Plasma membrane Ca^{2+}-ATPase overexpression depletes both mitochondrial and endoplasmic reticulum Ca^{2+} stores and triggers pancreatic β-cell apoptosis

by

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Supplemental Research Design and methods

Reseach Design and methods

Materials

RPMI-1640, L-glutamine, penicillin, streptomycin, foetal calf serum (FCS), G418, Lipofectamine, Zeocin™ and Hoechst 33342 were from Invitrogen (Merelbeke, Belgium). Coelenterazine wild-type (wt), *n* and *h*, MitoTracker® Green were from Molecular probe (Eugene, OR, U.S.A.). DePsipher™ was from TREVIGEN INC (Gaithersburg, MD, USA). Thapsigargin, cyclopiazonic acid (CPA), digitonin, propidium iodide and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and rabbit anti-tubulin were from Sigma Aldrich (Bornem, Belgium). Zeocin™ and Super Script™, Dynabeads mRNA DIRECT™ kit, Alexa fluor 546 and anti-rabbit Alexa fluor 488-conjugated antibodies were from Invitrogen SA (Merelbeke, Belgium). Pwo DNA polymerase kit was from Roche Diagnostic GmbH. IQ™ SYBR Green Supermix was from BIO-RAD (Nazareth Eke, Belgium). Rabbit anti-BiP, anti-phospho PERK, anti-phospho-eIF2α, anti-cleaved caspase 3, anti-Bcl-2 and anti-β-actin were from Cell Signaling**,** Bioké, Leiden, The Netherlands. Rabbit anti-eIF2α and anti-Bax were from Santa Cruz Biotechnology, Inc., Heidelberg, Germany. Monoclonal mouse anti-cytochrome c and anti-ATP-synthase-β was from BD Biosciences Europe (Erembodegem, Belgium).

Pancreatic islets isolation

Rat pancreatic islets were isolated using the collagenase technique as previously described

(29).

Assessment of cell viability

The percentage of viable, apoptotic and necrotic cells was determined using the DNA-binding dyes propidium iodide (PI, 5 µg/ml) and hoechst 33342 (HO, 5 µg/ml) as previously described (1).

mRNA extraction and quantitative RT-PCR

Poly(A)+ mRNA was isolated and reverse transcribed as previously described (2). Quantitative PCR was performed using the IQ™ SYBR Green Supermix, in an IQ5 instrument (BIO-RAD). Expression values were corrected to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). CPA treatment does not modify GAPDH expression in insulin producing cells under the present experimental conditions (2).

Western Blot Analysis

Cells were washed once with cold PBS and directly lysed with Laemmli buffer. Lysates were then resolved by SDS-PAGE and transferred to PVDF membrane. Immunoblot analyses were performed using the following antibodies : rabbit anti-PMCA2 (1/1000; Swant®, Bellinzona, Switzerland), rabbit anti-BiP (1/2000), anti-phospho PERK (1/1000), anti-phospho-eIF2α (1/1000), anti-eIF2α (1/2000), anti-Bax (1/10000), anti-cleaved caspase 3 (1/2000), anti-Bcl-2 (1/20000), anti-tubulin (1/10000) and anti-β-actin (1/5000).

References

1. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL: Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138: 2610-2614, 1997

2. Cardozo AK, Kruhøffer M, Leeman R, Orntoft T, Eizirik DL: Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. *Diabetes* 50:909-20, 2001.

Tables

Table S1. Primers sequences for RT-PCR

GENE	Forward (5'-3')	Reverse (5'-3')
Aequorin	ACTTCGACAACCCAAGATGG	ATACGGATGAGCGTTTC
PMCA2	AAGGAGACATATGGGGAC	TTCACCTTCATCTTCTGC
Beta-actin	GTTTGAGACCTTCAACACCCCA	CGGATGTCAACGTCACACTTCA
CHOP	CCAGCAGAGGTCACAAGCAC	CGCACTGACCACTCTGTTTC
Spliced XBP-1	GAGTCCGCAGCAGGTG	GCGTCAGAATCCATGGGA
BiP	CCACCAGGATGCAGACATTG	AGGGCCTCCACTTCCATAGA
Calnexin	CTTCCTCCCTCAAGGTTCTAT	CATGATTGGCAGTTCTACCTACT
Calbindin	AAGGAAAACAAGACCGTGGAT	CATCTCTGTCAGCTCCAGCTT
Calreticulin	AGATTCGAACCCTTCAGCAA	GGAAACAGCTTCACGTAGCC
GAPDH	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC
Caspase 12	GCGAGCTTAATCCTGAGCAA	GGCAATGTGACCTGCAAATA

Table S2. Doubling time of clones transfected or not with aequorin and/or PMCA2.

