doi:10.4149/gpb_2010_04_414

Short Communication

Expressional changes of RyR1 and RyR2 in PC12 cells after induction of apoptosis

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Abstract. Ca^{2+} released from endoplasmic reticulum through ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs) can trigger apoptotic or necrotic pathways in cooperation with proapoptotic and/or prosurvival proteins, as those of Bcl-2 family. In such regulatory pathways expressional modulation of these Ca^{2+} transporters could also be expected. Therefore, our aim was to determine the expressional changes of RyR1 and RyR2 after experimental induction of apoptosis in PC12 cells. Our results showed significant decrease of RyR1 and RyR2 expressions, while caspase-3 and Bax expression significantly increased. We conclude that induction of apoptosis in PC12 cells could result in RyR expression down regulation.

Key words: Ryanodine receptors — Gene expression — Apoptosis — PC12 cells

Abbreviations: ER, endoplasmic reticulum; IP3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATP-ase; ROS, reactive oxygen species; si-RyR2, small interfering RNA targeting RyR2; AIK, Apoptosis Inducer Kit.

Apoptosis as well as inappropriate activation of cell death pathways are involved in pathogenesis of many disorders such as cardiomyocyte injury, diabetes, cerebral ischemia, various neuronal disorders and others. There are multiple programmed cell death pathways and in many of those, endoplasmic reticulum (ER) is importantly involved. ER is the main intracellular calcium store that releases Ca^{2+} to the matrix *via* inositol 1,4,5-trisphosphate receptors (IP3Rs) and ryanodine receptors (RyRs) and re-uptakes Ca^{2+} by action of sarco(endo)plasmic reticulum Ca^{2+} ATP-ase (SERCA). RyRs and IP3Rs play a central role in cell survival (Ruiz et al. 2009), but it has been shown that these receptors can also be crucial in apoptotic and necrotic cell death (Hajnóczky et al. 2000).

RyRs are distributed evenly throughout the cell (Straub et al. 2000) and augment Ca^{2+}-signal after small increase of Ca^{2+} released from IP3Rs concentrated mainly in the apical region of the cell (Nathanson et al. 1994), phenomenon known as calcium-induced calcium release (CICR). Inhibition of RyRs and Ca^{2+} release from ER during excitotoxic insult results in attenuation of cytosolic Ca^{2+} increase, mitochondrial membrane depolarization, ROS generation and activation of caspase-3 (Ruiz et al. 2009). Guo et al. (2010) showed that si-RyR2 prevented cell injury associated with attenuating cytosolic Ca^{2+} and ROS production in cardiomyocytes. Intracellular Ca^{2+} overload as well as ROS generation appears to be crucial for induction of cell death during reperfusion and reoxygenation.

There are two main pathways through which ER can induce apoptosis. Mitochondrial-dependent pathway consisting on a Ca^{2+} overload from ER to mitochondria is the first. These organelles are functionally coupled by microdomains that involve RyRs and IP3Rs (Rizzuto et al. 2009) and Ca^{2+} signaling between them can induce apoptosis crosstalk followed by cytochrome *c* release (Ferreiro et al. 2008). The other pathway is activated by impairment of ER functioning. When unfolded proteins accumulate or ER calcium stores are depleted, highly conserved stress response is initiated and ER attempts to restore its capacity and shuts down translation and overexpress ER function-related proteins (Ruiz et al. 2009).

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Programmed cell death is regulated by number of „players" – prosurvival and proapoptotic. Major regulators of mitochondrial-mediated cell death pathways belong to the proteins of Bcl-2 family. This family includes proapoptotic proteins such as Bax, Bak and tBid. Bax and Bak form channels in the outer mitochondrial membrane allowing the release of proapoptogenic signals such as cytochrome *c* (Gerasimenko et al. 2010). On the other hand, prosurvival Bcl-xL and Bcl-2 bind to and sequester the proapoptotic proteins resulting in inhibition of apoptosis. It is known that Bcl-2 family proteins can also physically and functionally regulate release of Ca^{2+} from intracellular stores (Rong and Distelhorst 2008).

The aim of our study was to determine expressional changes of both RyR1 and RyR2 after experimental induction of apoptosis. In our experiments we used PC12 cells, a cell line derived from a rat pheochromocytoma cells. These cells are routinely used in our laboratory and express both RyR1 and RyR2 receptors. Cells were cultured in DMEM (Dulbeco Modified Eagle's Medium) with 10% FCS (fetal calf serum), in 6-well tissue culture plates at density of 1.0×10^6 in 3 ml of media and incubated at 37°C in a humidified atmosphere of 5% CO_2 air for 24 hours before apoptosis induction. Apoptosis was induced by Apoptosis Inducer Kit (Calbiochem, Merck Biosciences, Germany) diluted 1 : 1000 according to manufacturer's protocol during 3 and 6 hours. The kit contains 5 chemical reagents (actinomycin D, camptothecin, etoposide, cycloheximide and dexamethasone) inducing apoptosis through different mechanisms. After induction of apoptosis, cells were used for RNA isolation and RT-PCR analysis of gene expression as described by Jurkovicova et al. (2007). Exprimed proteins were analyzed by Western blot analysis (Hudecova et al. 2010) with subsequent immunodetection with specific antibodies against RyR1 and RyR2 (Chemicon, Millipore, USA). Results are presented as means ± S.E.M. Statistical analysis was done by Student's *t*-test. Statistical significance $p < 0.05$ was considered significant.

