Overexpression of Bcl-2 suppresses the calcium activation of a mitochondrial megachannel

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Abstract The molecular mechanism(s) by which Bcl-2 regulates apoptosis is poorly understood. Bcl-2 suppresses apoptosis by inhibiting calcium activation of the permeability transition of mitochondria. In this patch-clamp study, overexpression of Bcl-2 in mitochondria of cultured cells suppressed calcium activation of a high conductance channel that may underlie the permeability transition. All other single channel parameters were identical when multiple conductance channel activities of mitochondria from control and Bcl-2 overexpressing cells were compared. Bcl-2 forms channels in artificial membranes; however, no novel channel activities could be linked to Bcl-2 overexpression, suggesting Bcl-2 does not form channels in native inner membranes of mitochondria. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrion; Apoptosis; Bcl-2; Patch clamp; Channel

1. Introduction

Mitochondria play a pivotal role in the process of apoptosis. Release of various mitochondrial components, e.g. cytochrome *c*, from the intermembrane space of mitochondria is involved in the commitment step of the apoptotic cascade [1–5]. In addition, the anti-apoptotic factor, Bcl-2, is located in mitochondria [6] and its presence prevents the release of cytochrome *c* [5].

One model for initiation of apoptosis proposes that release of the mitochondrial components of the cascade is the result of opening a megachannel in the mitochondrial inner membrane (e.g. [8]). Opening of this megachannel or multiple conductance channel (MCC) (also referred to as the permeability transition pore or PTP, see [7]) causes swelling of the matrix space. This swelling ruptures the outer membrane and spills pro-apoptotic factors into the cytoplasm. The involvement of the PTP is supported by its binding of the pro-apoptotic factor Bax [9]. Conversely, Bcl-2 was found to inhibit the opening of the PTP [1–3,10]. The permeability transition is, in fact, triggered by many agents such as Ca^{2+} and activated oxygen that are implicated in apoptosis [11,12]. Experiments in which blockage of the permeability transition also prevented apoptosis support this model [1–3,13,14].

The effect of Ca^{2+} is of particular interest. Ca^{2+} participates in apoptosis and, in addition, has been shown to open the MCC [28,29]. Interestingly, Jonas et al. [15] demonstrated the opening of high conductance mitochondrial channels (possibly MCC) in pre-synaptic terminals of the squid following stimulation. The opening of the channels may be attributed to Ca^{2+} since Ca^{2+} is a well-known mediator of neurotransmitter release by exocytosis. Overexpression of Bcl-2 [16] increased the capacity of mitochondria to accumulate Ca^{2+} and resist Ca^{2+}-induced respiratory injury, e.g. inhibition of NADH-dependent respiration. Similar results were obtained comparing normal liver mitochondria to hepatoma mitochondria that overexpress Bcl-2 [17]. The different properties of the two sets of mitochondria were ascribed to the increased amount of Bcl-2 in the hepatoma mitochondria estimated by Western blotting. In addition, Bcl-2 antibodies stimulated the permeability transition of hepatoma mitochondria. More recently, experiments with Bcl-2 overexpressing cells were carried out using organelle-targeted aequorin, a Ca^{2+}-sensitive photoprotein [18]. The Bcl-2 overexpression reduced the Ca^{2+} levels in the endoplasmic reticulum and Golgi. In addition, Bcl-2 overexpression decreased the magnitude of cytoplasmic and mitochondrial Ca^{2+} transients induced by inositol 1,4,5-triphosphate generating compounds. Finally, Bcl-2 decreased a Ca^{2+} store depletion response that opens Ca^{2+} channels of the plasma membrane, the so-called capacitative calcium entry.

These experiments suggest that the protective effect of Bcl-2 may involve inhibition of the opening of mitochondrial channels induced by Ca^{2+}. Although experiments have suggested an interaction between Bcl-2 and mitochondrial channels, little is known of how this takes place. The present study was initiated to pursue this question using patch-clamp techniques. In addition, since Bcl-2 has been shown to form channels when added to artificial lipid bilayers [19], it was deemed important to find out whether Bcl-2 normally formed channels in mitochondrial native membranes. The results show that Bcl-2 overexpression decreased the activation of the MCC by Ca^{2+}. However, no novel channels were detected in the mitochondria from Bcl-2 overexpressing cells.