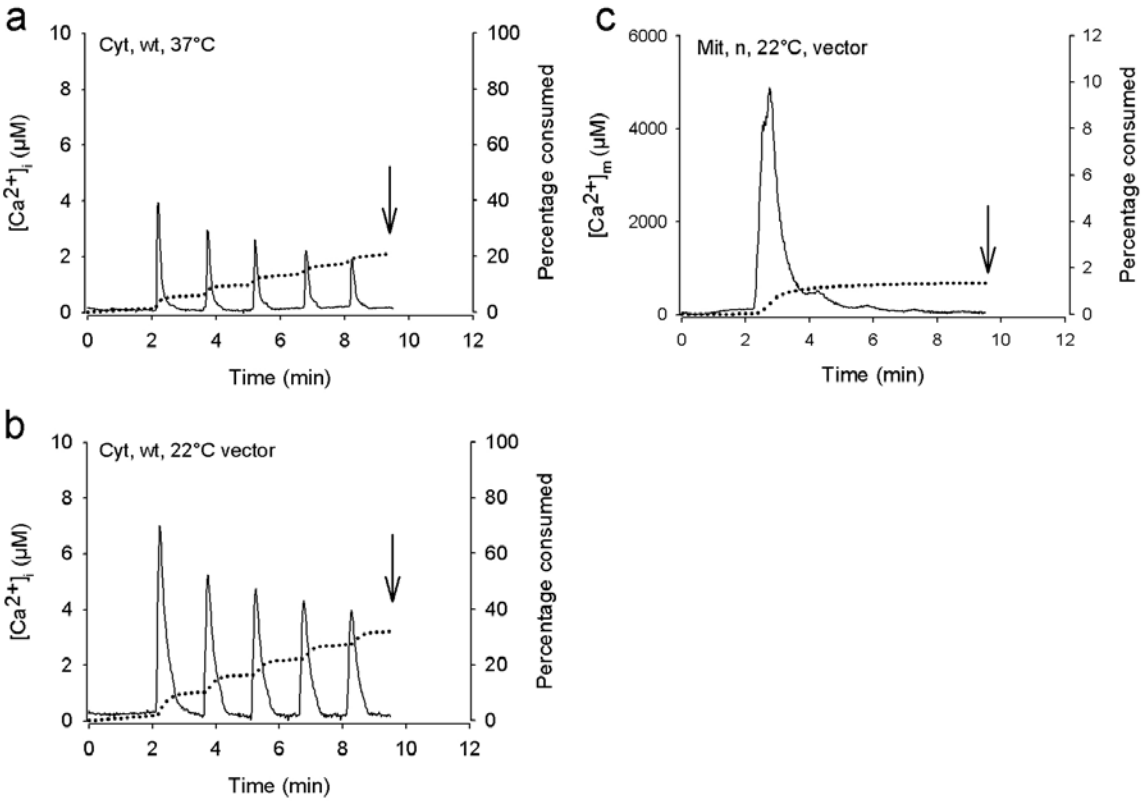
Cell clone	Doubling time (h)
Ctrl	*22.4±0.38*
Cyt-Aeq	*24.8±2.04*
Mit-Aeq	*24.9±1.93*
PMCA2-Cl2	*29.2±0.88* **
PMCA2-Cl5	*25.8±1.13*
Cyt-Aeq-Cl2	*29.3±1.36* *
Cyt-Aeq-Cl5	*24.6±0.94*
Mit-Aeq-Cl2	*28.6±1.43* *
Mit-Aeq-Cl5	*25.8±1.40*

* P>0.05 vs Ctrl; ** P<0.01 vs Ctrl.

