dopamine treatment showed no change in expression of VDAC1, 2, and 3 with 50 μM dopamine, but clear reduction in the three isoforms with 100 and 200 μM dopamine (Fig. 1b). These PCR data were also scanned, indicating a significant effect of dopamine at the two higher concentrations (Fig. 1c).

To verify whether the changes in mRNA also affect VDAC expression at the translation level, proteins were extracted and analyzed by western immunoblotting with

<table>
<thead>
<tr>
<th>Treatment [dopamine], μM</th>
<th>cDNA fragment (bp)</th>
<th>Identity (% Homology)</th>
<th>Expression profile</th>
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<tr>
<td>20</td>
<td>252</td>
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<tr>
<td>20</td>
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<td>360</td>
<td>Stathmin (99)</td>
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<td>463</td>
<td>AFIQ (100)</td>
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<td>20</td>
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<tr>
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<td>265</td>
<td>VPS4B (99)</td>
<td>Up</td>
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<tr>
<td>100</td>
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Differentially expressed DNA fragments were isolated from DNA sequencing gels, prepared from cells treated with 20 or 100 μM dopamine, cloned and sequenced as described in Materials and methods. Increased or decreased expression of each cDNA relative to control (untreated) cells are shown as Up or Down. GeneBank accession numbers for Tis 11D, VDAC-2, Stathmin, AFIQ, VPS4B and Elongation Factor 1 were U07802, L06328, J04991, U16954, AF282904 and X03558, respectively.
specific anti-VDAC antibodies. Unexpectedly, two protein bands with the size of about 33 and 34 kDa were identified (Fig. 2a). As to the effect of dopamine, it caused a time- and dose-dependent decrease in the expression of the 34 kDa protein band, with a much smaller effect on the 33 kDa protein (Fig. 2a,b). The specificity in dopamine effect on VDAC expression was indicated by that MPP⁺, an inhibitor of the mitochondrial complex I energy chain, that is also toxic to dopaminergic neurons, did not alter VDAC protein expression (Fig. 2c). Likewise, rotenone did not alter VDAC expression (data not shown).

Dopamine effect on the mitochondrial membrane potential; involvement of the mitochondrial transition pore, caspases and ATP

VDAC dysfunction associated with altered mitochondrial transmembrane potential \( (\Delta \Psi_m) \) is an early indication of apoptosis in several cell death systems (Harris and Thompson 2000; Nicholls and Ward 2000). To determine whether the dopamine-induced down-regulation of VDAC expression was also accompanied with changes in the mitochondrial membrane potential \( \Delta \Psi_m \), dopamine-treated cells were collected at different time points, incubated with Rh123 and analyzed by flow cytometry (see Materials and methods). Figure 3 shows a shift in Rh123 fluorescence to the left within 3 h, indicating that dopamine decreases the mitochondrial membrane potential \( \Delta \Psi_m \). The drop in \( \Delta \Psi_m \) persisted for 12 h, and returned to control values at 24 h after treatment.

As cyclosporin A interacts with the mitochondrial pore complex by binding to cyclophilin D, and close or maintains the pore closed, it was used to verify whether the dopamine-induced drop in the mitochondrial \( \Delta \Psi_m \) is mediated by the pore complex. Cells were therefore pre-incubated for 30 min with cyclosporin A before adding dopamine, and the \( \Delta \Psi_m \) was tested with Rh123. Figure 4 (panel 2) shows that cyclosporin A inhibited the dopamine-induced \( \Delta \Psi_m \) dissipation, but had no effect on \( \Delta \Psi_m \) on its own (Fig. 4, panel 3). The protonophore cccp, known to dissipate the mitochondrial membrane potential, was used as a positive control to verify Rh123 labeling of the mitochondrial.

Fig. 1 Time- and dose-dependent effect of dopamine on VDAC-1, -2 and -3 mRNA expression in NMB cells. (a) RT-PCR analysis of cells treated with 100 \( \mu \)M dopamine for 4, 12 or 24 h. (b) RT-PCR analysis of cells treated with 0–200 \( \mu \)M dopamine for 24 h. (c) Quantitative presentation of the RT–PCR data of (b) after normalization with 18S. Data are mean ± SD from four to six independent experiments.

Fig. 2 Time-course and dose-dependent effect of dopamine on VDAC protein expression, determined by western immunoblotting. (a) Cells were treated for 0–24 h with 100 \( \mu \)M dopamine. (b) Cells were treated with 0–200 \( \mu \)M dopamine for 24 h. The VDAC immunoreactive bands were scanned and results presented as mean ± SD. (c) VDAC western immunoblotting of cells treated with 0–100 \( \mu \)M MPP⁺, determined in triplicates.
Considering that at high concentrations cyclosporin A might have other effects, including induction of cell death, it was essential to verify the sensitivity of NMB cells to this compound. Figure 5 shows that cyclosporin A was not cytotoxic to NMB cells at 1–6 \( \mu \text{M} \), and it did not rescue cell death upon dopamine treatment at these concentrations.