Amplification of apoptotic genes caspase-3 and Bax by RT-PCR verified apoptosis induction in PC12 cells. After 3 hours of apoptosis induction, caspase-3 mRNA expression increased significantly compared to control from 100.0 ± 18.2 to 164.8 ± 9.1% ($p < 0.05$) and after 6 hours of induction from 100.0 ± 5.0 to 153.0 ± 11.8% ($p < 0.01$) (Fig. 1A). Controls were adjusted to 100%. Similarly Bax gene mRNA expression raised significantly after 3 hours from 100.0 ± 3.3 to 293.0 ± 45.0% ($p < 0.01$) and after 6 hours from 100.0 ± 7.2 to 183.0 ± 15.4% ($p < 0.01$) (Fig. 1B). These results point to entering of PC12 cells into apoptosis. Our experiments were focused on changes of mRNA and protein expression of RyR1 and RyR2. RyR1 mRNA expression significantly decreased after 3 hours of apoptosis induction from 100.0 ± 1.3 to 71.0 ± 7.4% ($p < 0.05$) and after 6 hours of treatment from 100.0 ± 11.8 to 59.0 ± 7.1% ($p < 0.05$) (Fig. 2A). We detected

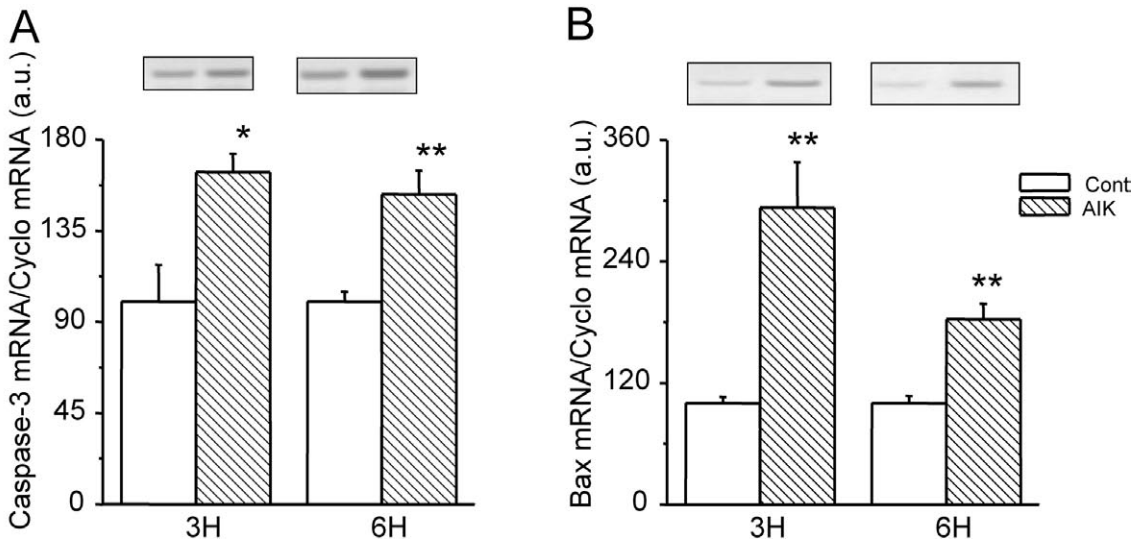

Figure 1. Comparison of mRNA levels of the caspase-3 (**A**) and Bax (**B**) in control PC12 cells and PC12 cells after 3 and 6 hours of apoptosis induction by AIK, as described in the text. Cyclophyline (Cyclo) expression was used as a housekeeper gene control for semi-quantitative evaluation of PCR. Duration of the treatment by AIK was chosen according to previous experiments (data not shown). Both, 3 and 6 hour treatments resulted in significant increase of caspase-3 and Bax mRNA levels in comparison with their time-corresponding controls. Each column is displayed as mean ± S.E.M ($n = 5$), * $p < 0.05$, ** $p < 0.01$. Cont, control cells; AIK, cells after apoptosis induction; 3H, apoptosis induction for 3 hours; 6H, apoptosis induction for 6 hours.

no changes in RyR1 protein amount (Fig. 2B). RyR2 gene expression was also down regulated by induction of apoptosis and mRNA decreased significantly from 100.0 ± 5.9 to 63.0 ± 0.5% ($p < 0.01$) after 3 hours and from 100.0 ± 10.3 to 68.0 ± 5.1% ($p < 0.01$) after 6 hours (Fig. 2C). The amount of RyR2 protein decreased approximately 20% after 3 hours of apoptosis induction compared to control (Fig. 2D).

