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VOLUME-DEPENDENT K^+ AND Cl^- FLUXES IN RAT THYMOCYTES

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SUMMARY

1. Hypotonic stress unmasked inward and outward K^+ and Cl^- movements in rat thymocytes. This KCl flux stimulation was reduced by DIOA (dihydroindenyl-oxy-alkanoic acid), but not by DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonate), quinidine, DPAC 144 (5-nitro-2-(2-phenylethyl-amino)-benzoic acid), bumetanide or ouabain.

2. In isotonic media (308 ± 5 mosmol kg^{-1}), the cells exhibited the following DIOA-sensitive fluxes: (i) a K^+ efflux of $42{\cdot}7 \pm 17{\cdot}1$ mmol (l cells.h)$^{-1}$ (mean ± S.D., $n = 7$), (ii) a Cl^- efflux of 68 ± 21 mmol (l cells.h)$^{-1}$ ($n = 3$), (iii) a Rb^+ influx of $9{\cdot}7 \pm 3{\cdot}9$ mmol (l cells.h)$^{-1}$ ($n = 6$) and (iv) a Cl^- influx of $9{\cdot}4 \pm 4{\cdot}1$ mmol (l cells.h)$^{-1}$ ($n = 6$).

3. Hypotonic shock (183–200 mosmol kg^{-1}) induced a sevenfold stimulation of DIOA-sensitive K^+ and Cl^- effluxes and a twofold stimulation of DIOA-sensitive Rb^+ and Cl^- influxes (with a Rb^+ to Cl^- stoichiometry of $1{\cdot}04 \pm 0{\cdot}31$; mean ± S.D., $n = 6$).

4. The DIOA-sensitive membrane carrier catalysed net outward KCl extrusion (the outward/inward flux ratio was 5–7 in isotonic media and 20 in hypotonic media at 189 mosmol kg^{-1}). Inhibition of DIOA-sensitive $^{36}Cl^-$ efflux by cell K^+ depletion suggested coupling of outward K^+ and Cl^- fluxes. Conversely, inward K^+ and Cl^- fluxes were found to be uncoupled in NO_3^- media and in K^+-free media.

5. The results clearly show that rat thymocyte membranes possess a 1:1 K^+–Cl^- co-transport system which is strongly activated by hypotonic shock and catalyses net KCl extrusion.

INTRODUCTION

The pioneering work of Kregenow (1971, 1981) revealed that duck (nucleated) red cells extrude KCl (and water) in response to hypotonic media. This phenomenon, namely 'regulatory volume decrease' (RVD), was subsequently found in erythrocytes from other species, including man (Dunham & Ellory, 1981; Lauf, Adragna & Garay, 1984; Garay, Nazaret, Hannaert & Cragoe, 1988), in Ehrlich ascites tumour cells (Hoffman, Simonsen & Dunham, 1984; Thornhill & Laris, 1984), in lymphocytes (for review see Grinstein, Rothstein, Sarkadi & Gelfand, 1984; Grinstein & Dixon, 1989) and in several other cells (for review see Hoffman & Simonsen, 1989; Lauf, 1988).

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In red blood cells, kinetic studies suggested that RVD was mediated by an outward K^+–Cl^- co-transport (Dunham & Ellory, 1981; Kregenow, 1981; Lauf *et al.* 1984; Garay *et al.* 1988). At that time, the observation that the Na^+–K^+–Cl^- co-transport system was able to catalyse K^+ fluxes in the absence of Na^+ (Canessa, Brugnara, Cusi & Tosteson, 1986) and other considerations suggested that outward K^+–Cl^- co-transport was perhaps one mode of operation of the Na^+–K^+–Cl^- co-transport system. However, outward K^+–Cl^- co-transport was found to be resistant to bumetanide, a potent and quite specific inhibitor of the Na^+–K^+–Cl^- co-transport system (Lauf *et al.* 1984). Moreover, it was also resistant to quinidine, quinine, DIDS (4,4′-diisothiocyanostilbene-2,-2′-disulphonate), ouabain, amiloride and EIPA (ethyl-isopropyl-amiloride) (Lauf *et al.* 1984; Garay *et al.* 1989).

A clear understanding of the RVD mechanism in red blood cells was provided by the development of a new ion transport inhibitor: DIOA (dihydroindenyl-oxy-alkanoic acid; see structure in Fig. 1), an RVD inhibitor without side effects on the Na^+–K^+–Cl^- co-transport system. It was found that RVD was mediated by a DIOA-sensitive K^+–Cl^- co-transport system having the following properties: (i) it was quiescent under physiological conditions, (ii) when the red cells were swollen it used the energy of the electrochemical K^+ gradient in order to catalyse a net efflux of both K^+ and Cl^-, thus helping the cells to extrude the excess intracellular water, and (iii) it was the only cell volume regulator in these cells.

DIOA is now being used for the investigation of the K^+–Cl^- co-transport system in red blood cells (see for instance Morle *et al.* 1989; Vitoux, Olivieri, Garay, Cragoe, Galacteros & Beuzard, 1989). Therefore, it is important to insist that, although DIOA is exempt of side-inhibitory action on the Na^+–K^+–Cl^- co-transport system, it inhibits the Cl^-–HCO_3^- exchanger (like most aromatic carboxylic acids), and the Ca^{2+}-sensitive K^+ channels (Garay *et al.* 1988 and Table 1 in Arrazola, Rota, Hannaert, Soler & Garay, 1993). Of course, side inhibitory effects are also observed with almost all classical transport inhibitors. For instance, furosemide was used for almost 15 years in order to characterize the Na^+–K^+–Cl^- co-transport system (see for instance Canessa *et al.* 1986), in spite of the fact that it also effectively inhibits the anion carrier (Brazy & Gunn, 1976; Garay, Hannaert, Nazaret & Cragoe, 1986).

The use of DIOA for studying the K^+–Cl^- co-transport system requires: (i) to avoid the side effects on the Cl^-–HCO_3^- exchanger and the Ca^{2+}-sensitive K^+ channels by working in the presence of DIDS and/or quinidine and (ii) to run parallel studies with at least DIDS and quinidine.

In lymphoid cells, Grinstein and co-workers (Grinstein *et al.* 1984; Grinstein & Dixon, 1989; Grinstein & Foskett, 1990) reported a very different RVD mechanism: i.e. RVD was suggested to be mediated by independent Cl^- and quinine-sensitive K^+ channels.