In several systems, VDAC malfunction resulting with apoptosis involves the release of cytochrome C through the mitochondrial transition pore. To verify whether dopamine-induced apoptosis is associated with activation of proteases of the caspase family, indicative for cytochrome C release, NMB cells were pre-treated with either the broad-spectrum caspase inhibitor zVAD or with a specific caspase 1 inhibitor. Figure 6 shows that zVAD partially (43%) rescued the cells from dopamine toxicity, whereas caspase 1 inhibitor had no such effect. zVAD had no effect on the mitochondrial \( \Delta \Psi_m \) in both dopamine-treated (Fig. 4, panel 4) and control cells (Fig. 4, panel 5).

In cooperation with another pore complex protein, adenine nucleotide translocator (ANT), VDAC is a key player in the exchange of ADP/ATP from cytosol to mitochondria. To verify whether the decreased VDAC expression impairs ATP homeostasis, cellular ATP level was measured at different time points after dopamine treatment. Figure 7 shows a significant drop in the overall ATP level within 4 h, and 52% reduction at 24 h after treatment. Examining ATP levels in the mitochondria showed a similar but a faster, effect of dopamine, with a 48% decrease in ATP levels within 1 h.

**Transfection of NMB cells with pcDNA–VDAC**

The role of VDAC was further studied with NMB cells transfected transiently with pcDNA–VDAC. As control, we used cells transfected with pcDNA–GFP, also useful to determine transfection efficiency. Using 2 \( \mu \text{g} \) plasmid DNA, the efficiency of pcDNA–GFP transfection after 36 h was 45 ± 5%. Figure 8 shows that in pcDNA–GFP-transfected cells, 100 \( \mu \text{M} \) dopamine decreased cell number by 64.4 ± 9.9%, similar to non-transfected cells (Gabbay et al. 1996; Simantov et al. 1996). Interestingly, in pcDNA–VDAC-transfected cells dopamine effect was significantly

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**Fig. 3** Mitochondrial membrane potential of NMB cells treated with 100 \( \mu \text{M} \) dopamine for 1, 3, 12 and 24 h. Rh123 fluorescence was determined by flow cytometry as described in Materials and methods. The heavy line shows the profile of control (untreated) cells.

**Fig. 4** Effect of cyclosporin A and zVAD on the mitochondrial membrane potential. Each panel shows the membrane potential of control cells (heavy line) compared with cells treated as follows: 1, dopamine (100 \( \mu \text{M} \); 2, cyclosporin A (5 \( \mu \text{M} \)) + dopamine; 3, cyclosporin A (6 \( \mu \text{M} \)); 4, zVAD (50 \( \mu \text{M} \)) + dopamine; 5, zVAD (50 \( \mu \text{M} \)); and 6, cccp (50 \( \mu \text{M} \)) as a positive control for fully dissipated membrane potential.

**Fig. 5** Effect of cyclosporin A on dopamine toxicity. Cells were treated without (□) or with (●) 100 \( \mu \text{M} \) dopamine and various concentration of cyclosporin A, and cell viability was determined by the MTT assay. Data are mean ± SD from six replicates. Cyclosporin A at 1–6 \( \mu \text{M} \) had no significant effect on dopamine toxicity (compared with 0 dopamine), and was not cytotoxic on its own.
more modest, a decrease of 7.8 ± 20.6% in the number of viable cells. It was also of interest that the growth rate of cells transfected with pcDNA–VDAC (without dopamine treatment) was slower than pcDNA–GFP transfected cells, being 25.5 ± 11.9% lower at 36 h after transfection (Fig. 8).

Discussion

The human VDAC gene family has three fully sequenced isoforms (Blachly Dyson et al. 1993, 1994). The isoform identified herein, hVDAC-2 has 66% and 71% sequence homology with hVDAC-1 and hVDAC-3, respectively. Tissue-specific alternative splicing of the VDAC gene resulted with two VDAC proteins apparently differing in one amino acid (Sampson et al. 1998). VDAC encodes for a pore-forming mitochondrial protein, which is involved in controlling cell death in association with other pore-forming mitochondrial proteins, including the adenine nucleotide translocator (Pastorino et al. 1998; Shimizu et al. 1999).

Other proteins, primarily of the Bcl-2 protein family, interact with the mitochondrial pore complex, modulate the release of cytochrome c, and act as pro- or anti-apoptotic stimulants (Vander Heiden et al. 1997; Narita et al. 1998; Shimizu et al. 1999; Harris and Thompson 2000).