2. Materials and methods

MDA-MB-231 (ATCC# HTB-26) human breast cancer cells were transfected by the calcium phosphate method with either plasmid pCΔj-SV-2 (vector-only) or pCΔj-bcl-2 (bcl-2) that had been generously provided by Y. Tsujimoto [20]. Following transfection, the cells were selected in 1 g/l G418 until individual colonies appeared. Clones were collected separately and analyzed for Bcl-2 expression by West-

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Abbreviations: MCC, multiple conductance channel

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ern blot. Antibodies were from Dako (Carpinteria, CA, USA; Cat# M0887). One of those clones, designated bcl2-1, expressed high levels of Bcl-2 protein in its mitochondrial fraction and was used for the present study. A vector-only-transfected clone that does not express detectable levels of Bcl-2 was designated SV2 and chosen as a control.

Cells, cultured in IMEM Richters modification with 25 mg/l gentamicin and 2 mM glutamine (5% CO_2, 37°C), were harvested with trypsin when the population density was close to confluence. Cells (pellet volume between ~0.3 and 1.2 ml) were re-suspended in 10 ml 1× isolation medium (230 mM mannitol, 70 mM sucrose, 5 mM HEPES pH 7.4) and homogenized by hand for three strokes. The remainder of the mitochondrial isolation was a modification of the method of Campo et al. [21]. Mitoplasts were prepared from mitochondria by passage through a French press at 2000 psi according to the method of Decker and Greenawalt [22] as previously described [23]. Mitochondria were morphologically distinct from mitoplasts and were easily avoided during patch-clamp experiments.

Micropipets (10–40 MΩ) were formed on a Sutter model PC-84 or PC-87 puller. High resistance seals (>750 MΩ) were obtained by pressing a micropipet onto the membrane of a mitoplast, with or without negative pressure. Patches were excised so that the matrix face of the inner membrane was exposed to the bath. The remainder of the conditions and procedures used were as described by Kinnally et al. [24]. Voltage-clamp conditions were maintained with a Dagan 3900A (Minneapolis, MN, USA) patch-clamp amplifier in the inside-out mode. Voltages across patches excised from mitoplasts were reported as bath (i.e. matrix) potentials, where $V = V_{bath} - V_{pipet}$. Voltage and current were digitized with a NeuroData Neurocorder Digitizer model DR390 (New York, NY, USA) and stored on videotape at 10 kHz. Computer analysis of current signals was bandwidth-limited to 2 kHz with a low pass filter (model 902 Frequency Devices, Haverhill, MA, USA) and sampling of 5 kHz. Analysis was done with Strathclyde Electrophysiological Software (PAT, courtesy of J. Dempster) [25] through a DT2801A (Data Translation, Marlboro, MA, USA) analog–digital board. Open probability, P_o, was calculated as the fraction of the total time spent in the fully open state from total amplitude histograms of current traces of 20–60 s in duration. Patches were included in surveys of MCC detection frequency if the seal was >0.75 GΩ. Typically, MCC was considered present if current transitions were >250 pS, the P_o was above 0.5 at negative voltages, and the activity was sustained for >10 min. Gating charge (the net charge needed to move across the membrane during a transition to the open state) and V_0 (the voltage at which open probability equals 0.5) were calculated from the voltage profiles of the open probability using the method of Moczydlowski [26]. The gating charge was calculated from the slope of $\ln(P_o/(1-P_o))$ vs. voltage plots.

