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Changes in K^+, Na^+, Cl^- Balance and K^+, Cl^- Fluxes in U937 Cells Induced to Apoptosis by Staurosporine: On Cell Dehydration in Apoptosis[1]

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Abstract—The K^+, Na^+, and Cl^- balance and K^+ (Rb^+) and $^{36}Cl^-$ fluxes in U937 cells induced to apoptosis by 0.2 or 1 μM staurosporine were studied using flame emission and radioisotope techniques. It is found that two-thirds of the total decrease in the amount of intracellular osmolytes in apoptotic cells is accounted for by monovalent ions and one-third consists of other intracellular osmolytes. A decrease in the amount of monovalent ions results from a decrease in the amount of K^+ and Cl^- and an increase in the Na^+ content. The rate of $^{36}Cl^-$, Rb^+ (K^+), and $^{22}Na^+$ equilibration between cells and the medium was found to significantly exceed the rate of apoptotic change in the cellular ion content, which indicates that unidirectional influxes and effluxes during apoptosis may be considered as being in near balance. The drift of the ion flux balance in apoptosis caused by 0.2 μM staurosporine was found to be associated with the increased ouabain-resistant Rb^+ (K^+) channel influx and insignificantly altered the ouabain-sensitive pump influx. Severe apoptosis induced by 1 μM staurosporine is associated with reduced pump fluxes and slightly changed channel Rb^+ (K^+) fluxes. In apoptotic cells, the 1.4–1.8-fold decreased Cl^- level is accompanied by a 1.2–1.6-fold decreased flux.

Key words: cell ion balance, apoptosis, ion transporter, monovalent ion flux, cell Cl^-.

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Abbreviations used: TCA-, trichloroacetic acid; Bum,bumetanide; DIDS, 4,4'-diisothiocyano-2,2'-stilbene-disulfonic acid; NPPB-, 5-nitro-2-(3-phenylpropylamino) benzoic acid; STS, staurosporine.

Programmed cell death is distinguished from non-programmed destruction by the lack of osmotic lysis. Monovalent ions play a critical role in the regulation of the water balance in apoptosis, as well as under normal conditions. It is commonly accepted that the intracellular K^+ content is reduced in apoptosis. However, the extent of the decrease in the K^+ content correlates to the decreased amounts of water and which mechanisms regulate the monovalent ion balance in apoptosis are still unclear. Currently, there is much interest in the role of monovalent ions in apoptosis (Yu, 2003; Burg et al., 2006; Okada et al., 2006; Shirokova, 2007; Bortner and Cidlowski, 2007; Lang et al., 2008; Hoffmann et al., 2009). Our previous papers focused on monovalent cation fluxes (Vereninov et al., 2004, 2007, 2008; Yurinskaya et al., 2005a, 2005b). Here, we present results on the intracellular content and fluxes of Cl^-, as well as K^+ and Na^+, which allowed one to evaluate the changes in the total balance of osmolytes in apoptosis. The examination of Cl^- and Rb^+ fluxes was necessary as a background for the analysis of the total balance of the Cl^-, K^+, and Na^+ fluxes in apoptotic cells. The total flux balance is considered in the next paper (Rubashkin et al., 2010).

MATERIALS AND METHODS

The study was performed on two sublines of human histiocytic lymphoma U937 cells, i.e., U937-160B2 cells obtained from Russian Cell Culture Collections and U937-DSMZ cells obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). We found that these sublines are distinguished by flux values through the Na^+/K^+ pump (Vereninov et al., 2008). However, the properties we studied in the present work are similar in both cell lines. Cells were grown in RPMI 1640 medium (Biolot, Russia) with 10% fetal calf serum (HyClone Standard, United States) at 37°C and 5% CO_2. The cell density in experiments was (1.0–1.5) × 10^6 cells/ml. Apoptosis was induced with 0.2 or 1.0 μM staurosporine (STS) (Sigma-Aldrich, Germany) added to cell cultures for 4–5 h. 0.2 mM STS stock solution was prepared in DMSO. Therefore, the final DMSO concentration in cell cultures did not exceed 0.1–0.5%. An inhibitor analysis of ion fluxes was performed with 0.1 mM ouabain, 0.05 mM bumetanide, 0.5 mM

[1] Dedicated to the memory of L.M. Chailakhyan.