The apparent differences between RVD mechanisms in erythrocytes and lymphoid cells pushed us to investigate if lymphoid cell membranes had a K^+–Cl^- co-transport system and if it was functional, i.e. stimulated by cell swelling. Therefore, we examined hypotonically stimulated K^+ and Cl^- fluxes in rat thymocytes by using DIOA and other ion transport inhibitors (see chemical structures in Fig. 1).

METHODS

Rats

Male Wistar rats (weighing 200–300 g) were supplied by Centre d'Elevage Roger Janvier (Le Genest, France). All rats were fed a standard diet containing 0·2% NaCl (UAR, Villemoisson, France) and were given tap water to drink.

Preparation of thymocytes

Animals were killed by rapid decapitation. Rat thymus was extracted and homogenized in Na^+–K^+ Ringer medium at room temperature, and the cells were separated by simple aspiration with a pipette. The Na^+–K^+ Ringer medium contained (mmol l^{-1}): 145 NaCl, 5 KCl, 10 3-(*N*-morpholino)propanesulphonic acid (Mops)–Tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7·4 at 20 °C), 1 $MgCl_2$ and 1 $CaCl_2$. The cell suspension was centrifuged at room temperature for 5 min at 800 *g* and then washed twice with Na^+–K^+ Ringer medium at room temperature. The obtained cell population contained almost 100% of thymocytes. Viability during incubation in all flux media was > 95% as assessed by Trypan Blue exclusion.

Ion fluxes

Washed fresh thymocytes were resuspended at room temperature in Na^+–K^+ Ringer medium containing 10 mmol l^{-1} glucose (and the pH adjusted to 7·4 at 37 °C). The osmolality of the suspending medium was measured by using a Knauer semi-micro-osmometer (Oberursel, FRG) and adjusted to 308 ± 5 mosmol kg^{-1}. The cell suspension was preincubated for 30 min at 37 °C, and then used for the different flux experiments.

K^+ efflux

Thymocytes were washed three times with 150 mmol l^{-1} NaCl at 4–10 °C and resuspended in the same medium at a thymocrit of 12–18%. Thymocrit was measured like a haematocrit (v/v) in an Adams Autocrit II (Clay Adams; for details see Senn & Garay, 1989). A portion of the cell suspension was set aside to measure thymocrit and intracellular K^+ in an Eppendorf flame photometer (Eppendorf Gerätebau, Hamburg, FRG). To measure intracellular K^+ the suspensions were frozen and thawed two or three times, sonicated, and trichloroacetic acid (TCA) was added up to a final concentration of 5%. The suspensions were then centrifuged for 10 min at 3000 *g*. K^+ content was measured in the supernatants by flame photometry. Intracellular levels of K^+ were calculated by dividing the K^+ readings by the final thymocrit (v/v).

Cell suspensions (100 μl) were added to duplicates of tubes containing 1 ml of Na^+–Rb^+ medium at 4–10 °C (final thymocrit = 1–1·6%). The Na^+–Rb^+ medium contained (mmol l^{-1}): 145 NaCl, 5 RbCl, 10 Mops–Tris buffer (pH 7·4 at 37 °C), 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 1 ouabain and 0·05 bumetanide. The osmolality of the Na^+–Rb^+ medium was adjusted to 308 ± 5 mosmol kg^{-1}.

The cell suspensions were incubated for times (s): 0, 20, 40, 60, 90, 120, 240 and 300 at 37 °C. The tubes were then transferred to 4–10 °C and spun down for 4 min at 800 *g* at 4 °C. The supernatants were rapidly removed for measuring K^+ contents by flame photometry. K^+ standards (checked with commercial standards, Merck, Darmstadt) were prepared in water and compared with those prepared in the different efflux media.

K^+ efflux was obtained from the initial (linear) slope relating external K^+ with time divided by the final thymocrit.

Rb^+ influx

Thymocytes were washed three times with 150 mmol l^{-1} NaCl at 4–10 °C and resuspended in the same medium at a thymocrit of 12–18%. Cell suspensions (100 μl) were added to duplicates of tubes containing 1 ml of Na^+–Rb^+ medium at 4–10 °C. The cell suspensions were incubated for times (min): 0, 2, 5, 10 and 30 at 37 °C. Then the tubes were transferred to 4–10 °C, washed three times with cold 150 mmol l^{-1} NaCl and resuspended in 2 ml of 0·02% Acationox. The suspensions were frozen and thawed two or three times, sonicated, and TCA was added up to a final concentration of 5%. The suspensions were then centrifuged for 10 min at 3000 *g*. Rb^+ and K^+ contents were measured in the supernatants by using an IL 457 atomic absorption spectrophotometer (Instrumentation Laboratory, Wilmington, MA, USA). Intracellular levels of Rb^+ and K^+ were calculated by dividing the Rb^+ and K^+ readings by the final thymocrit.

Rb^+ influx was obtained from the initial (linear) slope relating internal Rb^+ with time divided by the final thymocrit.

Cl^- efflux

Thymocytes were incubated for 45 min at 37 °C in Na^+–K^+ Ringer medium containing 2·5 μCi ml^{-1} of $^{36}Cl^-$ and 10 mmol l^{-1} glucose (pH adjusted to 7·4 at 37 °C). A portion of the flux media was set aside to measure specific $^{36}Cl^-$ activity.

$^{36}Cl^-$-loaded cells were washed three times with 150 mmol l^{-1} NaCl at 4–10 °C and resuspended in the same medium at a thymocrit of 12–18 %. A portion of the cell suspension was set aside to measure thymocrit and intracellular $^{36}Cl^-$ activity in a type 300 Packard beta-counter (Packard Instruments Company, Downers Grove, IL, USA). To measure intracellular $^{36}Cl^-$ the suspensions were frozen and thawed two or three times, sonicated, and TCA was added up to a final concentration of 5 %. The suspensions were then centrifuged for 10 min at 3000 *g*. The supernatants were removed, diluted in an optifluor high flash-point liquid scintillation cocktail (Packard Instruments Company, Downers Grove, IL, USA) and $^{36}Cl^-$ activity was measured in a beta-counter. Intracellular $^{36}Cl^-$ activities were calculated by dividing the $^{36}Cl^-$ readings by the final thymocrit.

Cell suspensions (100 μl) were added to duplicates of tubes containing 1 ml of Na^+–Rb^+ medium (final thymocrit 1–1·6 %) at 4–10 °C. The cell suspensions were incubated for times (s): 0, 20, 40, 60, 90 and 120 at 37 °C. The tubes were then transferred to 4–10 °C and spun down for 4 min at 800 *g* at 4 °C. The supernatants were rapidly removed for measuring $^{36}Cl^-$ activity in a beta-counter.