Figures

Legend of figures

Figure S1 : Effect of KCl (50 mM) on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ in control and vector-transfected cells



Same protocol as in Figure 1.

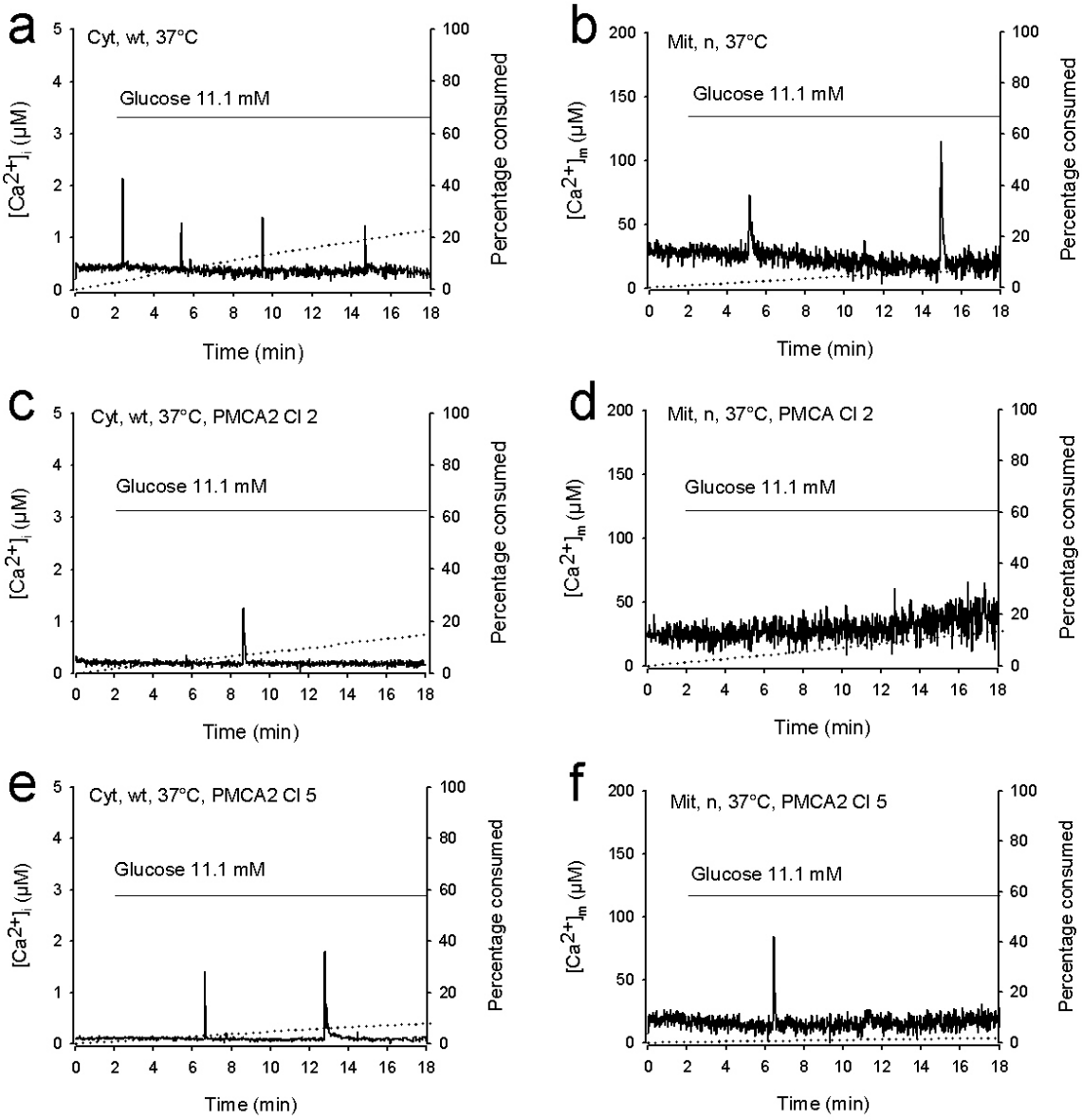
Panel a. Wild type or cytosolic aequorin (Cyt) was reconstituted with wild type coelenterazine (wt) in control non transfected cells and the experiment was carried out at 37°C. The first $[Ca^{2+}]_i$ peak value averaged 3.48 ± 0.25 µM.