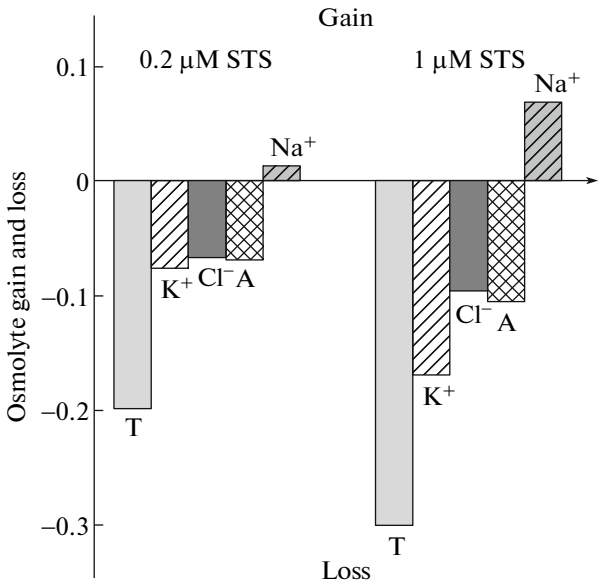

Fig. 1. Shift in the intracellular osmolytes in U937-160B2 cells induced to apoptosis with STS in different concentration. T, total osmolyte content ($K^+ + Na^+ + Cl^- + A^-$) in cells. A^-, other osmolytes (see Footnote to Table 1). Change in osmolyte content is expressed in mmol per 1 g protein.

DIDS, and 0.25 mM NPPB (Sigma-Aldrich, Germany).

Intracellular water was assessed by cell buoyant density in Percoll gradient. The density gradient was prepared from 35–40% Percoll (Pharmacia, Sweden) as described (Vereninov et al., 2004). The cell suspension ((3–5) × 10^6 cells in 100–200 μl) was layered on Percoll gradient and centrifuged at 400 *g* for 10 min. The density gradient was calibrated by markers 1.033, 1.049, and 1.062 g/ml (Sigma-Aldrich, Germany). The water content per 1 g protein (v) was calculated according to the formula $v = (1 - \rho/\rho_{dry})/[0.79(\rho - 1)]$, where ρ is the cell buoyant density and ρ_{dry} is the density of the dry mass of the cell. The density of the dry mass of the cell was taken as 1.35 g/ml and the percentage of protein in the dry mass was accepted as 0.79. The method we applied to estimate the intracellular water content is more sensitive than the standard method based on a comparison of radioisotope markers that do and do not penetrate pelleted cells. Thus, a 10% variation of the intracellular water content corresponds to a 0.005-g/ml change in the cell buoyant density and results in an easily registered 1-cm cell displacement in tubes.

For determination of intracellular ions the cells were pelleted, washed with 96 mM $MgCl_2$ five times without resuspending, and lysed with 5% trichloroacetic acid (TCA). Cations in the supernatant were assayed by flame emission with an AA 306 Perkin-Elmer spectrophotometer. $^{36}Cl^-$ radioactivity was determined in a liquid scintillator with a Beckman LS 6500 analyser. The intracellular content of Cl^- ions was evaluated by a $^{36}Cl^-$ distribution between cells and medium (4.4 × 10^3 bk/ml). Supernatant was removed and the cell pellet was dissolved in 0.1 N NaOH and analyzed for protein amount by Lowry method. The ion content was calculated as μmol per 1 g cellular protein.