Cl^- efflux was obtained from the initial (linear) slope relating (external) $^{36}Cl^-$ activity with time divided by the final thymocrit and the specific $^{36}Cl^-$ activity.

Cl^- influx

Thymocytes were washed three times with 150 mmol l^{-1} NaCl at 4–10 °C and resuspended in the same medium at a thymocrit of 12–18 %. Cell suspensions (100 μl) were added to duplicates of tubes containing 1 ml of a Na^+–Rb^+ medium containing 0·5 μCi ml^{-1} of $^{36}Cl^-$ at 4–10 °C.

The cell suspensions were incubated for the following times (min): 0, 2, 5, 10, 20, 40 and 80 at 37 °C. The tubes were then transferred to 4–10 °C, washed three times with cold 150 mmol l^{-1} NaCl and resuspended in 2 ml of 0·02 % Acationox. The suspensions were frozen and thawed two or three times, sonicated, and TCA was added up to a final concentration of 5 %. The suspensions were then centrifuged for 10 min at 3000 *g*. The supernatants were removed for measuring $^{36}Cl^-$ activity in a beta-counter. Intracellular $^{36}Cl^-$ activity was calculated by dividing the $^{36}Cl^-$ readings by the final thymocrit.

Unidirectional Cl^- influx was obtained from the initial (linear) slope relating (internal) $^{36}Cl^-$ activity with time divided by the specific $^{36}Cl^-$ activity.

Cytosolic free calcium

Cytosolic free calcium content was measured by using the fluorescent probe fura-2 AM (fura 2 tetrakis acetoxymethyl-ester). Fresh thymocytes were incubated for 15 min at 37 °C in Na^+–K^+ Ringer medium. The Na^+–K^+ Ringer medium contained (mmol l^{-1}): 145 NaCl, 5 KCl, 10 Mops–Tris (pH 7·4 at 37 °C), 1 $MgCl_2$, 1 $CaCl_2$ and 10 glucose. Then, the cells were centrifuged and resuspended in Na^+–K^+ Ringer medium containing 2·5 μmol l^{-1} of fura-2 AM at a concentration of $5–8 \times 10^7$ cells ml^{-1}. The cell suspensions were incubated for 30 min at 37 °C. The cells were then washed twice with Na^+–K^+ Ringer medium and suspended in the same medium at a concentration of $5–8 \times 10^7$ cells ml^{-1}. To measure cytosolic free calcium content, the suspensions were diluted in the fluorescent cuvette of a Shimatzu RF 5000 spectrofluorimeter. The suspensions were magnetically stirred at 37 °C (excitation wavelengths = 340 and 380 nm and emission wavelength = 505 nm). Fluorescent readings were calibrated by using ionomycin and EGTA.

Membrane potential

Membrane potential was measured by using the fluorescent probe DiS-C_3-(5) (3,3′-dipropyl-thyadicarbocyanine). Fresh thymocytes were incubated for 45 min at 37 °C in Na^+–K^+ Ringer medium, washed twice with NaCl (150 mmol l^{-1}) and resuspended in the same NaCl medium. To measure membrane potential the suspensions were diluted tenfold in the cuvette of the spectrofluorimeter (final cell concentration = 2×10^6 cells ml^{-1}) containing 2 ml of Ringer medium

with different Na^+ and K^+ concentrations ($Na^+ + K^+$ was constant) and 1 μmol l^{-1} DiS-C_3-(5). The suspensions were magnetically stirred at 37 °C (excitation wavelength = 620 nm and emission wavelength = 665 nm). Five minutes later, valinomycin was added up to a final concentration of 2 μmol l^{-1}. Membrane potential was calculated from the equilibrium K^+ concentration, i.e. the K^+ concentration where valinomycin was not able to induce a change in fluorescence ('null-point').

