Blebbing of Thymocyte Plasma Membrane and Apoptosis are Related to Impairment of Capacitance Ca^{2+} Entry into Cells

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We studied the role of disturbances in cell Ca^{2+} homeostasis in plasma membrane blebbing and death of thymocytes. Capacitance Ca^{2+} channels of the plasma membrane and intracellular Ca^{2+} stores are involved in the induction and progression of changes in the membrane and cytoskeleton and apoptosis induced by acrylonitrile.

Key Words: *blebbing; apoptosis; necrosis; acrylonitrile; calcium*

Blebbing of the plasma membrane is a typical sign of apoptosis and necrosis. Progression of blebbing is related to disturbances in the membrane-cytoskeleton interaction, oxidation of functional groups in membrane and cytoskeletal proteins, changes in protein kinase and protease activities, and disturbances in the energy and ionic homeostasis in the near-membrane area of cells [4,5]. Blebbing of the plasma membrane (PM) usually develops in the initial stage of cell damage and is reversible. However, blebbing can cause cell lysis or release of membrane microparticles with procoagulant and antigenic activity into the extracellular medium. These processes are of considerable pathogenetic importance.

Ca^{2+} imbalance is a component of the effect of apoptogenic and necrogenic factors on the cell and a result of disturbed activity of ion channels and intracellular Ca^{2+} stores [2,6,12]. Much recent attention is given to the mechanisms that coordinate replenishment of intracellular Ca^{2+} stores and activity of potential-dependent and capacitance Ca^{2+} channels. Exhaustion of intracellular Ca^{2+} stores increases activity of store-activated (capacitance) Ca^{2+} channels, which initiates Ca^{2+} influx into cells [14]. Exhaustion of Ca^{2+} stores is a strong stimulus for apoptosis, which does not depend on Ca^{2+} entry into cells. On the other hand, excessive accumulation of Ca^{2+} in the endoplasmic reticulum (EPR) leads to so-called endoplasmic stress, which activates transcription factor NFκB. Reactive oxygen species have a co-stimulating effect on NFκB [13].

Cytotoxicity of xenobiotics, inductors of oxidative stress, is mediated by several mechanisms, including induction of mitochondrial dysfunction, disturbances in intracellular ionic homeostasis, damage to biological membranes, and oxidative modification of proteins and nucleic acids [7,15]. The cause-effect relations between these events remain unclear.

Lymphocytes express a variety of ion channels involved in the regulation of functional activity, proliferation, differentiation, and cell death [3,9]. Expression of these channels depends on the development of immunocompetent cells. Therefore lymphocytes and thymocytes are convenient models for studying of ionic homeostasis in nonexcitable cells.

Here we studied the Ca^{2+}-dependent mechanisms of blebbing and death of thymocytes under the in-

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fluence of acrylonitrile (AN), which acts as a xenobiotic and inductor of oxidative stress.

MATERIALS AND METHODS

Experiments were performed on male outbred albino mice weighing 16-18 g. Isolation and short-term culturing of thymocytes were performed routinely in serum-free medium 199 (10^6 cells/ml).

Viability of thymocytes was determined by exclusion of 0.1% trypan blue (vital dye) from cells.

Apoptosis and necrosis were detected by phosphatidylserine expression on the outer membrane (FITC-labeled annexin V) and membrane permeability for propidium iodide, respectively (Annexin V Apoptosis Detection Kit, Caltag Laboratories). The relative number of cells of four subpopulations (viable cells, cells in the early stage of apoptosis, cells in the state of secondary necrosis, and necrotic cells) was determined by fluorescent microscopy.

Blebbing of thymocyte PM was recorded by phase contrast microscopy. The cells were incubated at 37°C for 60 min. We detected cells that were characterized by initial blebbing (small bubbles on the membrane surface) and terminal blebbing (large bubbles, more than $^1/_3$ of cell diameter).

The intensity of oxidative stress was estimated by the content of malonic dialdehyde [1].

The results were analyzed by Student's *t* test.

RESULTS

Spontaneous blebbing of PM was induced in a short-term culture of thymocytes. The dynamics of initial and terminal blebbing correlated with the dynamics of MDA accumulation (r=0.85 and r=0.87, respectively). Up to 90% cells retained viability throughout the incubation period (Table 1).