The rate constants of ion exchange between the cells and medium were obtained by analyzing the time course of ion gain and release. Rb^+ and Li^+ influx was estimated by their uptake from the culture medium with an addition of 2.5 mM RbCl and 5 mM LiCl. To estimate the pump component of the Rb^+ influx, cells were incubated with 0.1 mM ouabain for 10 min. Longer incubation with ouabain resulted in a significant increase in intracellular Na^+ (Vereninov et al., 2007). To study the $^{36}Cl^-$ gain, a radioisotope was added to the medium with an activity of ≈4.4 × 10^3 bk/ml (Isotope, Russia). Experiments with $^{22}Na^+$ gain were described previously (Vereninov et al., 2007). To assess ion release, cells were preliminary incubated for 20 h in RPMI medium with 1 mM RbCl and then for 1.5 h in the same medium with an addition of 5 mM LiCl, $^{36}Cl^-$, and $^{22}Na^+$. It was suggested that tracers are equilibrated between the cells and medium according to the simple exponential kinetics $y(t) = y_{t=\infty}(1 - \exp(-kt))$ for the ion gain and $y(t) = y_0\exp(-kt)$ for ion release, where $y(t)$ is the tracer content at the moment t (5, 10, 20 min); $y_{t=\infty}$ and y_0 are the final and initial tracer contents, respectively; and k is the rate constant in both equations. The values of k were found as the fitting parameters for equations related to the curves in Fig. 2 or by averaging the rate constants calculated from single points by the same equations. The results were analyzed with Student's t test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Osmolyte content in normal and apoptotic U937 cells. The water balance between easily distensible animal cells and the medium is attained by equal intracellular and external osmolyte concentrations. Therefore, the total intracellular amount of osmolytes may be calculated from the total cellular water content. If the contents of K^+, Na^+, and Cl^- are known, it is possible to estimate the total amount of other intracellular osmolytes by the simple subtraction of the monovalent ions from the total osmolytes. Unfortunately, the publications on the quantitative evaluation of the osmolyte and water content in apoptotic cells are scanty because of the difficulties in reliably and sensitively determining the intracellular water. In the present study, intracellular water was assayed by the most sensitive method based on measuring the cell buoyant density in the Percoll gradient. It was found

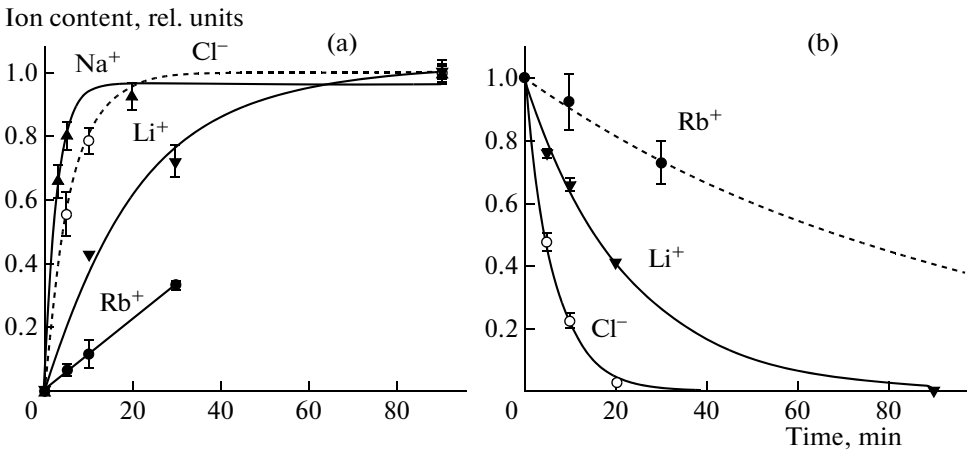

Fig. 2. Time course of $^{36}Cl^-$, $^{22}Na^+$, Li^+, and Rb^+ gain (a) and release (b) in normal U937 cells. Cells were incubated in RPMI medium with $^{36}Cl^-$ and $^{22}Na^+$ isotopes, 5 mM LiCl and 2.5 mM RbCl. Each point is the mean with its standard error for three to four experiments with duplicate or triplicate determinations in each one. Rate constants corresponding to the equations fitted the curves are presented in Table 2. Curves of $^{36}Cl^-$ gain (a) and Rb^+ release (b) were obtained with DMSZ subline; others were obtained with 160B2 subline.

previously that the radioisotope method, which is less sensitive, gives the same result when the change in cell water content is fairly high (Vereninov et al., 2008).