The main finding of the current study is that, in neuronal NMB cells, dopamine at concentrations inducing apoptosis down-regulates the expression of VDAC-1, -2 and -3 at the mRNA level. Moreover, these cells express two immuno-reactive VDAC proteins of about 33 and 34 kDa, and expression of the 34 kDa protein is more vulnerable to dopamine treatment. As the alternative splicing of the VDAC gene (Sampson et al. 1998) results in only one additional methionine, it is unlikely that the two proteins detected in the current work, differing by about 1 kDa, reflect this divergence. The gene origin and molecular nature of these two VDAC immunoreactive proteins, and the mechanism underlying the selective effect of dopamine on expression of the larger protein needs further examination. It is unclear, e.g. whether the 33 kDa protein, not regulated by dopamine, is...
expressed by one or two VDAC genes, and controlled at the mRNA but not the protein level, or whether the three genes express both proteins, regulated differentially at the translation level. Thus, post-translational regulation including different turnover rate and half-life cannot be excluded. MPP\(^+\) and rotenone, other models of dopaminergic cell death, did not induce down-regulation of VDAC, evidently involving alternative death processes. Considering the interaction between Bcl-2 and VDAC (Shimizu et al. 1999, 2000; Tsujimoto and Shimizu 2000) and the observation that transfection of NMB cells with Bcl-2 protects from dopamine-induced apoptosis (Porat and Simantov 1999), the interplay between VDAC and Bcl-2 in controlling dopamine neurotoxicity needs further analysis.

The decrease in VDAC expression was accompanied with a decrease in the mitochondria membrane potential \(\Delta \Psi_m\), detected by the dye Rh123. Dose- and time-dependent studies, as well as treatment with the protonophore cccp, confirmed that Rh123 reading reflects mitochondrial membrane potential. Dopamine-induced drop in Rh123 reading indicative of the mitochondria \(\Delta \Psi_m\) was confirmed recently in human B-lymphocytes (Elkashef et al. 2002). Moreover, we recently studied dopamine effect on permeability transition pore opening with fluorescent calcein–AM, according to the method of Petronilli et al. (1999). Using a fluorescent microscope, we observed profound changes in fluorescence intensity in the mitochondria upon dopamine application, indicating that dopamine keeps the pore open, and drops the mitochondria \(\Delta \Psi_m\) (A. Premkumar and R. Simantov, unpublished data), therefore confirming the results obtained herein with Rh123.

Based on the time-course analysis of the change in mitochondria membrane potential \(\Delta \Psi_m\), it is unlikely that the decrease in VDAC is responsible for the drop in \(\Delta \Psi_m\); this drop precedes the decreased expression of VDAC, and then recovers. Treatment with cyclosporin A, which binds to cyclophilin D and blocks the mitochondrial pore complex, prevented the decrease in \(\Delta \Psi_m\) induced by dopamine. Yet, cyclophilin A failed to rescue cell death. It appears therefore that the dopamine-induced decrease in mitochondrial \(\Delta \Psi_m\) may depend on an active (open) mitochondrial pore complex, but this drop in \(\Delta \Psi_m\) is not essential for dopamine-induced cell death. The drop in the mitochondrial \(\Delta \Psi_m\), apparently transient as it recovers after 24 h, may reflect the dopamine-induced drop in ATP (Fig. 7), although a modest increase in \(\text{Ca}^{2+}\) uptake by the mitochondria cannot be ruled out. Indeed, a recent study showed that dopamine, but not MPP\(^+\), enhanced \(\text{Ca}^{2+}\) uptake by the mitochondria (Lee et al. 2002). Also, it has been widely documented that free radicals, prevalent products of dopamine, can disturb the mitochondrial proton pump, and thus contribute to the drop in the \(\Delta \Psi_m\).

The observation that the caspase inhibitor zVAD diminished dopamine toxicity, but not the drop in mitochondrial \(\Delta \Psi_m\), is in line with the notion that activation of caspases is downstream in the programmed cell death process (e.g. Fraser and Evan 1996; Vander Heiden et al. 1997). As indicated in several experiments, again the dopamine-induced apoptosis differed from rotenone-induced apoptosis, which was inhibited by cyclosporin A (Isenberg and Klaunig 2000).

Transfection of NMB cells with pcDNA–VDAC decreased their vulnerability to the toxic effect of dopamine. Although it is premature to speculate about the mechanism responsible for this apparent protection, one should consider the possibility that overexpression of VDAC can alter ADP/ATP exchange and ion transport. Dopamine-induced damage to the mitochondrial membrane potential and cellular ATP levels may reflect a restrained or defective activity of VDAC, and the mitochondrial pore. Overexpression of VDAC may provide protection by restoring normal activity of the mitochondrial pore complex. Whether VDAC play a role in mitochondrial malfunction in neurodegenerative disorders, including Parkinson’s disease, is worth further examination.

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