Panel b. Wild type or cytosolic aequorin (Cyt) was reconstituted with wild type coelenterazine (wt) in vector-transfected cells and the experiment was carried out at 22°C.

Panel c. Mutated or mitochondrial aequorin (Mit) was reconstituted with n coelenterazine (n) in vector-transfected cells and the experiment was carried out at 22°C.

The curves shown are representative of 8 individual experiments in each case.

Figure S2. Effect of PMCA2 overexpression on glucose (11.1 mM)-induced increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$

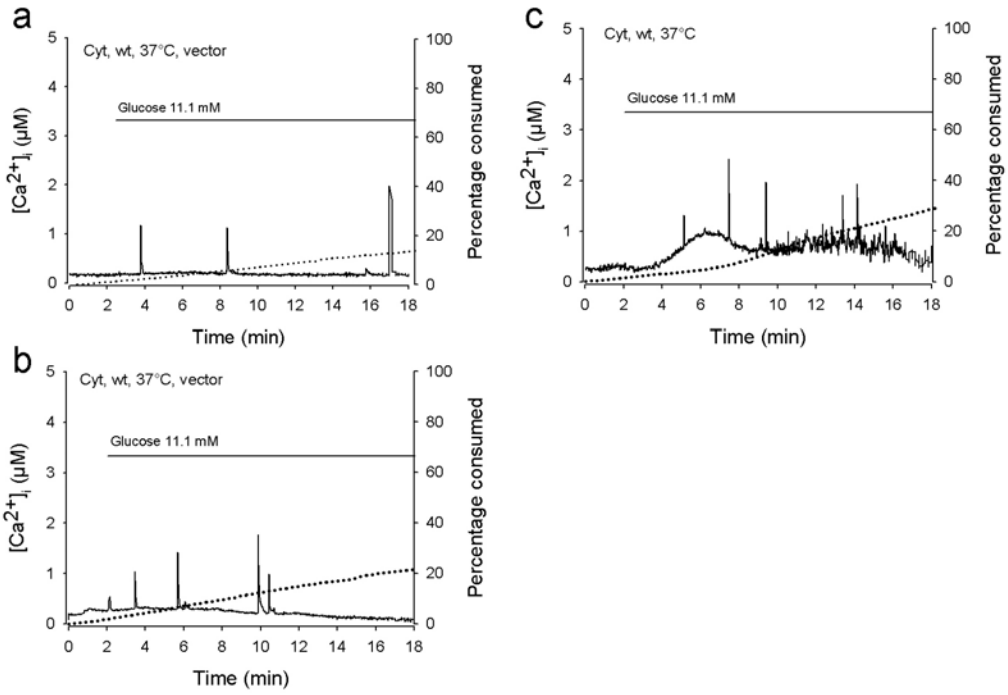


Left panels: Wild type aequorin (Cyt) was reconstituted with wild type coelenterazine (wt) in control non transfected cells (panel a) and in PMCA2-tranfected cells, clone 2 (Cl 2, panel c), clone 5 (Cl 5, panel e). The experiments were carried out at 37°c and the exposure to the sugar was continuous (from min 2 to the end of the experiment)

Right panels Mutated aequorin (Mit) was reconstituted with n coelenterazine (n) in control non transfected cells (panel b) and in PMCA2-tranfected cells, clone 2 (Cl 2, panel d), clone 5 (Cl 5, panel f). The experiments were carried out at 37°c and the exposure to the sugar was continuous (from min 2 to the end of the experiment). In control cells and clone 5 of PMCA2-overexpressing cells the number of spikes measured were 2.06 ± 017 and 1.28 ± 0.29, respectively ($P<0.05$) while the values for the largest spikes were 162.32 ± 16.96 μM and 53.30 ± 8.45 μM, $P<0.001$).

The curves shown are representative of 8 individual experiments in each case.