Incubation of thymocytes with AN for 1 h was followed by induction of apoptosis and necrosis. It was detected by expression of phosphatidylserine on the outer membrane and increase in cell membrane permeability, respectively (Fig. 1, *a*). The number of cells with blebbing increased 2-fold. The initial and terminal blebbing peaked by the 15th and 45th minutes of incubation, respectively (Fig. 2). The dynamics of terminal (but not initial) blebbing correlated with accumulation of MDA in the incubation medium (r=0.82, Table 1).

The cytotoxic effect of AN (5 mM) was *in vitro* modulated with compounds regulating activity of intracellular Ca^{2+} stores (Table 2). These data suggest that AN induces the release of Ca^{2+} from EPR.

TABLE 1. Blebbing of PM in Cells (% of Total Cell Number) and MDA Accumulation in Thymocytes *in Vitro* ($M\pm m$)

Series	Incubation time, min				
	5	15	30	45	60
Control					
n	8	9	8	7	7
initial blebbing	2.80±0.85	2.78±0.64	2.70±0.63	3.16±0.44	2.86±0.55
terminal blebbing	1.74±0.37	1.41±0.22	1.83±0.32	3.19±0.27	3.20±0.51
necrosis	1.61±0.13	2.03±0.26	2.65±0.46	3.57±0.25	4.00±0.19
MDA, μmol/liter	1.24±0.13	1.35±0.08	1.62±0.10	1.95±0.12	2.06±0.17
AN, 5 mM *in vitro*					
n	10	10	10	10	10
initial blebbing	3.56±0.82	4.81±0.47*	3.13±0.43	2.94±0.46	2.83±0.61
terminal blebbing	1.60±0.25	2.70±0.31**	3.15±0.26**	5.13±0.4***	3.80±0.37
necrosis	2.16±0.20	3.56±0.13	4.65±0.21***	7.91±0.28****	11.09±0.26****
MDA, μmol/liter	1.43±0.19	2.46±0.16	2.99±0.15**	4.73±0.33***	5.22±0.15****
Isoptin (10^{-5} M) and AN (5 mM)					
n	4	4	4	4	4
initial blebbing	2.90±0.24	1.00±0.14^{++}	1.25±0.19^{+}	1.18±0.25^{+}	1.00±0.14
terminal blebbing	1.75±0.32	1.58±0.20	2.25±0.32	2.75±0.32^{+}	2.83±0.25
necrosis	3.93±0.17^{++}	5.00±0.14^{++}	6.03±0.27	8.00±0.33	12.00±0.56
MDA, μmol/liter					3.40±0.17^{+++}

Note. Here and in Tables 2 and 3: *p<0.05, **p<0.02, ***p<0.01, and ****p<0.001 compared to the control; ^{+}p<0.05, ^{++}p<0.02, ^{+++}p<0.01, and ^{++++}p<0.001 compared to the effect of AN.

