Biochemical and Biophysical Research Communications 375 (2008) 501–505

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Akt kinase reducing endoplasmic reticulum Ca^{2+} release protects cells from Ca^{2+}-dependent apoptotic stimuli

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ARTICLE INFO

Article history:
Received 30 July 2008
Available online 22 August 2008

Keywords:
Calcium homeostasis
Apoptosis
Mitochondria
Cell death
Bcl-2

ABSTRACT

The proto-oncogene Akt is a potent inhibitor of apoptosis, and it is activated in many human cancers. A number of recent studies have highlighted the importance of the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) in mediating calcium (Ca^{2+}) transfer from the endoplasmic reticulum (ER) to the mitochondria in several models of apoptosis. Akt is a serine-threonine kinase and recent data indicate the IP3R as a target of its phosphorylation activity.

Here we show that HeLa cells, overexpressing the constitutively active myristoylated/palmitylated AKT1 (m/p-AKT1), were found to have a reduced Ca^{2+} release from ER after stimulation with agonist coupled to the generation of IP3. In turn, this affected cytosolic and mitochondria Ca^{2+} response after Ca^{2+} release from the ER induced either by agonist stimulation or by apoptotic stimuli releasing Ca^{2+} from intracellular stores.

Most importantly, this alteration of ER Ca^{2+} content and release, reduces significantly cellular sensitivity to Ca^{2+} mediated proapoptotic stimulation. These results reveal a primary role of Akt in shaping intracellular Ca^{2+} homeostasis, that may underlie its protective role against some proapoptotic stimuli.

Protein kinase B or Akt, is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKB alpha (Akt1), PKB beta (Akt2), and PKB gamma (Akt3) [1].

Forced membrane localization of Akt through its myristoylation/palmitylation is sufficient for maximal phosphorylation of key aminoacids and in turn for its full activation. The activity of this plasma membrane targeted Akt chimera (m/p-AKT1) is 60-fold higher compared with the unstimulated wild-type enzyme [2] and it is widely utilized in studying the effects of Akt in several signal transduction pathways [3–6].

Key roles for this enzyme can be found in cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration [7,8] . Akt functions to promote cell survival through several pathways [8–10]. The precise mechanisms by which Akt prevents apoptosis are not completely understood.

Akt is a growth-factor-regulated protein kinase that phosphorylates in vitro and in vivo the IP3Rs, thanks to the presence of a consensus substrate motif that is conserved in all three IP3R isoforms [11]. The IP3R is involved in Ca^{2+} mobilization from intracellular stores, where channel activity is largely under the control of the IP3 binding. IP3R is involved in fundamental processes such as fertilization, meiosis, and mitosis and it has been reported that several proteins interact with IP3Rs [12]. Some of these have a modulatory role. Indeed, in addition to Akt mentioned above, the IP3R is also a substrate for several other protein kinases including calmodulin dependent protein kinase II [13], protein kinase C [14,15] and MAP kinases. As mentioned before, the main role of the IP3R is releasing Ca^{2+} into the cytoplasm and to other effector systems (such as mitochondria). Ca^{2+} is recognized to be a fundamental second messenger involved in fertilization, proliferation, muscle contraction and secretion [16,17]. As a second messenger, Ca^{2+} exerts its role inside the cell, where its concentration is highly controlled. The extent of Ca^{2+} signal is also a key determinant of the intrinsic apoptotic pathway, thus controlling cell fate under physiological and pathological conditions [18].

There is general agreement in the literature that Ca^{2+} efflux from the ER and Ca^{2+} accumulation into the mitochondria are linked to the effects of various apoptotic stimuli [18].

The task of this work was to evaluate the effect of the overexpression of a constitutively active AKT1 on subcellular Ca^{2+} homeostasis in transiently transfected HeLa cells, i.e. the same cell model in which the effects of Bcl-2 and Bax on Ca^{2+} signaling were previously detected and analyzed [19–21]. We then analyzed the effects of Akt overexpression on the cytosolic Ca^{2+} signal elicited

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doi:10.1016/j.bbrc.2008.07.153

by apoptotic stimuli such as oxidative stress and arachidonic acid, as they act through mitochondrial Ca^{2+} overload and activation of the intrinsic apoptotic pathway following to Ca^{2+} release from the ER.