Figure S3. Effect of glucose (11.1 mM)-induced increase in $[Ca^{2+}]_i$

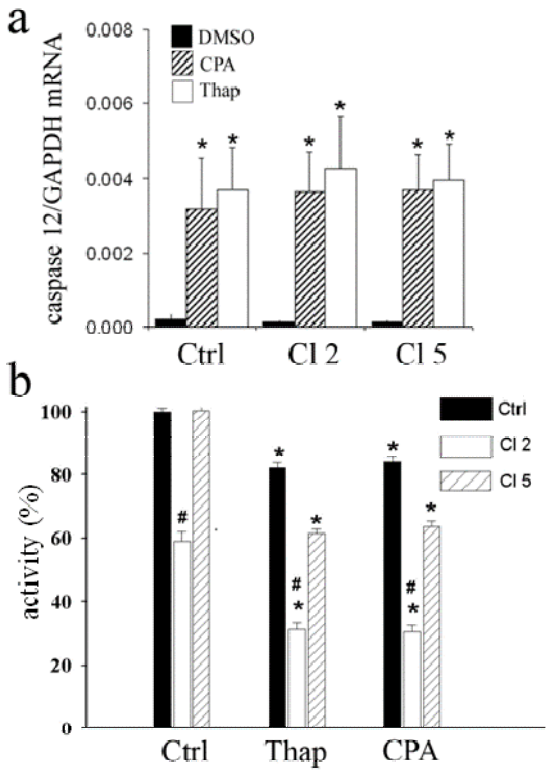


Wild type aequorin (Cyt) was reconstituted with wild type coelenterazine (wt) in vector-transfected cells (panel a and b) and control non-transfected cells (panel c), the experiments were carried out at 37°c and the exposure to the sugar was continuous (from min 2 to the end of the experiment).

Panel a: the curve shown is representative of 8 individual experiments.

Panel b and c : individual experiments showing a rise in baseline value in response to glucose.

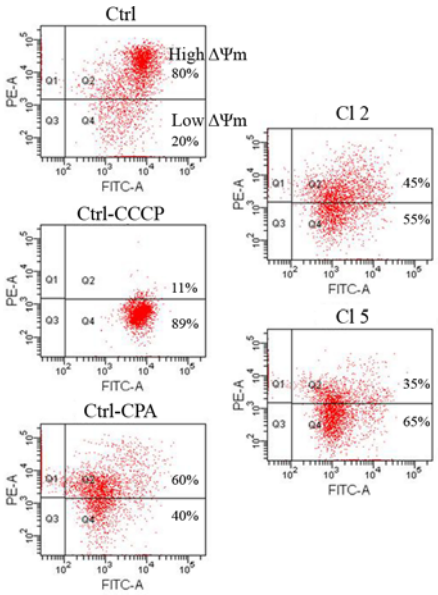
Figure S4. Effect of PMCA2 overexpression on caspase 12 cleavage and mitochondrial activity.



Panel a: Caspase 12 cleavage. BD-Brin11 cells (ctrl) or two cells-lines overexpressing PMCA2: Cl 5 and Cl 2 were treated or not (black bars) for 24h with CPA (stripe bars) or thapsigargin (white bars). Data represent expression of caspase 12 normalized per GAPDH expression. Results are means ± SEM of four independent experiments.