There is strong evidence suggesting that enhanced calcium release from internal stores plays an important role in the progression of apoptosis. ER serves as a rapidly exchanging Ca^{2+} store that contributes to cytosolic calcium cascade by releasing Ca^{2+} mainly through RyRs and IP3Rs (Verkhratsky and Petersen 2002). Critical role of these receptors in regulation of apoptosis is supported by several studies. Ruiz et al. (2009) showed that blocking of RyRs and IP3Rs attenuated NMDA (N-methyl D-aspartate)-induced ER stress promoting apoptosis in neuronal cells. Guo et al. (2010) showed that si-RyR2 reduces cell death in neonatal cardiomyocytes. Other studies showed that genomic deletion of IP3Rs (Sugawara et al. 1997) or antisense knockdown (Jayaraman and Marks 1997) significantly attenuates apoptosis.

According to results of other authors, after induction of apoptosis, we expected that the gene expression of both RyR1 and RyR2 would increase consequently triggering massive release of Ca^{2+} from ER leading cells into apoptosis. We observed the gene expression of both RyRs significantly decreased in PC12 cells. Decrease in RyR2 expression was also observed by Salas et al. (2010) during cardiac ischemia. This decrease was similar to that observed at the onset of reperfusion-associated with an increase in the rate of SR Ca^{2+} release. This paradoxical result was explained by an ischemic damage of RyR2 leading to an increase in the open probability and/or conductance of Ca^{2+}-release channels (Salas et al. 2010).

Zhou et al. (1998) presented that apoptosis can also result from ER stress evoked by inhibition of Ca^{2+} uptake to ER, by