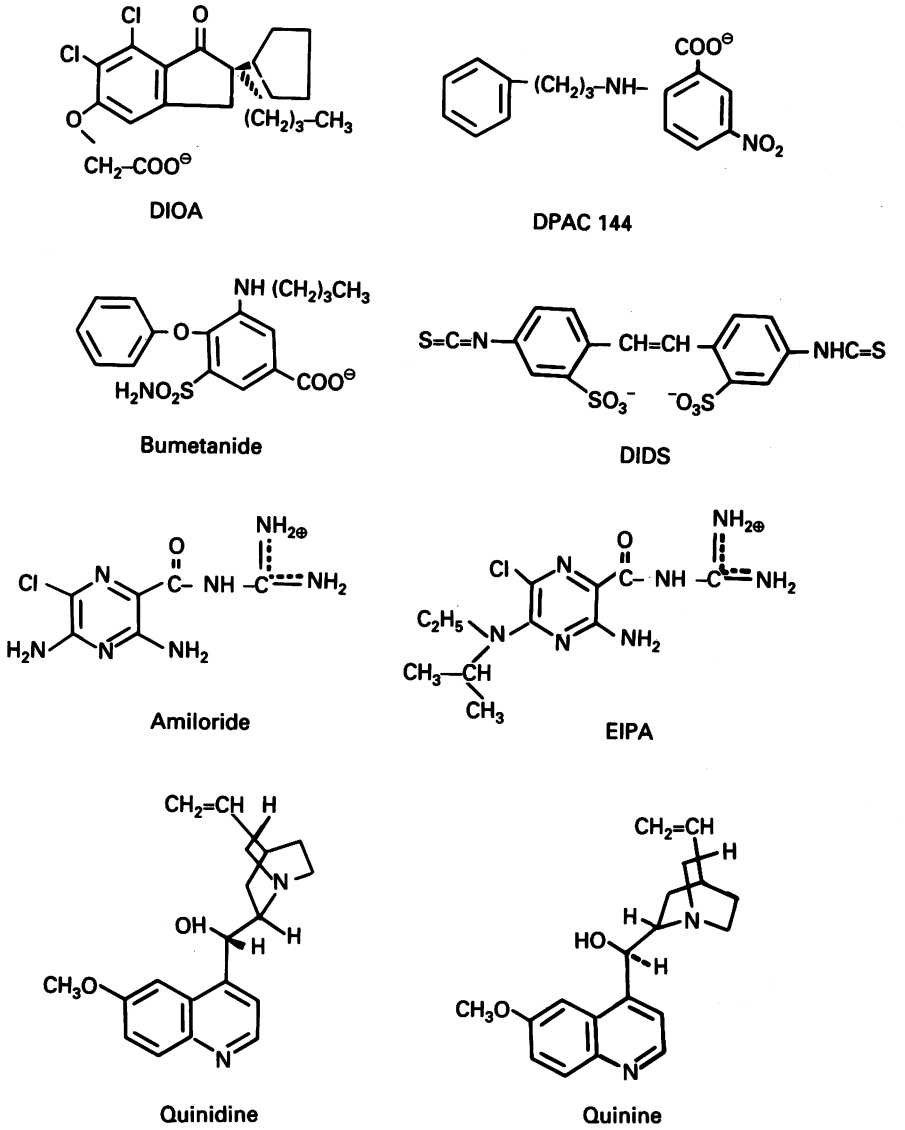

Fig. 1. Chemical structures of ion transport inhibitors used in the study (see also Burgess, Claret & Jenkinson, 1981; Berkowitz & Orringer, 1982; Hugues, Romey, Duval, Vincent & Lazdunski, 1982; Garay *et al.* 1986, 1988; Traore, Cognard, Poitreau & Raymond, 1986; Wangeman *et al.* 1986; Cragoe, 1987).

Cell pH

Cell pH was measured by using the fluorescent probe BCECF AM (acetoxy-methyl 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein). Fresh thymocytes were incubated for 15 min at 37 °C in Na^+–K^+ Ringer medium, centrifuged and resuspended in the same medium containing 2·5 μmol l^{-1}

of BCECF AM at a concentration of $5–10 \times 10^6$ cells ml^{-1}. The cell suspensions were incubated for 30 min at 37 °C. The cells were then washed three times with cold NaCl (150 mmol l^{-1}) and suspended in the same medium at a concentration of $5–10 \times 10^6$ cells ml^{-1}. To measure cytosolic pH, the suspensions were diluted in the fluorescent cuvette of the spectrofluorimeter (final cell concentration $\approx 10^6$ cells ml^{-1}). The suspensions were magnetically stirred at 37 °C (excitation wavelengths = 505 nm and emission wavelength = 530 nm). pH readings were calibrated by using nigericin.

The effect of hypotonicity, ion substitutions and drugs

In experiments with anisotonic media, the incubation media contained a Na^+–K^+ Ringer media of the following composition (mmol l^{-1}): $(140+x)$ NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Mops–Tris (pH 7·4 at 37 °C) and 10 glucose; where x varied from -140 to $+180$ mmol l^{-1}. The osmolality of the solutions was measured by using a Knauer semi-micro-osmometer (Oberursel, FRG).

To study the effect of internal K^+ (K_i^+) on $^{36}Cl^-$ efflux, Na^+-loaded–K^+-depleted cells were prepared by using a previously published nystatin technique (Senn & Garay, 1989). Cell volume of nystatin-treated cells was measured by using a Coulter counter ZM (Coulter Electronics, Luton, UK). Differences in mode of cell volume distribution between nystatin-treated and untreated cells never exceeded ± 5 %. The cells were loaded with $^{36}Cl^-$ in a K^+-free Ringer medium and $^{36}Cl^-$ efflux was measured in hypotonic Na^+–K^+ Ringer media (183 ± 5 mosmol kg^{-1}) containing (mmol l^{-1}): 0·05 DIDS, 0·05 bumetanide and 1 ouabain, by using the above described protocol.

To study the effect of external Cl^- (Cl_o^-) on Rb^+ influx, cells were washed three times with cold 150 mmol l^{-1} $NaNO_3$ and resuspended in hypotonic Na^+–Rb^+ medium (183 ± 5 mosmol kg^{-1}) where Cl^- was substituted mole by mole with NO_3^-. Rb^+ influx was measured by using the above described protocol.

To study the effect of external K^+ (K_o^+) on Cl^- influx, cells were incubated in hypotonic Na^+ Ringer media (183 ± 5 mosmol kg^{-1}) containing (mmol l^{-1}): 0·05 DIDS and 0·05 bumetanide, and where K^+ was substituted mole by mole with Na^+. $^{36}Cl^-$ influx was measured by using the above described protocol.

DIOA and other compounds used in the study have been described previously (Berkowitz & Orringer, 1982; Cragoe, 1987; Garay *et al.* 1986, 1988; Wangeman *et al.* 1986; see Fig. 1 for chemical structures). DIOA was provided by E. Cragoe Jr (Nacogdoches, TX, USA). DPAC 144 was synthesized by G. Moinet and Th. Imbert (Lab. Anphar Rolland, Chilly Mazarin, France). Bumetanide was a gift from Leo Laboratories (Vernouillet, France). Cetiedil was obtained from Innothera Laboratories (Arcueil, France). Fura-2 AM, DiS-C_3-(5) and BCECF AM were obtained from Molecular Probes (Eugene, OR, USA). Nigericin and ionomycin were obtained from Calbiochem (distributed through France Biochem, Meudon, France). Acationox (Monoject Scientific, St Louis MO, USA) was supplied by American Scientific Products (McGaw Park, IL, USA). The other compounds were either from Merck or Sigma (distributed through Coger, Paris, France).

To study the effect of transport inhibitors on ion fluxes in rat thymocytes, the free acid (or base) form was neutralized with Tris base (or with Mops), and the compounds were added from freshly prepared, concentrated stock solutions in water or dimethyl sulphoxide (DMSO), provided that the final concentrations of these solvents had no effect *per se* on ion fluxes (final DMSO concentration in the flux media was always lower than 0·1 %). DIOA was tested in concentration–response curves from stock solutions containing 50–500 mmol l^{-1} of compound in DMSO.

RESULTS

Inhibitory action of DIOA on hypotonically stimulated K^+ and Cl^- fluxes

DIOA-sensitive K^+ efflux

Figure 2 shows the response of rat thymocytes to hypotonic shock. It can be seen that: (i) hypotonic media strongly stimulated the release of internal K^+ content, (ii) K^+ efflux remained at the initial rate for about 2 min, (iii) the K^+ extrusion was fully blocked by 44 μmol l^{-1} DIOA and (iv) even in isotonic media DIOA blocked a small K^+ efflux.