The amount of major osmolytes in normal and STS-treated apoptotic U937 cells is presented in Table 1; their apoptotic shift is shown in Fig. 1. The total amount of osmolytes is reduced in apoptotic cells treated with 0.2 or 1.0 μM STS by 0.2 and 0.3 mmol/g, respectively. In these cases, dehydration by 11.3 and 17.6% takes place. These shifts are the result of the loss of both the monovalent ions and other intracellular osmolytes. The loss of K^+ and Cl^- content taken together yield 0.14 and 0.27 mmol/g, whereas the loss of other osmolytes gives 0.07 and 0.1 mmol/g. An increase in the amount of Na^+ content diminishes the total loss of the intracellular osmolytes by 0.01 and 0.07 mmol/g for 0.2 and 1 μM STS, respectively. The reduction of the electric charge caused by changes in the total Na^+ and K^+ content is almost entirely compensated for by the reduction of the Cl^- content. Thus, two-thirds of the total decrease in the amount of intracellular osmolytes in apoptotic cells is accounted for by monovalent ions and one-third is associated with other intracellular osmolytes. There are numerous indications on altered nonelectrolyte transport, in particular taurine, in apoptotic cells (see reviews Lang et al., 2008; Hoffmann et al., 2009). However, the impact of the intracellular osmolytes other than monovalent ions on the total loss of osmolytes in apoptotic cells was estimated only recently (Poulsen et al., 2010).

The simultaneous estimation of ion and water content that allows one to evaluate the change in ion concentration showed that K^+ and Cl^- concentrations in intracellular water are only slightly altered. It is doubtful that this affects apoptosis regulators, more specifically caspases, as some workers believe (Hughes et al., 1997). Our results show that K^+ and Cl^- implication in the regulation of apoptosis is not associated with changes in their concentration in cell water.

Changes in monovalent ion fluxes during apoptosis. Changes of the $^{22}Na^+$, Li^+, and Rb^+ fluxes during apoptosis caused by STS were described previously (Vereninov et al., 2007). Here, the flame emission analysis of the intracellular monovalent ion content and Rb^+ fluxes in U937 cells was combined with measurements of $^{36}Cl^-$ content and fluxes. Figure 2 shows the gain and release of $^{36}Cl^-$, $^{22}Na^+$, Li^+, and Rb^+. The latter is a proper K^+ physiological analog and its movement is used as an indicator of K^+ transport. In proliferating cells, the rate of equilibration of K^+ (Rb^+), Na^+, and Cl^- between the cytoplasm and medium under both normal and apoptotic conditions is much higher than in cells used in classical studies on ion transport, such as erythrocytes, skeletal muscle, or squid axon (Vereninov and Marakhova, 1986). In proliferating U937 cells, about 15% intracellular Cl^-, 40% Na^+, 5% Li^+, and 1% Rb^+(K^+) are exchanged for 1 min. Exchange fluxes in U937-160B2 cells are: Cl^-—30–40, K^+—about 4, and Na^+—about 50–75 mmol/min per g protein (Table 1).

It is interesting to compare the exchange fluxes of ions with the net fluxes, i.e., with the rate of change in ion content caused by apoptosis. The data on K^+, Na^+, and Cl^- net fluxes are presented in Table 1; it can be seen that these fluxes are substantially less than exchange fluxes. One should bear in mind that the intracellular ion content changes mostly at the beginning of apoptosis (Figs. 3a, 3b). Therefore, in fact, the net fluxes in a 3–5-h interval should be much less than

Table 1. Water and ion balance in normal and staurosporine (STS)-induced apoptotic U937 cells