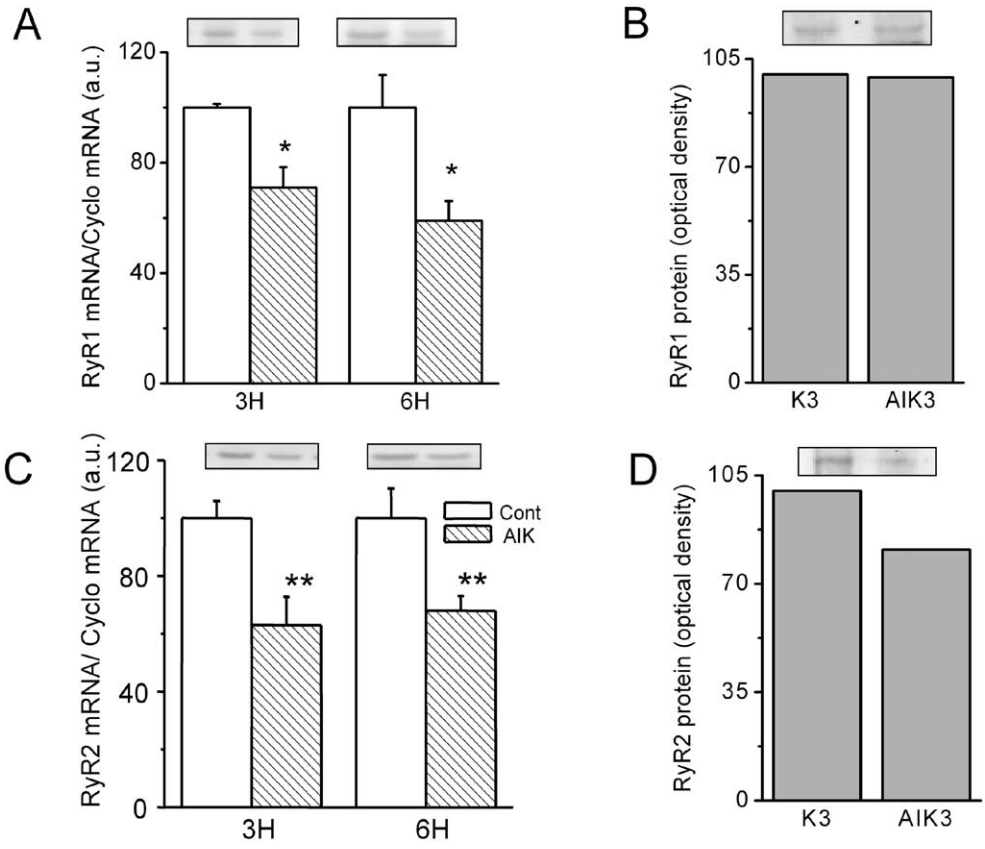

Figure 2. Comparison of the RyR1 mRNA (**A**) and RyR2 mRNA (**C**) levels in control PC12 cells and PC12 cells after 3 and 6 hours of apoptosis induction by AIK and typical RyR1 (**B**) and RyR2 (**D**) protein levels after 3 hours of apoptosis induction. Both, 3 and 6 hour treatments resulted in significant decrease of RyR1 and RyR2 mRNA levels in comparison with their time-corresponding controls. Cyclophyline (Cyclo) expression was used as a housekeeper gene control for semi-quantitative evaluation of PCR. There was no change observed on protein level of RyR1 compared to corresponding control. RyR2 protein level decreased by 20% after 3 hours of AIK treatment compared to its time-corresponding control. Each column is displayed as mean ± S.E.M ($n = 5$), * $p < 0.05$, ** $p < 0.01$. Cont, control cells; AIK, cells after apoptosis induction; 3H, apoptosis induction for 3 hours; 6H, apoptosis induction for 6 hours.

blocking SERCA pump. In our experiments decreased RyR1 and RyR2 expression was accompanied by significantly decreased mRNA levels of SERCA2 (data not shown). Luciani et al. (2009) point that both SERCA and RyR inhibition can activate programmed cell death in β-cells and that disruption of β-cell ER Ca^{2+} handling can trigger multiple apoptosis pathways. Based on these observations it might be possible that induction of apoptosis in PC12 cells inhibited SERCA2 first and observed down regulation of both RyR1 and RyR2 was only following feedback event. More studies need to be performed to elucidate this possibility.

On the other hand there are numerous studies showing the role of Bcl-2 proteins in calcium metabolism (White et al. 2005). Gerasimenko et al. (2010) reported results of their experiments with small molecule inhibitors of Bcl-2/Bcl-xL. Their study on pancreatic acinar cells showed that ER contains significant quantities of Bcl-2 family proteins. Molecule inhibitors of Bcl-2/Bcl-xL caused dissociation of proapoptotic Bax from prosurvival Bcl-2 and Bcl-xL and this association was accompanied with the ability of these agents to cause release of Ca^{2+} from intracellular stores. Interpretation of the findings of Gerasimenko is that dissociating proapoptotic Bcl-2 proteins such as Bax from prosurvival Bcl-2 proteins such as Bcl-2 and Bcl-xL increases the sensitivity of IP3Rs and RyRs to activation by calcium as described previously in pancreatic acinar cells (Leite et al. 2002). Works of Chen et al. (2004) and White et al. (2005) demonstrated that Bcl-2 and Bcl-xL physically bind to the IP3R and alters its ability to release Ca^{2+}. Addition of Bax prevented this release. Taken together, when associated with proapoptotic proteins, the prosurvival Bcl-2 proteins inhibit the Ca^{2+} releasing channels, while when dissociated from proapoptotic Bcl-2 proteins, Bcl-2 and Bcl-xL increase the sensitivity of the channels to Ca^{2+} release by increasing their sensitivity for activation by Ca^{2+} (Gerasimenko et al. 2010).

Also in neuronal cells evidence is accumulating that Bcl-2 constitutes a rheostat for the fine tuning of Ca^{2+} at the ER by affecting content and release of Ca^{2+} from ER (Hetz and Glimcher 2008). In cerebral granule cells Lossi et al. (2009) showed that depending on upstream signal, Ca^{2+} released from the ER signals back and acts as a second messenger that plays a crucial role in the post translation regulation of Bcl-2.

We could possibly speculate that our observation of decreased mRNA and protein expression of RyR1 and RyR2 receptors after induction of apoptosis with strong apoptosis inducer set of chemicals could be explained in concordance with observations of mentioned authors. Decreased expression of RyRs observed in PC12 cells could be also due to Bcl-2 protein increased sensitivity of RyR1 and RyR2 to Ca^{2+} resulting in increased ER Ca^{2+} release. By negative feedback regulation mechanism cell could down-regulate mRNA and protein expression of these receptors.

In conclusion we can state that experimental induction of apoptosis for 3 and 6 hours in PC12 cells diminished the gene expression of main ER Ca^{2+} release transporter – RyR. There are many more experiments necessary to be accomplished to explain the mechanism and regulatory pathway. Possible explanation could be searched in modulation effect of apoptotic Bcl-2 family proteins physically and functionally regulating ER Ca^{2+} transporters and in transcriptional modulation of ER Ca^{2+} transporters according to their modulation function on intracellular Ca^{2+} concentration triggering apoptotic or necrotic cell death. There is growing evidence that inappropriate transport activity of these ion channels can both negatively and positively modulate ER stress and trigger multiple apoptosis pathways (Luciani et al. 2009).

Acknowledgements. The study was supported by grant APVV 51-0397.

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Received: September 21, 2010
Final version accepted: October 19, 2010