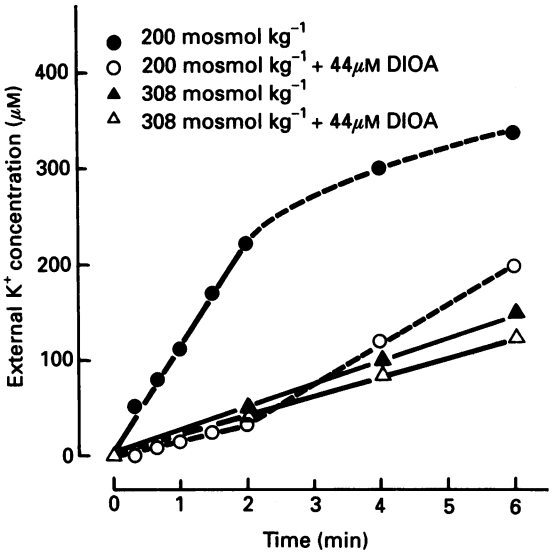

Fig. 2. K^+ release induced by a hypotonic medium in rat thymocytes (medium at osmolality of 200 ± 5 mosmol kg^{-1} and containing 1 mmol l^{-1} ouabain and 50 μmol l^{-1} bumetanide). The stimulated K^+ efflux remained at the initial rate for about 2 min and was fully blocked by 44 μmol l^{-1} DIOA. Note the presence of a small DIOA-sensitive K^+ efflux in isotonic medium.

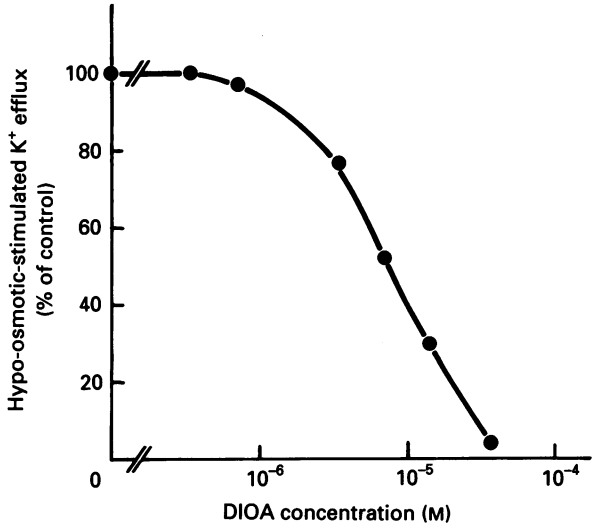

Fig. 3. Inhibition of hypotonically stimulated K^+ efflux by DIOA (medium at osmolality of 200 ± 5 mosmol kg^{-1}). It can be seen that DIOA inhibited the hypotonically stimulated K^+ release with an IC_{50} of about 8×10^{-6} mol l^{-1}.

In isotonic media (308 ± 5 mosmol kg^{-1}), the initial rate of DIOA-sensitive K^+ efflux was $42{\cdot}7 \pm 17{\cdot}1$ mmol (l cells.h)$^{-1}$ (mean $\pm$ S.D., $n = 7$). This value was sevenfold stimulated up to 289 ± 105 mmol (l cells.h)$^{-1}$ (mean $\pm$ S.D., $n = 5$) in hypotonic medium (200 ± 5 mosmol kg^{-1}).

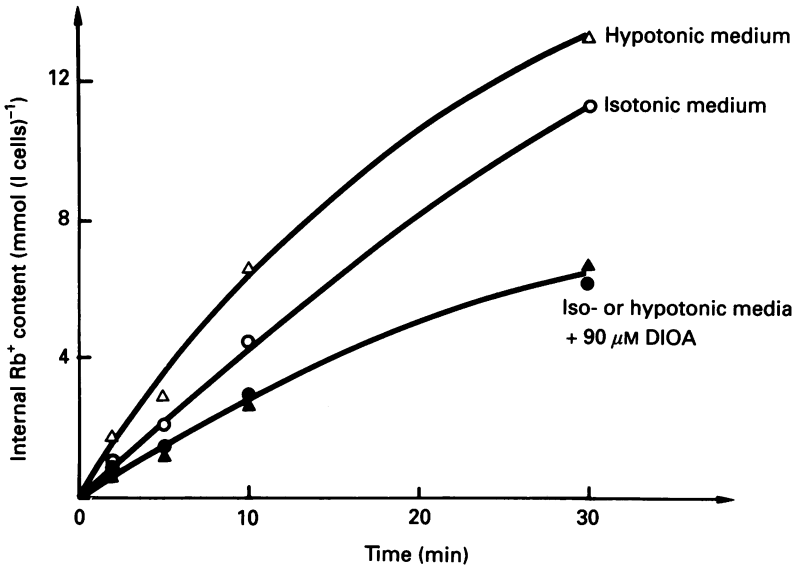

Fig. 4. Stimulation of DIOA-sensitive Rb^+ uptake by hypotonic shock (medium at osmolality of 183 mosmol kg^{-1} and containing 1 mmol l^{-1} ouabain and 50 μmol l^{-1} bumetanide). The hypotonic shock was unable to modify DIOA-resistant Rb^+ uptake.

TABLE 1. DIOA-sensitive Rb^+ and Cl^- influx in rat thymocytes

Osmolality	Rb^+ influx*	Cl^- influx*	Rb^+/Cl^- ratio
Isotonic	9·7±3·9	9·4±4·1	1·10±0·32
Hypotonic**	17·0±6·6	16·3±4·2	1·04±0·31

Values are given as means±S.D. of 6 experiments. Fluxes were measured in Na^+–Rb^+ medium containing 50 μmol l^{-1} DIDS. DIOA was used at a concentration of 90 μmol l^{-1}.
* In mmol (l cells.h)$^{-1}$, ** 183±5 mosmol kg^{-1}.

Regarding DIOA-resistant K^+ efflux, Fig. 2 shows that it was almost unchanged by the hypotonic shock (a modest increase in K^+ efflux was observed after 2 min, a phenomenon certainly due to an increase in K^+ leak; see Garay *et al.* 1988).

Figure 3 shows in a concentration–response curve the inhibitory action of DIOA on the hypotonically stimulated K^+ efflux. It can be seen that DIOA inhibited the K^+ release with an IC_{50} of about 8×10^{-6} mol l^{-1}.

DIOA-sensitive Rb^+ influx

Figure 4 shows that a hypotonic shock stimulated Rb^+ uptake in rat thymocytes. It can be seen that: (i) internal Rb^+ content increased with constant slope (initial rate) for 5–10 min, (ii) the stimulatory action of the hypotonic media was fully blocked by 90 μmol l^{-1} DIOA and (iii) DIOA blocked a relatively significant Rb^+ influx in isotonic media.

Table 1 summarizes the results of six experiments showing that the initial rate of DIOA-sensitive Rb^+ influx was twofold stimulated by hypotonic medium (183±5 mosmol kg^{-1}).