Altogether our data suggest that the alteration of Ca^{2+} fluxes through the IP3R is a key component of the protective action of Akt against apoptosis induced by stimuli acting in a Ca^{2+}-sensitive manner.

Materials and methods

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in 75-cm^2 Falcon flasks. For the aequorin experiments, the cells were seeded onto 13-mm glass coverslips and allowed to grow to 75% confluence.

At this stage, transfection with 4 μg of total DNA (3 μg of the indicated expression plasmids and 1 μg of aequorin) was carried out as described previously [22]. All measurements were performed 36 h after transfection.

Aequorin measurement. All measurements were carried out as previously described [22]. Briefly, for the experiments with cytAEQ and mtAEQmut, cells were incubated with 5 μM coelenterazine for 1–2 h in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum. To reconstitute erAEQmut with high efficiency, the luminal Ca^{2+} concentration ($[Ca^{2+}]$) of the ER ($[Ca^{2+}]_{er}$) first had to be reduced. This was achieved by incubating cells for 1 h at 4 °C in Krebs–Ringer buffer (KRB) supplemented with 5 μM coelenterazine, 5 μM Ca^{2+} ionophore ionomycin, and 600 μM EGTA. After this incubation, cells were extensively washed with KRB supplemented with 2% bovine serum albumin and then transferred to the perfusion chamber.

Fura-2/AM measurements. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) was evaluated using fluorescent Ca^{2+} indicator Fura-2/AM (Molecular Probes, Inc.). Briefly, cells were incubated in medium supplemented with 2.5 μM Fura-2/AM for 30 min, washed with KRB to remove extracellular probe, supplied with preheated KRB (supplemented with 1 mM $CaCl_2$), and placed in a thermostated incubation chamber at 37 °C on the stage of an inverted fluorescence microscope (Zeiss Axiovert 200). Dynamic video imaging was performed using MetaFluor software (Universal Imaging Corp.). $[Ca^{2+}]_c$ was calculated as previously described [23].

Apoptotic counts. Cells were seeded on 24 mm coverslips and co-transfected 1:1 ratio with m/p-AKT1 and mtGFP, mtGFP as control. On a fluorescent field picture, took before apoptotic stimulus, was recorded the number of GFP-positive cells and the same was done after treatment with arachidonic acid and hydrogen peroxide. Fifty different fields per condition were analyzed and a mean per condition was extracted. Ratio between the mean after and before treatment is shown in figure.

Caspase-3 assay. Cells were centrifuged (1000g for 5 min) and washed once in phosphate-buffered saline. The enzcheck® caspase-3 assay kit # 2 by Molecular Probes was used for determination of caspase-3 activity. Lysate (20 μg), resuspended in a final volume of 100 μl, was assayed using Wallac 1420 Victor3 multitask plate reader (Perkin Elmer, Inc.).

Results

Akt drastically reduces ER Ca^{2+} release, while leaving ER Ca^{2+} levels unaffected

We measured the ER $[Ca^{2+}]$ ($[Ca^{2+}]_{er}$) in HeLa cells transfected with ER-targeted aequorin (erAEQmut) [22]. In these experiments, the ER Ca^{2+} store was first depleted of Ca^{2+}, during the phase of aequorin reconstitution, performed in Ca^{2+}-free medium (as described in Materials and methods section). When Ca^{2+} was added back to the KRB perfusion medium, $[Ca^{2+}]_{er}$ rose from <10 μM to a plateau value of ~400 μM, as previously reported [24,25]. No significant difference was observed between m/p-AKT1 overexpressing and control cells (348 ± 20.08 μM and 357.16 ± 19.68 μM, $n = 21$; $p > 0.1$) (Fig. 1A).