3. Results and discussion

The Bcl-2-transfected cells (Bcl-2) had a much higher Bcl-2 content than the vector-only control cells (SV2), as shown by the Western blot of the mitochondrial fraction in Fig. 1A. The effect of overexpressing Bcl-2 on MCC activity was determined in patch-clamp studies of mitoplasts (mitochondria whose inner membrane was exposed) isolated from these two cell lines. Current traces typical of MCC from the mitoplasts of SV2 and Bcl-2 overexpressing cells were indistinguishable in minimal medium (Fig. 1B). The voltage dependence of the MCC from the two cell lines was the identical (Fig. 1C). Furthermore, the single channel analysis of MCC from the SV2 and Bcl-2 cell lines summarized in Table 1 revealed no significant differences. For example, the ion-selec-

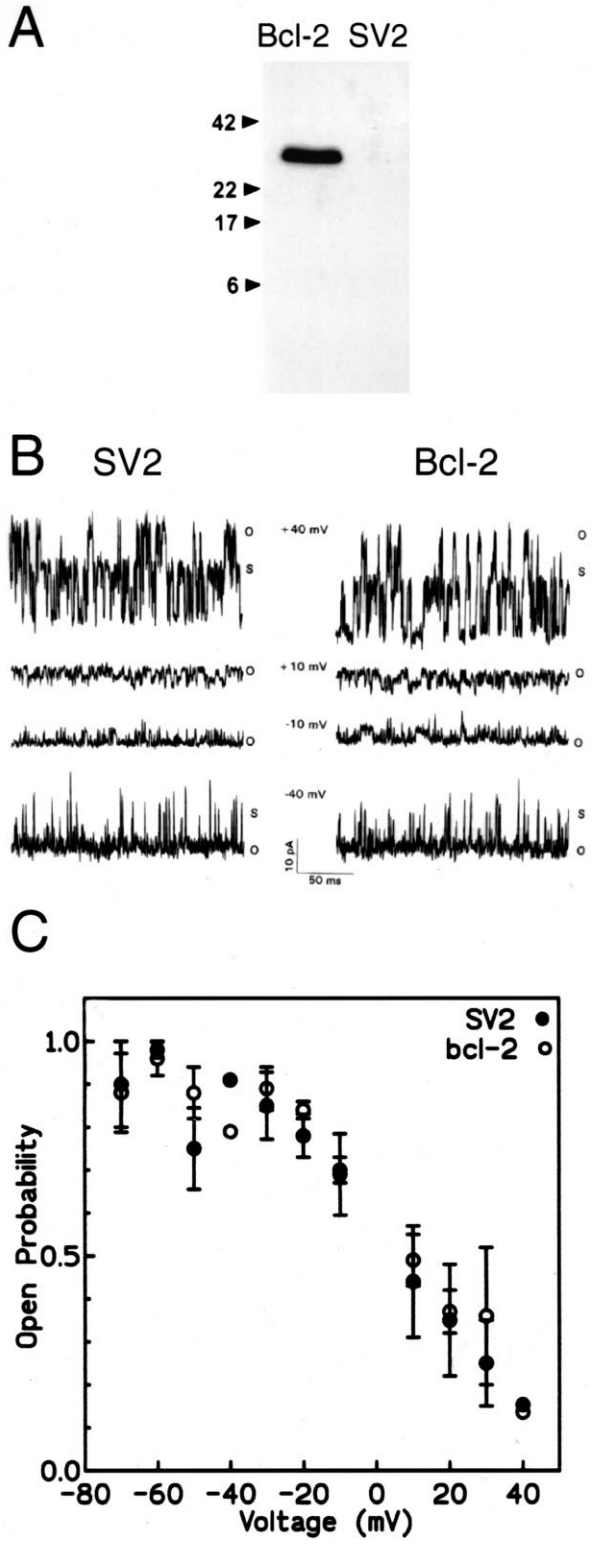


Fig. 1. MCC activity mitoplasts from SV2 and bcl2-1 cells. A: Western blot analysis with a monoclonal antibody that recognizes human Bcl-2 was performed on the mitochondrial fraction (1.5 μg protein) isolated from MDA231 breast epithelial cells that were vector-only-transfected (SV2) or overexpressing Bcl-2 (bcl2-1). Bcl-2 protein was detected by enhanced chemiluminescence. B: Typical current traces of MCC in membrane patches excised from mitoplasts isolated from SV2 and bcl2-1 are shown at various voltages. Medium was symmetrical 150 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.23 mM $CaCl_2$ (10^{-8} M free Ca^{2+}) at pH 7.4. Computer analysis of current signals was bandwidth-limited to 2 kHz with a low pass filter, and sampled at 5 kHz. O and S correspond to the open and substate conductance levels. C: Open probabilities (P_o) were calculated from total amplitude histograms of 20–30 s of current data at each voltage for MCC of mitoplasts isolated from the two cell types. The correlation coefficient (r) for data from −40 mV to +40 mV was 0.98. Means ± S.D. are shown.