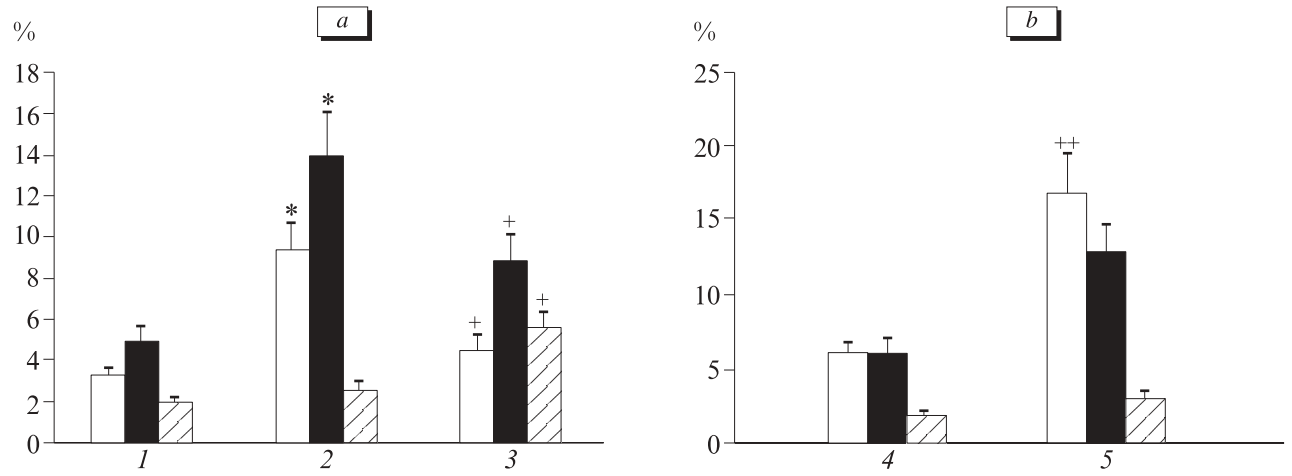

Fig. 1. Ratio between apoptotic and necrotic cells in thymocyte culture after *in vitro* treatment with acrylonitrile (AN) and isoptin (*a*) and during *in vitro* blockade of store-activated Ca^{2+} entry (*b*). Control (*1*), AN (*2*), isoptin and AN (*3*), SKF96365 (*4*), SKF96365 and AN (*5*). Light bars: apoptosis. Dark bars: necrosis. Shaded bars: secondary necrosis.

Preincubation of thymocytes with the blocker of potential-dependent ion channels isoptin (10 μM) decreased apoptogenic and necrogenic activity of AN, but the number of cells in secondary necrosis increased and the intensity of oxidative stress decreased (Fig. 1, *a*). Isoptin alone had no effect on thymocyte viability (Table 1).

The effect of isoptin can be interpreted as its ability to suppress xenobiotic-induced generation of free radicals (there are published data that isoptin possesses antioxidant activity [11]). Isoptin blocks voltage-dependent Ca^{2+} channels in cells of various types. Lymphocytes do not express these channels, but are sensitive to specific blockers, because they inhibit potential-dependent K^+ channels (*Kv*). These channels are maximally expressed in immature T cells and are physically associated with cytoskeletal proteins. They maintain membrane potential in thymocytes, which is essential for activity of calcium release-activated channels (CRAC) present in lymphocytes and involved in activation of these cells [3].

TABLE 2. Viability of Thymocytes *in Vitro* Cultured with Various Agents (*M*±*m*)

Conditions	*n*	Count of necrotic cells, %
Control	15	4.70±0.34
AN, 5 mM	15	2.14±0.76****
+sodium nitroprusside, 1 mM	8	10.50±1.24
+caffeine, 10 mM	8	8.05±0.57+++
+ruthenium red, 10^{-5} M	8	9.74±0.66+
+procaine, 1 mM	8	30.3±3.69+++
+EDTP, 0.5 mM	8	18.70±0.98+++

SKF96365 that acts as a CRAC blocker in PM was used to study the role of store-activated Ca^{2+} influx in the progression of blebbing, apoptosis, and necrosis in thymocytes. SKF96365 in a dose of 10 μM produced blebbing (incubation for 5-15 min) and apoptosis. SKF96365 had low prooxidant activity (Table 3).

SKF96365 increased apoptogenic, but not necrogenic activity of AN. This effect was not related to changes in prooxidant activity of the xenobiotic. The cells treated with SKF96365 and AN were characterized by intensive blebbing at various periods of incubation (Fig. 1, *b*).

Activity of *Kv* channels indirectly modulates the mechanism of capacitance Ca^{2+} entry into lymphocytes, which is mediated by membrane hyperpolarization and maintenance of Ca^{2+} current through CRAC [3]. Since isoptin increased the number of cells in secondary necrosis after treatment with AN, it prevents completion of the apoptosis program in damaged cells, rather that suppresses its induction. By contrast, CRAC blocker potentiated the apoptogenic effect of AN, but did not modulate necrosis and secondary necrosis. As seen from these data, AN-induced massive release of Ca^{2+} from EPR is quite expected. Hence, isoptin via K^+ channels only partially inhibits store-activated Ca^{2+} entry into cells through CRAC (in response to exhaustion of stores), whereas direct inhibition of these channels with SKF96365 completely blocks Ca^{2+} entry. This is why isoptin blocks, while SKF96365 potentiates apoptosis and blebbing.

Partial decrease in intracellular Ca^{2+} concentration and inhibition of capacitance Ca^{2+} entry protect cells from apoptosis [14], whereas complete inhibition of Ca^{2+} influx in cells through CRAC promotes progression of apoptosis [10].