We then investigated the ER response to histamine, which acts on Gq-coupled plasma membrane receptors and causes the production of IP3, thus releasing Ca^{2+} from the ER through the IP3Rs, followed by sustained influx from the extracellular medium through plasma membrane Ca^{2+} channels (Fig. 1B). When the $[Ca^{2+}]$ in the lumen of the ER reached a plateau value, the cells were treated with histamine. As expected, a decreases in the $[Ca^{2+}]$ of the ER compartment was observed both in control and m/p-AKT1 expressing cells (Fig. 1B), but the decrease of $[Ca^{2+}]$ in control cells was extremely larger and faster (V_{max}: 23.03 ± 2.02 μM/s vs 3.87 ± 0.5 μM/s; mean of first 50 s of Ca^{2+} released: 3.67 ± 0.2 μM/s vs 1.56 ± 0.1 μM/s, $n = 21$), thus reflecting a more rapid flow through the IP3-gated channels, in controls compared to m/p-AKT1 expressing cells. Then we investigated if m/p-AKT1 overexpression could affect the IP3Rs

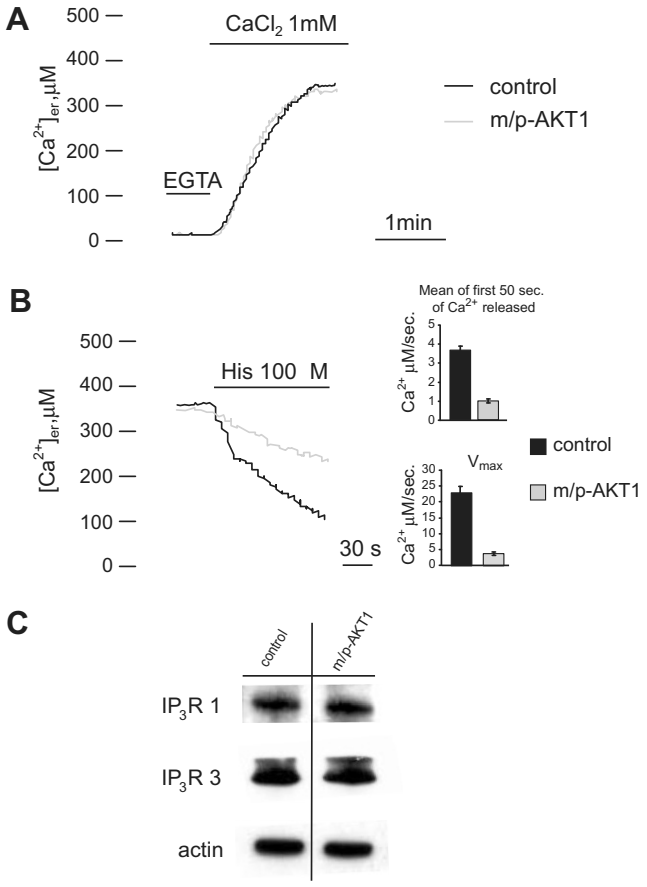

Fig. 1. Modifications of ER Ca^{2+} kinetic releases by overexpression of Akt in HeLa cells. (A) Effects of m/p-AKT1 on the ER Ca^{2+} concentration ($[Ca^{2+}]_{er}$) in control and in m/p-AKT1-overexpressing HeLa cells. (B) Reduced ER Ca^{2+} release in m/p-AKT1-overexpressing cells when stimulated with 100 μM histamine. To induce Ca^{2+} release from ER, the cells were challenged with an agonist that, trough interaction with G protein coupled receptors, evokes a rapid discharge from inositol 1,4,5-phosphate receptors (IP3Rs). The bars in the graph show the extent of the reduction in the mean rate of Ca^{2+} released induced by overexpressing m/p-AKT1 after cell stimulation, both for the first 50 s (upper graph) and for the maximum rate (lower graph). (C) HeLa cells were transfected with m/p-AKT1 or mock transfected in control cells. Western blot analysis shows that overexpression of constitutively active Akt does not affect the expression of different types of IP3 receptors.

expression level: as shown in Fig. 1C this was not the case. Indeed, both type 1 and type 3 IP3R, are expressed at comparable levels in m/p-AKT1 transfected and control cells.