Characteristic	Normal	STS 0.2 μM	Normal	STS 1 μM
Cell density, g/ml	1.046–1.055	1.052–1.059	1.048–1.055	1.055–1.064
Water content, ml/g	5.64 ± 0.12 (8)	5.00 ± 0.07 (8)	5.52 ± 0.13 (4)	4.55 ± 0.06 (4)
Dehydration, %		–11.3		–17.6
Cl_i^-, mmol/g	0.23 ± 0.01 (32)	0.16 ± 0.01 (28)	0.23 ± 0.01 (14)	0.13 ± 0.01 (14)
K_i^+, mmol/g	0.59 ± 0.01 (34)	0.51 ± 0.01 (28)	0.70 ± 0.03 (16)	0.53 ± 0.02 (16)
Na_i^+, mmol/g	0.14 ± 0.01 (34)	0.15 ± 0.01 (28)	0.21 ± 0.01 (16)	0.28 ± 0.01 (16)
Total osmolyte amount, mmol/g	1.75	1.55	1.71	1.41
$Na_i^+ + K_i^+ + Cl_i^-$, mmol/g	0.95	0.82	1.14	0.94
Others	0.80	0.73	0.57	0.47
$[Na^+]_i$, mM	24.5	30.2	38.6	62
$[K^+]_i$, mM	104	102	126	116
$[Cl^-]_i$, mM	40	32	41	29
k_{Cl}, min^{-1}	0.19 ± 0.03 (5)	0.17 ± 0.02 (8)	0.14 ± 0.01 (18)	0.21 ± 0.01 (18)
I_{Rb}	1.71 ± 0.07 (31)	1.87 ± 0.10 (28)	1.88 ± 0.08 (23)	1.04 ± 0.07 (23)
I_{Rb}^G	0.27 ± 0.01 (31)	0.44 ± 0.03 (28)	0.30 ± 0.02 (23)	0.33 ± 0.01 (23)
Net fluxes				
K^+		–0.32		–0.70
Na^+		+0.054		+0.29
Cl^-		–0.28		–0.40
Total exchange stationary fluxes				
I_{Cl}	43	27	33	28
I_K	4.0	4.3	4.4	2.4
Partial fluxes				
I_K^P	3.3	3.3	3.7	1.6
I_K^G	0.6	1.0	0.7	0.8

Note: I_{Rb}, I_K, total fluxes Rb^+ and K^+; I_{Rb}^G, I_K^G, ouabain-resistant component of Rb^+ and K^+ influx; I_K^P, K^+ influx inhibited with 0.1 mM ouabain (pump). All fluxes are presented as μmole per 1 g protein per 1 min. K^+ fluxes are estimated as Rb^+ fluxes multiplied on K^+ and Rb^+ concentration ratio in medium (5.8 and 2.5 mM, respectively). Rate constant of Cl^- exchange in experiments with 0.2 μM STS is estimated from the curves of $^{36}Cl^-$ gain similar to those shown in Fig. 2a and in experiments with 1.0 μM STS is estimated from the curves of $^{36}Cl^-$ release (Fig. 2b). Total Cl^- exchange flux is estimated by multiplying of intracellular Cl^- content with exchange rate constant (k_{Cl}). To calculate the total osmolyte content in cells, it was accepted that osmolarity of the medium is 310 mosmol/l. Other intracellular osmolytes are calculated as the difference between the total osmolytes content and K^+, Na^+, and Cl^- content. Net fluxes were calculated assuming linear change of ion content in apoptotic cells for 4 h. Mean values and their standard errors are presented; the number of determinations is shown in brackets.

the exchange fluxes. This means that the influx and efflux of each of species of monovalent ions are in near balance. Of course, apoptosis progresses according to the time course of Rb^+ fluxes (Figs. 3c, 3d). However, slow changes in the intracellular ion content during apoptosis should be considered as the drift of the balanced state, which is important because it allows one to utilize the balance equations for the mathematical