Table 2 shows that, in contrast with DIOA, quinidine was unable to inhibit the

TABLE 2. Lack of inhibition of Rb^+ uptake by quinidine

Condition	Rb^+ uptake (mmol (l cells.h)$^{-1}$) Isotonic medium	Hypotonic medium*
Control	19·1 ± 3·7	30·4 ± 5·3
DIOA, 90 μmol l^{-1}	9·7 ± 2·0	9·6 ± 2·7
Quinidine, 100 μmol l^{-1}	19·3 ± 3·5	37·6 ± 7·1
Quinidine, 200 μmol l^{-1}	20·3 ± 4·5	38·4 ± 9·3
Quinidine, 500 μmol l^{-1}	20·6 ± 5·1	34·3 ± 5·3

Values are given as means ± S.D. of 4 experiments. * 183 ± 5 mosmol kg^{-1}.

TABLE 3. Distinction between DIOA- and DIDS-sensitive $^{36}Cl^-$ efflux in rat thymocytes

Condition	Cl^- efflux (mmol (l cells.h)$^{-1}$) Isotonic medium	Hypotonic medium*
Control	267 ± 32	814 ± 154
DIDS, 47 μmol l^{-1}	175 ± 14	737 ± 157
ΔDIDS	92 ± 27	76 ± 25
DIDS + DIOA, 93 μmol l^{-1}	106 ± 36	267 ± 74
ΔDIOA	68 ± 21	471 ± 28

Values are given as means ± S.D. of 3 experiments. Initial flux rates were measured in Na^+–Rb^+ medium. * 183 ± 5 mosmol kg^{-1}.

hypo-osmotically simulated Rb^+ influx (see also that DIOA-resistant Rb^+ influx was not significantly stimulated by hypotonic media).

DIOA-sensitive Cl^- efflux

Fresh thymocytes were loaded with $^{36}Cl^-$ and the unidirectional $^{36}Cl^-$ efflux was measured at initial rate conditions (30–40 s) in the presence of bumetanide.

Table 3 shows that a hypotonic shock (183 ± 5 mosmol kg^{-1}) induced a threefold stimulation of Cl^- efflux, which mostly resulted from a sevenfold stimulation of DIOA-sensitive Cl^- efflux. DIDS-sensitive Cl^- efflux was unaffected by the hypotonic shock (Table 3).

A careful inspection of Table 3 shows that DIOA-resistant $^{36}Cl^-$ efflux was slightly but significantly higher in hypotonic than in isotonic media (267 ± 74 *vs.* 106 ± 36 mmol (l cells.h)$^{-1}$, mean ± S.D., $n = 6$, $P < 0{\cdot}05$, Student's t test). This small $^{36}Cl^-$ efflux component was lower than the DIOA-sensitive $^{36}Cl^-$ efflux (Table 3).

Finally, it is important to mention that: (i) in isotonic media, DIDS and DIOA were equally potent inhibitors of $^{36}Cl^-$ efflux (Table 3) and (ii) DIOA-sensitive Cl^- efflux was comparable in magnitude to DIOA-sensitive K^+ efflux (note that the incubation media for K^+ efflux in Fig. 2 was slightly less hypotonic than the Cl^- efflux media in Table 3).

DIOA-sensitive Cl^- influx

Unidirectional $^{36}Cl^-$ uptake and Rb^+ influx were measured at initial rate conditions (5–10 min). Table 1 shows that DIOA-sensitive Cl^- influx was: (i) twofold stimulated by the hypotonic media (183 ± 5 mosmol kg^{-1}) and (ii) very similar in magnitude

TABLE 4. DIOA-sensitive Cl^- efflux as a function of internal K^+ content

Internal K^+ content (mmol (l cells)$^{-1}$)	DIOA-sensitive Cl^- efflux (mmol (l cells.h)$^{-1}$)
117 ± 5 (5)†	518 ± 126 (5)
68·6 ± 7·8 (3)	227 ± 45 (3)*
39·7 ± 8·5 (3)	52 ± 45 (3)**

Values are given as means ± S.D. The number of experiments is indicated in parentheses. Internal K^+ was replaced by equivalent amounts of Na^+ by using the nystatin technique (Senn & Garay, 1989). Differences in mode of cell volume distribution between nystatin-treated and untreated cells never exceeded ±5%. $^{36}Cl^-$ efflux was measured in hypotonic Na^+–K^+ Ringer media (183 ± 5 mosmol kg^{-1}) containing (mmol l^{-1}): 0·05 DIDS, 0·05 bumetanide and 1 ouabain. For all cell samples, exchangeable Cl^- content was between 22 and 28 mmol (l cells)$^{-1}$.

* $P < 0·02$, ** $P < 0·01$ (Student's t test).

† Fresh cells.

TABLE 5. Effect of ion substitutions on inward fluxes

Chloride-free medium Condition	DIOA-sensitive Rb^+ uptake (mmol (l cells.h)$^{-1}$)
NO_3^- medium	18·0 ± 4·2 (3)
Cl^- medium	16·0 ± 2·1 (3)
Potassium-free medium	
$[K_o^+]$ (mmol (l cells)$^{-1}$)	DIOA-sensitive Cl^- influx (mmol (l cells.h)$^{-1}$)
0	20·4 ± 2·8 (3)
5	15·4 ± 3·7 (3)

Values are given as means ± S.D. The number of experiments is indicated in parentheses. Fluxes were measured in hypotonic media (183 ± 5 mosmol kg^{-1}; see Methods for further details).

to the DIOA-sensitive Rb^+ influx. In addition, hypotonically stimulated $^{36}Cl^-$ influx was resistant to DPAC 144 (5-nitro-2-(2-phenylethyl-amino)-benzoic acid; 10^{-6} mol l^{-1}) and DIDS (data not shown). DIOA-resistant $^{36}Cl^-$ influx was significantly lower in hypotonic media than in isotonic media (18·3 ± 3·6 *vs.* 38·8 ± 2·7 mmol (l cells.h)$^{-1}$, mean ± S.D., $n = 6$, $P < 0·001$, Student's t test).

Ion substitutions

Cis-interactions between volume-dependent K^+ and Cl^- fluxes were explored by means of ion substitutions. Interactions between outward K^+ and Cl^- movements were investigated by using K^+-depleted cells (prepared with the nystatin technique, see Methods section). Table 4 shows DIOA-sensitive Cl^- efflux as a function of internal K^+ content. It can be seen that cell K^+ depletion induced a marked reduction in Cl^- efflux.