Thus, we conclude that m/p-AKT1 is able to inhibit agonist-induced ER Ca^{2+} release into the cytoplasm.

Cytosolic and mitochondrial Ca^{2+} response to agonist stimulation is strongly impaired in m/p-AKT1 expressing cells

The effect of m/p-AKT1 expression on the cytosolic Ca^{2+} signal elicited by agonist stimulation was investigated using cytosolic aequorin. Both in control and m/p-AKT1-transfected cells, histamine stimulation causes a rapid rise in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$), followed by a gradually declining sustained plateau. In m/p-AKT1-transfected cells, the $[Ca^{2+}]_c$ increases evoked by stimulation with histamine are significantly smaller than in controls (peak amplitude 1.07 ± 0.03 vs 2.72 ± 0.04 μM; $n = 14$) (Fig. 2A).

Then we evaluated whether m/p-AKT1 could affect Ca^{2+} handling in mitochondria, an important site for decoding cellular Ca^{2+} signals and in particular Ca^{2+} signals regulating apoptosis. Both in control and m/p-AKT1-transfected cells, histamine stimulation caused a large, rapid rise in mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$), that returned to almost basal levels in approx 1 min. However, in m/p-AKT1-transfected cells, the $[Ca^{2+}]_m$ increases evoked by stimulation with histamine were drastically smaller than in controls (peak amplitude 76.82 ± 3.4 μM control, 22.84 ± 2.2 μM m/p-AKT1, $n = 18$) (Fig. 2B).

Taken together these data show that m/p-AKT1 globally affects cellular Ca^{2+} signaling probably acting on the ER Ca^{2+} release machinery.

Akt overexpression protects against Ca^{2+}-mediated cell death

Mitochondrial Ca^{2+} uptake has been shown to be a fundamental factor in the activation of the intrinsic apoptotic pathway and in sensitizing the response to several apoptotic stimuli [19,20,26–28]. Reduction of mitochondrial Ca^{2+} accumulation by m/p-AKT1 thus might result in diminished cellular sensitivity to Ca^{2+}-mediated apoptosis. Therefore, we applied oxidative stress and arachidonic acid to induce apoptosis in HeLa cells, as they act through mitochondrial Ca^{2+} overload and ensuing activation of the intrinsic apoptotic pathway. HeLa cells were transiently transfected with mitochondrially targeted GFP alone (control) or in combination with m/p-AKT1 (m/p-AKT1), and 36 h after transfection the cells were treated with (1 mM) H_2O_2 or (80 μM) arachidonic acid. After 3 h incubation with the apoptotic stimulus of interest, and elimination of dead cells we observed a substantial increase in the proportion of m/p-AKT1-expressing cells after treatment (H_2O_2: 25.4 ± 5.11% increase in m/p-AKT1/mtGFP-overexpressing cells, $n = 11$; arachidonic acid: 19.67 ± 6.04% increase in m/p-AKT1/mtGFP-overexpressing cells, $n = 9$. $p < 0.05$) (Fig. 3A). In this experimental setup positive changes in the percentage of surviving transfected cells indicates protection against the apoptotic stimulus by the overexpressed protein, while reduction provides evidence for its proapoptotic effect, thus Akt protected a significant number of cells from H_2O_2 and arachidonic acid. These data were