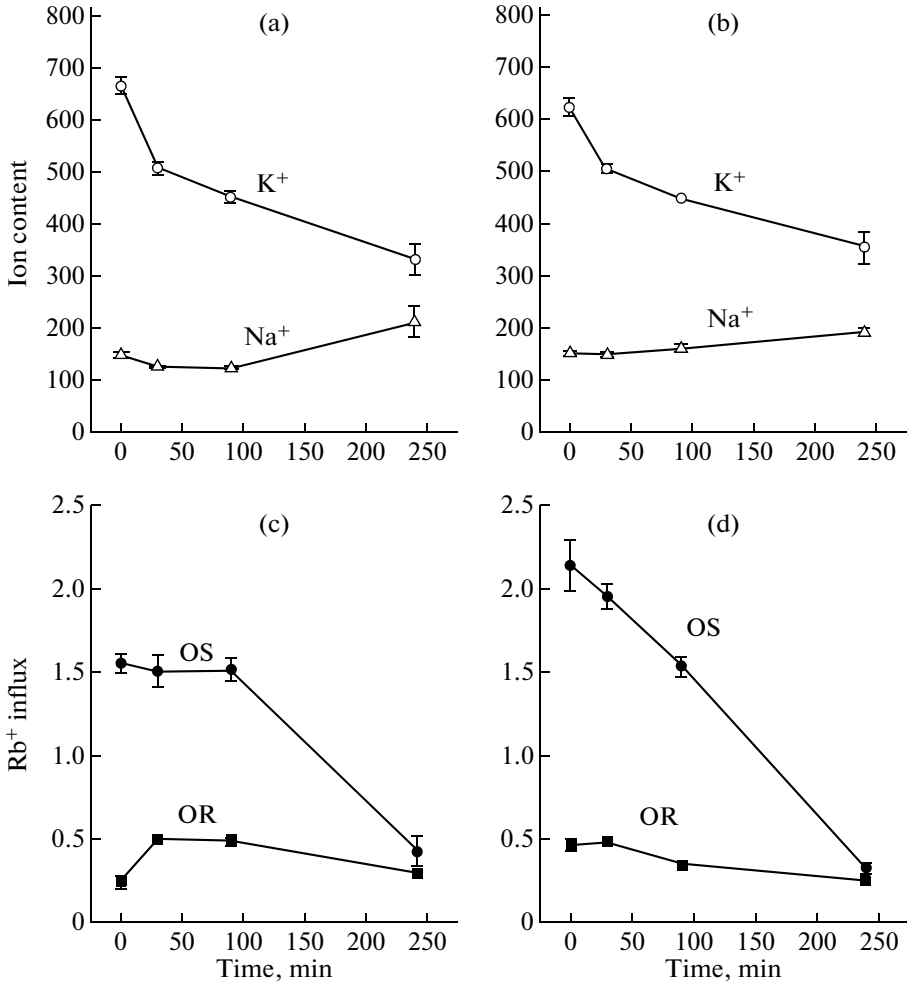

Fig. 3. Time course of intracellular K^+ and Na^+ ion content (a, b) and Rb^+ influxes (c, d) during apoptosis of U937 cells caused with 1 μM STS. 2.5 mM RbCl was added into the medium for 10 min. OS, Rb^+ influx component inhibited by ouabain (0.1 mM); OR, ouabain-resistant component. (a, c) U937-160B2 subline; (b, d) U937-DSMZ subline. The results of 3 experiments with triplicate determinations in each one are presented ($n = 9$). Ion content is presented as μmole per 1 g protein; Rb^+ influx given in μmol/g protein per 1 min.

modeling of the apoptotic change in ionic homeostasis discussed in the following paper (Rubashkin et al., 2010).

Based on the data presented here, some conclusions could be reached regarding the mechanisms of altered K^+ and Cl^- fluxes in apoptosis. Ouabain-sensitive K^+ influx mediated by the pump (I_K^P, Table 1) in apoptosis induced with 1 μM STS when dehydration was about 18% diminished (2.3-fold), whereas the ouabain-resistant component only increased by about 10%. Apoptosis caused by 0.2 μM STS was associated with less dehydration and enhanced ouabain-resistant component of the K^+ influx, but no decrease in the pump-mediated ouabain-sensitive K^+ influx. This implies that changes in different ion transporting mechanisms were responsible for the drift of ion balance in cells induced to apoptosis with 0.2 μM and 1 μM STS. It was found previously that the mechanism underlying the change in the ionic balance is determined not only by the STS concentration, but also the cell properties (Vereninov, 2008).