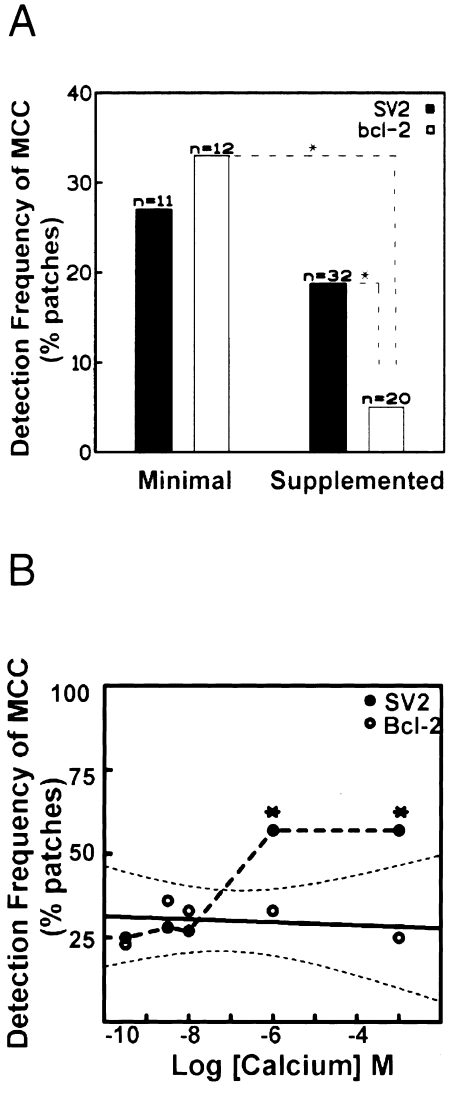
Table 1
Single channel parameters of MCC of mitochondria from control and Bcl-2 overexpressing cells

Cell type	SV2	Bcl2-1
Peak conductance (pS)	1127 ± 77[a]	1045 ± 28
Substates (pS)	338 ± 5, 739 ± 12	346 ± 8, 728 ± 25
Predominant transitions (pS)	~350	~350
Mean open time −30 mV (ms) (events)	5.4 ± 0.1 (7536)	4.9 ± 0.1 (13004)
Gating charge	−1.9 ± 0.1	−1.7 ± 0.2
V_0 (mV)	12 ± 3	11 ± 2
Selectivity	sl. cationic	sl. cationic
P_K/P_{Cl}^b	7	7

[a]Mean ± S.E.M. on a minimum of four determinations.
[b]P_K/P_{Cl} (permeability ratio of K^+ over Cl^-), determined with a 30 mM KCl (bath):150 mM KCl (pipet) gradient.

tivity and gating charge of the MCC from the two cell lines were identical. However, Bcl-2 expression altered other properties of the MCC.

MCC activity was suppressed in mitoplasts from the Bcl-2 overexpressing cells compared to that of the control cells when the medium was supplemented (Fig. 2A). The frequency of detecting MCC was significantly lower in the patches from mitoplasts from Bcl-2 overexpressing cells than the SV2 cells at physiological calcium levels in the presence of Mg^{2+}, succinate and phosphate (P value = 0.05; Fisher's exact statistical test) [27]. In previous studies, MCC was opened, and therefore



detectable, in mitoplasts of rat and mouse liver by the addition of μM Ca^{2+} [28,29]. As expected, the detection of MCC in mitochondria of SV2 cells increased when the Ca^{2+} concentration was above 10^{-8} M (Fig. 2B). However, the detection of MCC in mitoplasts of the Bcl-2 overexpressing cells did not increase at these higher Ca^{2+} concentrations. These results indicate Bcl-2 blocked the activation of MCC by Ca^{2+}.