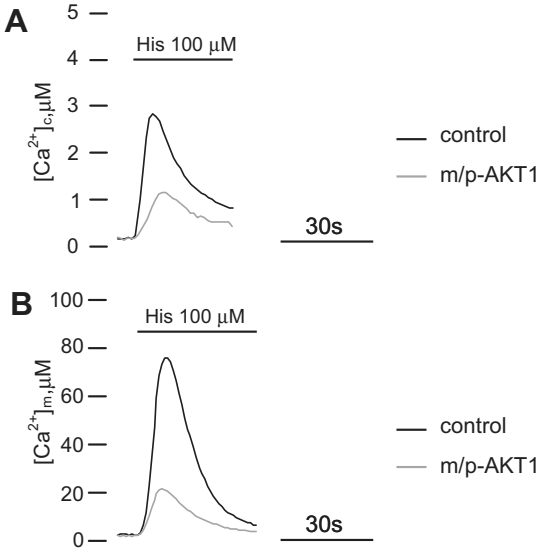

Fig. 2. Alteration of cytosolic and mitochondrial Ca^{2+} homeostasis in Akt-overexpressing cells. (A) Cytosolic Ca^{2+} homeostasis in control and m/p-AKT1-overexpressing HeLa cells. Where indicated, the cells were stimulated with 100 μM histamine. (B) Mitochondrial Ca^{2+} homeostasis in control and m/p-AKT1-overexpressing HeLa cells. Where indicated, the cells were stimulated with 100 μM histamine.

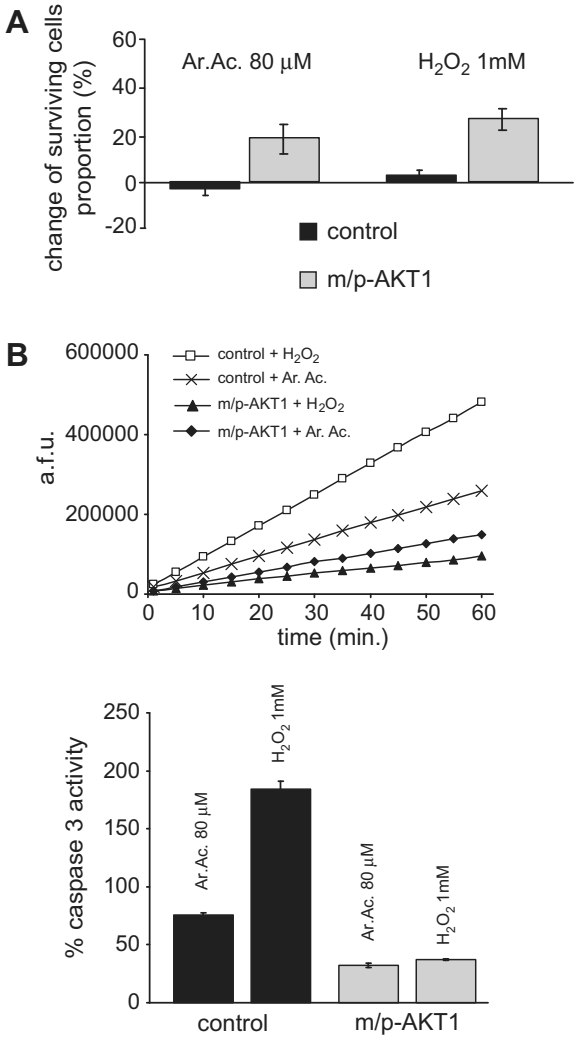

Fig. 3. Effect of Akt on cell death induced by Ca^{2+}-dependent apoptotic stimuli. (A) HeLa cells were co-transfected with mtGFP and m/p-AKT1 or mtGFP alone in control cells. At 36 h post-transfection, cells were treated with arachidonic acid (80 μM) and H_2O_2 (1 mM) for 4 h. The data show the change of percentage of GFP fluorescent cells among the whole cell population (determined by phase contrast microscopy), averaging values obtained by analyzing more than 50 fields. (B) HeLa cells were transfected and treated at the same manner of (A), and then aliquots of cells were centrifuged and lysates were assayed for caspase-3 activity as described in Materials and methods. The upper panel shows representative traces of a singular experiment. The data are the mean of different angular coefficients ± SE. of five independent experiments; the bars in the graph (lower panel) show the change of percentage of caspase-3 activation compared to untreated cells.