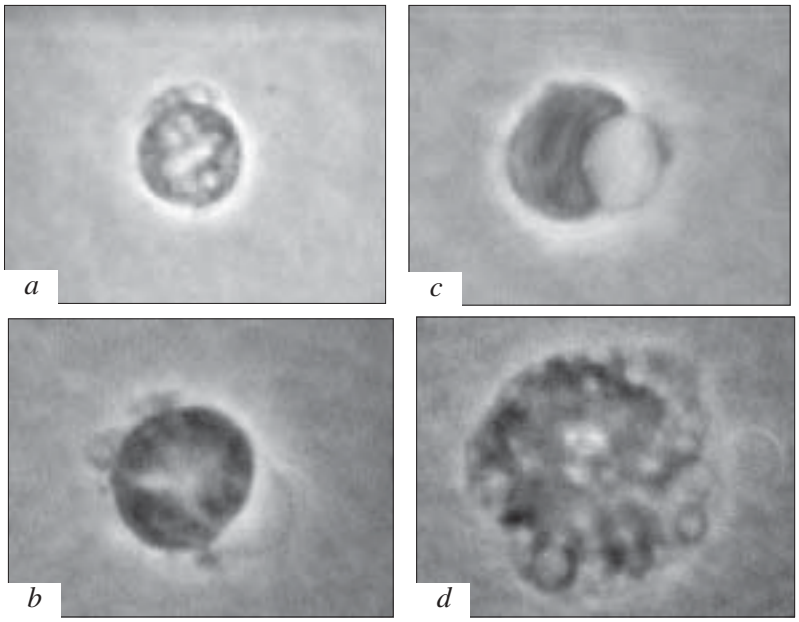

Fig. 2. Blebbing of the plasma membrane in thymocytes incubated with 5 mM acrylonitrile for 60 min: 15th minute of incubation, initial blebbing (*a*); 30th minute of incubation, initial and terminal bubbles at the upper and lower poles of the cell, respectively (*b*); 45th minute of incubation, terminal blebbing (*c*, *d*). ×400 (*a-c*); ×900 (*d*).

The intensity of blebbing in thymocyte PM significantly increases after exhaustion of Ca^{2+} stores (effect of AN) and more so after suppression of Ca^{2+} entry through CRAC (individual effect of SKF96365, combined effect of SKF96365 and AN). Thus, inhibition of blebbing in PM is associated with blockade of potential-dependent membrane channels and decrease in the intensity of oxidative stress.

Secondary necrosis (the process associated with cell inability to form apoptotic bodies) develops in thymocytes with suppressed blebbing. Partial inhibition of CRAC inhibits blebbing of PM, prevents apoptosis in cells, and contributes to the progression of secondary necrosis. Hence, the notions that blockade of mechanisms underlying the increase in intracellular Ca^{2+} concentration and free radical generation can reduce toxicity of xenobiotics [6,12,15] has to be revised, because adequate replenishment of intracellular Ca^{2+} stores via Ca^{2+} entry into cells can produce a potent protective effect.

AN-induced blebbing of PM, apoptosis, and necrosis of thymocytes are pathogenetically related to CRAC activity.

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TABLE 3. Blebbing of PM in Thymocytes and MDA Accumulation during *in Vitro* Blockade of Store-Activated Ca^{2+} Influx (% of Total Cell Number, *M±m*)

Series	Incubation time, min				
	5	15	30	45	60
SKF96365, 10 μM					
n	8	8	8	8	8
initial blebbing	7.01±0.66	4.95±0.32	5.43±0.79	4.01±0.51	3.66±0.74
terminal blebbing	7.20±1.66	5.95±0.79	6.04±0.43	4.44±0.55	4.66±0.61
necrosis	4.59±0.93	4.68±0.52	4.69±0.41	4.61±0.53	4.99±0.85
MDA, μmol/liter			2.8±0.31*		3.05±0.09**
SKF96365 (10 μM) and AN (5 mM)					
n	10	12	10	12	12
initial blebbing	8.14±0.93^{+++}	7.70±0.94^{+}	9.86±0.96^{++++}	10.05±0.83^{++++}	9.64±0.70^{++++}
terminal blebbing	5.92±0.88^{+++}	8.70±1.00^{++++}	9.36±0.79^{++++}	10.13±0.68^{++++}	11.89±1.03^{++++}
necrosis	5.04±078^{+++}	6.54±0.80^{+++}	6.94±1.09	9.23±1.09	10.08±0.91
MDA, μmol/liter			2.84±0.24		4.16±0.32^{++}

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