Interactions between inward K^+ and Cl^- fluxes were investigated by measuring K^+ or Cl^- influx in the absence of the respective counter-ion. Table 5 shows that: (i) DIOA-sensitive Rb^+ influx was almost unchanged by replacing external Cl^- for NO_3^- and (ii) DIOA-sensitive Cl^- influx was slightly increased in the absence of external K^+. Lack of effect of chloride removal on DIOA-sensitive Rb^+ uptake was also

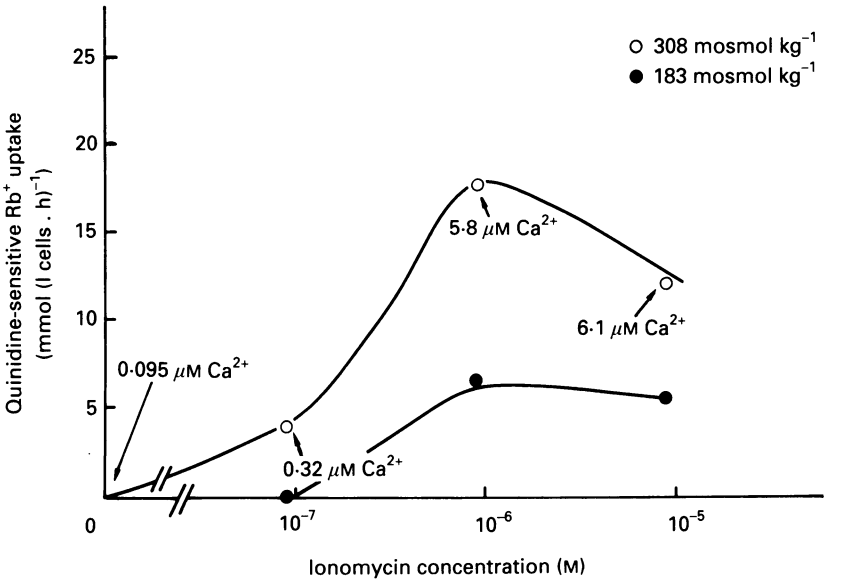

Fig. 5. Quinidine-sensitive Rb^+ influx as a function of ionomycin and cytosolic free calcium concentrations. Cytosolic free calcium contents were measured by using fura-2 AM. Quinidine was used at concentration of 2×10^{-4} mol l^{-1}. Medium contained 1 mmol l^{-1} ouabain and 50 μmol l^{-1} bumetanide. It can be seen that quinidine-sensitive Rb^+ influx was stimulated in the range of 0·3–6 μmol l^{-1} of cytosolic free calcium concentration. Moreover, quinidine-sensitive Rb^+ influx was less sensitive to free cytosolic calcium in hypotonic (183 mosmol kg^{-1}) than in isotonic media. Similar results were obtained in two other experiments.

TABLE 6. Stimulation of Rb^+ uptake by the calcium ionophore ionomycin

	Rb^+ uptake (mmol (l cells.h)$^{-1}$)	
Condition	Total	Quinidine, 200 μmol l^{-1}
Control	18·9±3·2	19·0±3·5
Ionomycin, 1 μmol l^{-1}	44·9±6·0	34·5±4·1*

Values are given as means±S.D. of 4 experiments. Fluxes were measured in isotonic Na^+–Rb^+ medium in the presence of 1 mmol l^{-1} ouabain and 50 μmol l^{-1} bumetanide.

* $P < 0.05$ (Student's t test), *vs.* ionomycin alone.

observed: (i) after 1 h preincubation of cells with nitrate media and (ii) in isotonic media (data not shown).

Ca^{2+}-sensitive K^+ channels

Table 6 shows that the calcium ionophore ionomycin strongly stimulated Rb^+ influx in rat thymocytes (similar results were obtained with A-23187). Moreover, it can be seen that quinidine inhibited ionomycin-stimulated Rb^+ influx by 40–50% (similar results were obtained with quinine).

Figure 5 shows quinidine-sensitive Rb^+ influx as a function of ionomycin and cytosolic free calcium concentrations. It can be seen that quinidine-sensitive Rb^+ influx was stimulated in the range of 0·3 to 6 μmol l^{-1} of cytosolic free calcium

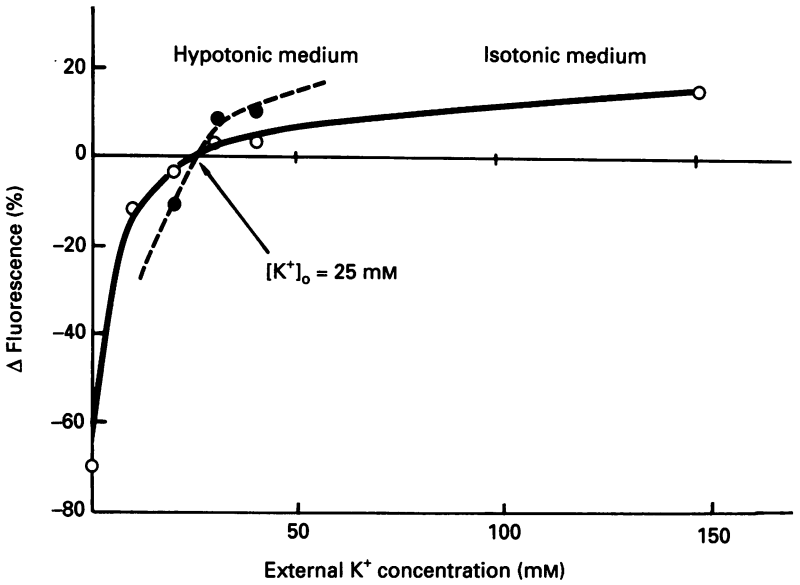

Fig. 6. Lack of change in membrane potential in hypotonically stimulated thymocytes. The change in fluorescent signal of DiS-C_3-(5) was studied as a function of external K^+. It can be seen that: (i) in isotonic media, the null-point corresponds to an external K^+ of about 25 mmol l^{-1}, i.e. to a membrane potential of about −45 mV and (ii) the hypotonic media (183 mosmol kg^{-1}) was unable to change this null-point. Similar results were obtained in two other experiments.

TABLE 7. Lack of action of hypotonic media on cytosolic free Ca^{2+}, membrane potential and cytosolic pH

Media	Cytosolic free Ca^{2+} (nM)	Membrane potential (mV)	Cytosolic pH
Isotonic	102±29 (3)	−45·4±3·8 (3)	7·06±0·07 (2)
Hypotonic	107±25 (3)	−45·6±1·7 (3)	7·00±0·12 (2)

Values are given as means±S.D. or range of variability. The number of experiments is indicated in parentheses. Hypotonic media at 183±5 mosmol kg^{-1}.

concentration. Moreover, quinidine-sensitive Rb^+ influx was less sensitive to cytosolic free calcium in hypotonic (183 mosmol kg^{-1}) than in isotonic media.