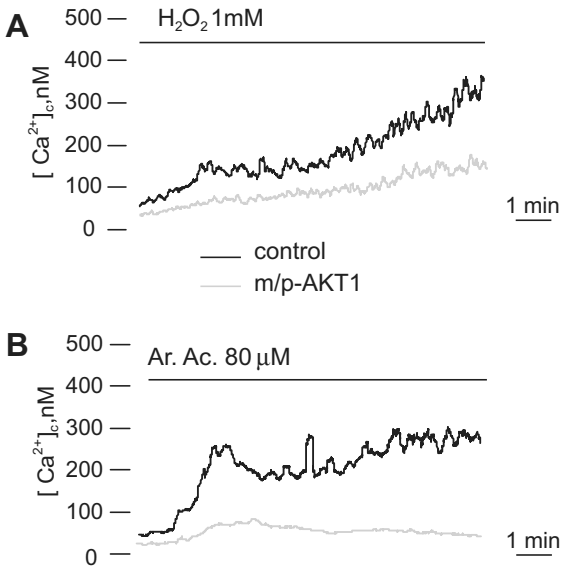

Fig. 4. Akt reduces $[Ca^{2+}]_c$ elevation generated by H_2O_2 and arachidonic acid. Effects of m/p-AKT1 on $[Ca^{2+}]_c$ increase induced by H_2O_2 (A) and arachidonic acid (B). HeLa cells were loaded with the Ca^{2+} indicator Fura-2/AM and $[Ca^{2+}]_c$ changes were measured as details in Materials and methods. The coverslips with the cells were maintained in 1 mM Ca^{2+}/KRB and, where indicated, the cells were challenged with 1 mM H_2O_2 or 80 μM arachidonic acid. The traces show the calibrated $[Ca^{2+}]_c$ values. Experiments were repeated at least five times.

confirmed by the direct measurement of caspase activity in H_2O_2 and arachidonic acid treated cells, showing a marked reduction of caspase-3 activity in m/p-AKT1 overexpressing cells (Fig. 3B). These results provided evidence that the m/p-AKT1-induced reduction of ER Ca^{2+} release increases the threshold for Ca^{2+}-mediated apoptosis.

Akt inhibits $[Ca^{2+}]_c$ increases induced by H_2O_2 and arachidonic acid

Apoptosis induced by H_2O_2 and arachidonic acid is triggered, or enhanced, by the release of Ca^{2+} from ER [29,30]. Thus, we investigated whether m/p-AKT1, acting on IP3Rs, had a direct effect on cytosolic apoptotic Ca^{2+} signals triggered by these apoptotic stimuli. Given that aequorin is not accurate enough to reveal small increases of $[Ca^{2+}]$, i.e. those expected in the cytosol after apoptotic stimulation, the $[Ca^{2+}]_c$ was measured with the fluorescent indicator Fura-2/AM [23]. In these experiments, in order to identify m/p-AKT1-overexpressing cells in single cell imaging experiments, the cells were co-transfected with mtGFP [31]. GFP-positive cells were distinguished from controls by the typical fluorescence emitted upon illumination with blue light. As shown in Fig. 4, treatment with 1 mM H_2O_2 (Fig. 4A) or 80 μM arachidonic acid (Fig. 4B) caused a $[Ca^{2+}]_c$ elevation, that gradually increased with time (Fig. 4). The simplest explanation for these results is that, as well as in the case of ceramide treatment [20], also these apoptotic stimuli caused a progressive release of Ca^{2+} from intracellular stores, thereby directly causing a $[Ca^{2+}]_c$ rise and activating capacitative Ca^{2+} influx, that in turn is responsible for maintaining a long-lasting $[Ca^{2+}]_c$ plateau. Interestingly, in m/p-AKT1-transfected cells, the $[Ca^{2+}]_c$ increases evoked by stimulation with H_2O_2 and arachidonic acid were significantly smaller than in controls.