Radioisotope measurements of Cl^- fluxes in apoptotic cells have not been performed yet. The concept on critical role of modified Cl^- fluxes via channels in apoptotic cell shrinkage is based on electrophysiological examinations. Our results show that the balanced distribution of $^{36}Cl^-$ is established in 5–7 min. The level of Cl^- in apoptotic cells is 1.4 or 1.8 times lower than in normal cells depending on whether apoptosis was induced with 0.2 or 1.0 μM STS. Steady state Cl^- exchange flux is also 1.2–1.6 times diminished. The rate constant of Cl^- exchange rate in apoptosis caused by 1 μM STS is 1.5 times increased.

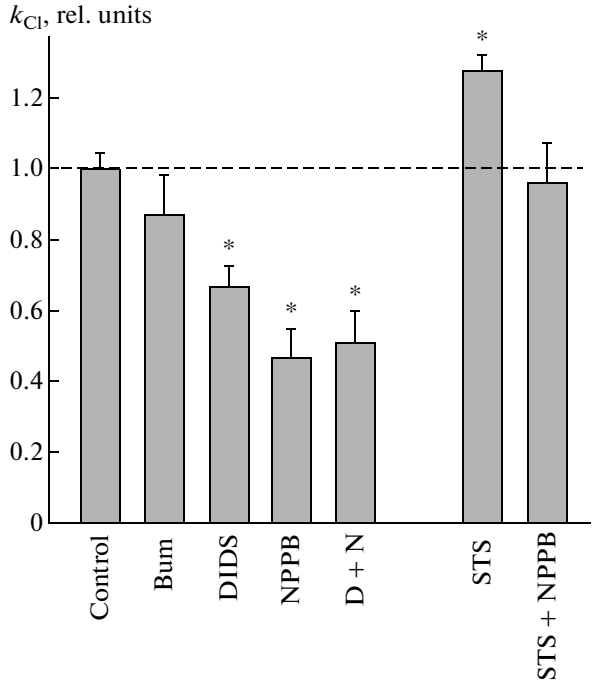

Fig. 4. Bumetamide, DIDS, and NPPB effects on rate constant of Cl^- exchange in U937-DSMZ cells. Inhibitors were added to the culture medium 20 min before $^{36}Cl^-$. Asterisks mark statistically significant differences. D + N—DIDS and NPPB were added simultaneously.

Figure 4 demonstrates the effect of known inhibitors of cotransport and chloride channels (bumetanide, DIDS and NPPB) on the rate constant of Cl^- exchange. According to electrophysiological data, NPPB blocks Cl^- channels and DIDS blocks both Cl^- channels and transporters, particularly Cl^-/HCO_3^-. In U937 cells, the constant of the rate of Cl^- exchange was decreased by DIDS by a digit of 1.5, while NPPB caused a twofold decrease.

The DIDS and NPPB effects were not cumulative with simultaneous application (Fig. 4, D+N). Hence, it is possible to conclude that both inhibitors block the same pathways of Cl^- carriage. Bumetanide did not change Cl^- flux in the examined cells. Therefore, even if the NKCC cotransporter is involved in Cl^- transport, its partial flux is so small that cannot be detected in the bulk Cl^- flux by the effect of bumetanide. The data presented are not sufficient to understand whether the changes in ion and water balance in apoptosis are associated with altered Cl^- channels or with altered Cl^- cotransport. The attempt to answer this question with use of mathematical modeling of ion balance is made in our next paper (Rubashkin et al., 2010).

Table 2. Rate constants of ion exchange (k) in U937 cells calculated from curves of ion gain or release

Ion	k values, min^{-1}	
	gain $y = 1 - \exp(-kt)$	release $y = \exp(-kt)$
$^{36}Cl^-$	0.158 ± 0.004	0.151 ± 0.003
$^{22}Na^+$	0.372 ± 0.033	
Li^+	0.048 ± 0.007	0.045 ± 0.002
Rb^+	0.011 ± 0.001	0.010 ± 0.001

Note: Constants are presented with standard errors of approximation.

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