Lack of action of hypotonic media on cytosolic calcium, membrane potential and cell pH

Table 7 shows the effect of hypotonic media (183±5 mosmol kg^{-1}) on cytosolic free calcium content. It can be seen that cytosolic calcium in hypotonic media was not significantly different from that in isotonic media. A similar lack of effect was found for cytosolic pH (Table 7).

Membrane potential was studied by measuring the fluorescent signal of DiS-C_3-(5) as a function of external K^+ (see Methods). Figure 6 shows that in isotonic media, the null-point corresponds to an external K^+ of about 25 mmol l^{-1}, i.e. to a membrane potential of about −45 mV. It can be seen that the hypotonic media was unable to change this null-point (see also Table 7).

Inhibition of DIOA-sensitive Rb^+ influx by hypertonic media

DIOA-sensitive Rb^+ influx was inhibited by 23 ± 5 and 42 ± 8% in media of 403 and 509 mosmol kg^{-1} respectively (mean ± range of two experiments).

DISCUSSION

Our results clearly show that rat thymocytes possess a K^+–Cl^- co-transport system similar to that previously described in red blood cells. First, DIOA fully blocked all K^+ movements and most of the Cl^- fluxes activated by hypotonic shock. Such fluxes were resistant to bumetanide, DIDS, quinidine, DPAC 144 and ouabain. Second, the K^+ to Cl^- stoichiometry of DIOA-sensitive fluxes was close to unity. Third, the system catalysed net outward KCl extrusion (the outward/inward flux ratio was 5–7 in isotonic media and 20 in hypotonic media at 189 mosmol kg^{-1}), as expected from the *chemical gradient* ratio, favourable for potassium extrusion relative to chloride entry. Finally, the inhibition of DIOA-sensitive Cl^- efflux by cell K^+ depletion suggested coupling of outward K^+ and Cl^- fluxes.

The physiological role of the K^+–Cl^- co-transport system is to extrude KCl, and this mode of operation seems to couple outward K^+ and Cl^- fluxes. Conversely, *inward* K^+–Cl^- co-transport fluxes were found to be uncoupled, both in NO_3 media and in K^+-free media. This suggested other modes of operation for the K^+–Cl^- co-transport system, i.e. uncoupled K^+ or Cl^- influx, K_o^+–K_i^+ or Cl_o^-–Cl_i^- exchange, or co-transport with other ionic species. Such types of non-physiological modes of operation were previously described for other co-transport systems such as the Na^+–K^+–Cl^- co-transport system (see for instance Canessa *et al.* 1986).

In rat thymocytes, DIOA-sensitive KCl fluxes were two orders of magnitude higher than in human erythrocytes, suggesting that the K^+–Cl^- co-transport system could play a role in the RVD mechanism of these cells. This and the lack of action of quinidine on hypotonically stimulated Rb^+ influx contrasted with the results of Grinstein and co-workers (1984, 1989, 1990). Therefore, we investigated quinidine (or quinine)-sensitive K^+ channels in rat thymocytes.

Rat thymocytes have quinine (or quinidine)-sensitive K^+ channels. These channels were quiescent at basal conditions, and were stimulated by modest increases in cytosolic free calcium contents, *but not by hypotonic media*. Indeed, hypotonic media reduced quinidine-sensitive Rb^+ influx. However, this was not decisive evidence against a role for K^+ channels in RVD because the well known long-pore effect predicts that Rb^+ entry could be inhibited by the increased exit of K^+ ions through the channels under hypotonic conditions.

Hypotonic shock was unable to modify cytosolic free calcium content, further suggesting that quinidine-sensitive K^+ channels were not involved in RVD in rat thymocytes. We also excluded the participation of potential-operated or H^+-dependent K^+ channels, since the hypotonic media was unable to modify membrane potential and cytosolic pH. However, it is important to state that our membrane potential measurements were performed at equilibrium conditions, i.e. after 5 min of equilibration (the response time was less than 1 min).

Contrasting with this lack of evidence for volume-dependent K^+ channels, we

found some results compatible with volume activated Cl^- channels. Thus, the hypotonic shock stimulated DIOA-resistant Cl^- efflux and inhibited DIOA-resistant Cl^- influx. This was in agreement with an increased exit of Cl^- ions through the channels under hypotonic conditions and an inhibition of Cl^- entry by the long-pore effect. However, it is important to stress that the chloride fluxes catalysed by this DIOA-resistant mechanism were smaller than those catalysed by the K^+–Cl^- co-transport system.

Most of the studies of Grinstein and co-workers (1984, 1989, 1990) were performed in mature lymphocytes. Therefore, one explanation for the discrepant results can be that cell maturation is associated with the replacement of a K^+–Cl^- co-transport system by independent K^+ and Cl^- channels.

Some minor aspects of our experiments deserve a comment. First, DIOA-sensitive Cl^- efflux was a non-saturable function of internal K^+ content (Table 4). This can be explained by the fact that K^+-depleted cells had high internal Na^+ contents, and non-translocating Na^+ ions can competitively inhibit the co-transport system at the inner K^+ sites. Second, a fraction of the calcium-stimulated Rb^+ influx was resistant to quinidine (or quinine). Whether this represents another Ca^{2+}-sensitive K^+ channel or some K^+ transport system remains to be elucidated.

A new aspect of the K^+–Cl^- co-transport system revealed by DIOA in thymocytes, is that it catalyses significant outward fluxes in isotonic media, i.e. (i) K^+ efflux by the K^+–Cl^- co-transport system was similar in magnitude to K^+ influx by the Na^+–K^+ pump (for references see Senn & Garay, 1989), (ii) Cl^- efflux by the K^+–Cl^- co-transport system was similar in magnitude to Cl^- efflux by the DIDS-sensitive anion carrier (Table 3). Moreover, in isotonic media outward K^+–Cl^- co-transport was 5–7 times higher than inward K^+–Cl^- co-transport. This results in a non-negligible net KCl extrusion.

Interestingly, a hypertonic stress inhibited DIOA-sensitive Rb^+ influx. This suggested a decreased KCl extrusion that should be translated into a small RVI (regulatory volume increase, see Arrazola *et al.* 1993).

In conclusion, rat thymocyte membranes possess a 1:1 K^+–Cl^- co-transport system which is strongly activated by hypotonic shock and catalyses net KCl extrusion. The role of this transport system in RVD deserves further investigation.

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