Discussion

Virtually in all eukaryotic cells the dynamic regulation of $[Ca^{2+}]_c$ is fundamental for cell life [32,33]. In distinction to the other messengers, several organelles diffused throughout the cell can sequester Ca^{2+} and, in response to appropriate signals, release it back into the cytoplasm. Intracellular Ca^{2+} stores play an essential role in completing this regulation. The most important intracellular Ca^{2+} store is represented by the ER in non-muscle cells, and by its specialized counterpart, the sarcoplasmic reticulum, in muscle cells [34]. The Ca^{2+} channels responsible for the rapid release of Ca^{2+} from the ER in response to extracellular stimuli are the families of IP_3Rs [12]. Coordinated activation of these receptors is responsible for the generation of elementary and global (oscillating or persistent) Ca^{2+} signals observed in the cytosol [35–37].

Strong evidence has been also accumulated supporting a central role of Ca^{2+} in the regulation of cell death [38]. Recently it has been recognized, that modification of the ER Ca^{2+} pool may itself be involved in the initiation and regulation of the apoptotic process. As to ER Ca^{2+} release, it has been shown that owing to the efficient Ca^{2+} signal transmission between ER and mitochondria, ER Ca^{2+} release via IP3R play a pivotal role in transmitting apoptotic Ca^{2+} signals into mitochondria [39,40] and can sensitize to ceramide-induced apoptosis [28,41]. The involvement of IP3Rs in sensitisation to apoptotic stimuli has been shown also by transgenic and anti-sense techniques. Thus, IP3R-deficient lymphocytes are resistant to a large panel of apoptosis inducers [42] and anti-sense oligonucleotide-mediated downregulation of IP3R decreases the cell death of glucocorticoid-treated T cells [43] and NGF-depleted neurons [39]. Akt has been found associated to ER membranes [44] and IP3Rs can be phosphorylated by the well known apoptotic inhibitor Akt kinase [11].

The data presented here show that overexpression of m/p-AKT1 did not change the steady-state $[Ca^{2+}]_{er}$ level. In contrast, after stimulation with histamine we observed a dramatic reduction in the speed of Ca^{2+} release, without any changes of the IP3R expression levels. Thus we can conclude that Akt was regulating IP3R activity. This modulation of IP3R activity resulted in an inhibition of agonist-induced Ca^{2+} release as we can observed measuring the cytosolic and mitochondrial Ca^{2+} responses.

More interestingly for an antiapoptotic protein such as Akt, its overexpression significantly blunted the $[Ca^{2+}]_c$ increases induced by H_2O_2 and arachidonic acid. This apoptotic protocol was chosen because shown to act through Ca^{2+} and, in our hands, Akt was highly efficient in protecting cells from death induced by these stimuli.

At this stage we can not ascribe the alterations of ER Ca^{2+} release to a direct Akt-dependent phosphorylation of the IP3R. Indeed, in a recent work, Joseph and colleagues reported as the Ca^{2+} flux properties of IP3R mutants in their Akt substrate motif did not reveal any modification of channel function under agonist stimulation [11]. A possibility is that m/p-AKT1 phosphorylates other proteins interacting with the IP3Rs and in turn the phosphorylation state of these proteins regulates the IP3-mediated Ca^{2+} channel activity of IP3Rs. However, and probably more interestingly for a pro-survival protein, the Akt-dependent IP3R phosphorylation appears functional to suppress the activation of cell death program triggered by apoptotic stimuli [11]. In agreement with this observation, our data clearly demonstrated that the overexpression of m/p-AKT1 chimera is efficient in reducing significantly the apoptotic Ca^{2+} signals induced by H_2O_2 and arachidonic acid. Thus, these observations may provide one mechanism of Akt to restrain the apoptotic effects of Ca^{2+}.

Acknowledgments

This work was supported by the Italian Association for Cancer Research, Telethon, local funds from the University of Ferrara, the Italian University Ministry, the PRRIITT program of the Emilia Romagna Region, the Italian Space Agency, NIH (Grant #1P01AG025532-01A1) and the United Mitochondrial Disease Foundation (UMDF).

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