The PTP is implicated in apoptosis by several studies [1–3,7,8]. Compounds that induce the permeability transition in isolated mitochondria often cause apoptosis. MCC is blocked by the anti-apoptotic drug cyclosporin [29,32] and several other compounds known to inhibit the PTP [33,34], and opened by uncouplers [35]. In general, the pharmacology of MCC is consistent with its assignment as the pore underlying the PTP. Hence, the effect of Bcl-2 on this channel activity is consistent with its link to the PTP.

An ion channel activity was detected by Schendel et al. [19] upon reconstitution of Bcl-2 in planar bilayers. However, no novel channel activities were detected in the mitoplast membrane during this study. Furthermore, no novel channel activities were found in studies of the outer membranes of mitochondria from FL5.12 cells overexpressing Bcl-2 (Pavlov and Kinnally, manuscript in preparation). These results suggest that when normal cells insert it into native mitochondrial membranes, Bcl-2 does not form channels.

Proteins associated with the outer membrane at contact sites can influence the permeability properties of the inner membrane when assayed in mitoplasts. These preparations

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Fig. 2. Bcl-2 overexpression suppresses MCC activity. A: The detection frequency of MCC in membrane patches from mitoplasts prepared from SV2 and bcl2-1 cells was determined in minimal media (as in Fig. 1) and media containing Mg^{2+}, succinate and phosphate. A statistical difference ($P = 0.05$) as indicated by * in the detection levels of MCC was found for the mitoplasts from the two cell lines under supplemented conditions and between the levels of MCC from bcl2-1 cells under supplemented and minimal conditions. Supplemented medium was 150 mM KCl, 1 mM EGTA, 5 mM succinic acid, 0.2 mM KH_2PO_4, 0.23 mM $CaCl_2$ (10^{-8} M free Ca^{2+}), 5 mM $MgCl_2$, 5 mM HEPES (pH 7.4). B: MCC was detected in patches by patch-clamp techniques that were excised from mitoplasts in 150 mM KCl, 5 mM HEPES, 1 mM EGTA, with varied free $CaCl_2$ levels at pH 7.4. The frequency of detecting MCC was lower in the bcl2-1 cells at higher calcium levels when compared to that of the SV2 cells ($\geq 10^{-6}$ Ca^{2+}). Data were compiled from 7–14 independent patches per point (average 10). The long dashed line connects the data points from SV2 cells and the solid line is best fit to the data from bcl2-1 cells. The short dashed lines show the 95% confidence interval and * indicates a statistical difference between the detection levels of MCC of the two cell lines at and above 1 μM free calcium.

are ideal for studying the possible interaction of inner and outer membrane proteins since bits of outer membrane are retained at contact sites in mitoplasts. For example, the voltage dependence of the inner membrane channel MCC recorded from mitoplasts was altered in mutants lacking the outer membrane protein VDAC, or mitochondrial porin [23]. In the present study, the overexpression of Bcl-2 had no effect on the voltage dependence of MCC. However, Bcl-2 modified the Ca^{2+} dependence of MCC. Presently, there are conflicting reports localizing Bcl-2 to both the inner and outer membranes. A possible explanation for this disagreement may be that Bcl-2 is associated with regions where the inner and outer membranes are in close juxtaposition, i.e. contact sites, and that MCC's properties are determined by both inner and outer membrane elements.

In summary, the single channel characteristics of the MCC of mitochondria from control and Bcl-2 overexpressing cells were identical in minimal medium at low Ca^{2+} concentrations (10^{-8} M). However, overexpression of Bcl-2 decreased the detection of MCC in medium containing Mg^{2+}, succinate and phosphate at low Ca^{2+} concentrations. Importantly, overexpression of Bcl-2 blocked the opening of MCC induced by μM Ca^{2+}. Furthermore, no novel channels were detected in the mitochondria from the Bcl-2 overexpressing cells.

While no evidence was found that supports the existence of novel channels due to the presence of Bcl-2 in mitochondrial membranes, the present findings support a functional role for Bcl-2 in regulating the channel MCC. Previous studies implied that the anti-apoptotic activity of Bcl-2 was associated with preventing the opening of a channel that allows the downhill flow of calcium and possibly other cellular effectors [16,18,30,31]. Thus, this study supports the notion that Bcl-2 suppresses cell death by modifying calcium-activated channels, in particular, the MCC of mitochondria.

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