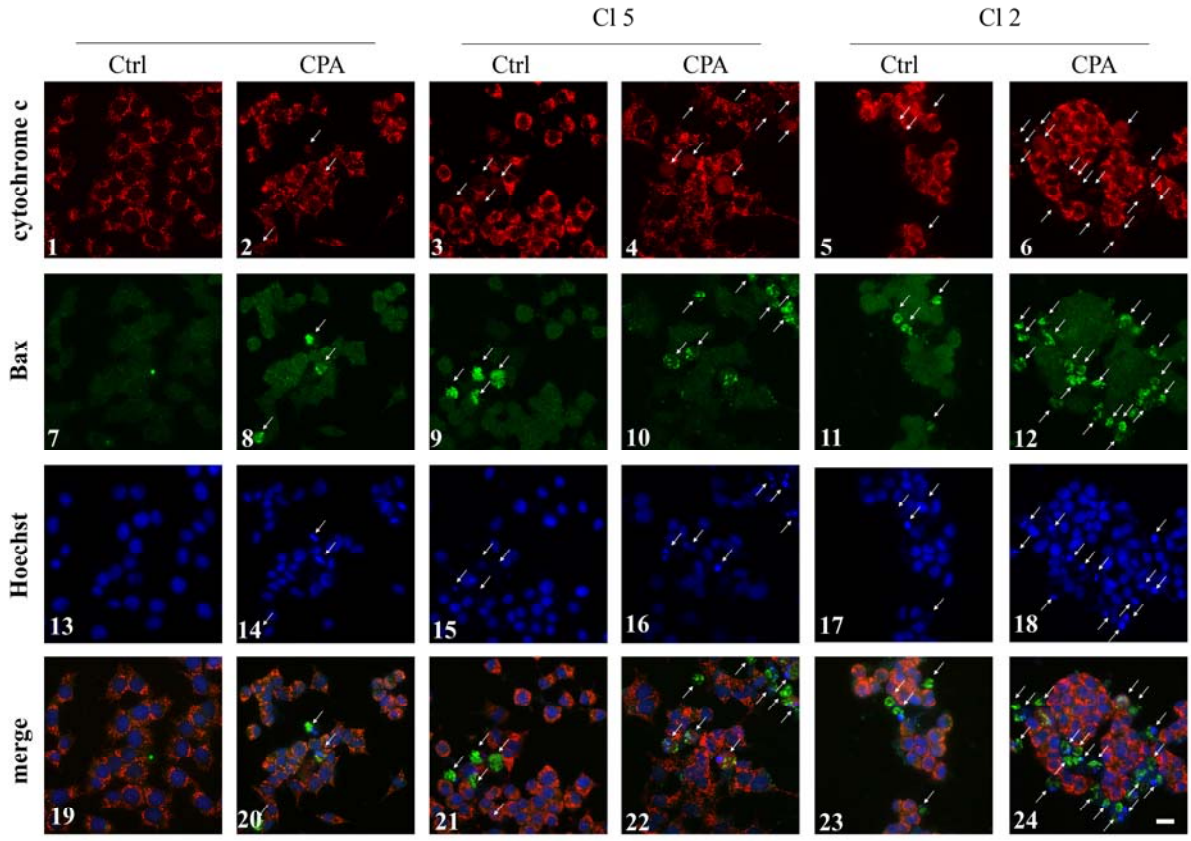
Panel b: mitochondrial activity. The MTT assay was used to measure mitochondrial activity. Closed columns: non-transfected cells; open columns: PMCA2-transfected cells (clone 2: Cl 2); hatched columns: (clone 5: Cl 5). First group of columns : untreated cells; second and third group of columns in the presence of thapsigargin (1 μM) and cyclopiazonic acid (CPA, 25 μM). Data are given as means ± SEM from 5 individual experiments comprising each at least 4 replicates. *P<0.05 vs. respective non-treated condition. #P<0.05 vs. respective non-transfected Brin-BD11 (Ctrl).

Figure S5 : Mitochondrial membrane potential



BD-Brin11 cells (Ctrl) or PMCA2-overexpresing cells (Cl 2 and 5) were treated or not for 24h with CPA or for 30min with CCCP. The cell's ΔΨm was determined by fluocytometry in cells stained with the DePsipher dye. Two population of high and low ΔΨm were separated using the positive control (CCCP-treated cells) condition. Data are representative of 3 independent experiments.

Figure S6. Bax Translocation and cyt c release.



BD-Brin11 cells (Ctrl) or cells transfected with PMCA2 (Cl 2 and Cl 5) were treated or not for 24h with CPA (50µM). Cells were stained using anti- cytochrome c (panels 1-6) and Bax (panels 7-12) and counterstained with Hoechst (panels 13-18).

Panels 1-6: Arrows indicate cells that have lost mitochondrial cytochrome c staining.

Panels 7-12: arrows indicate cells with Bax mitochondrial staining, as opposed to normal cytosolic staining.

Panels 13-18: arrows indicate typical apoptotic nuclei or cells which have lost DNA staining.

Panels 19-24: Merged image of cytochrome c, Bax and Hoechst staining.

Representative fields of f experiments (the white tab stands for 40 µm).