Biochimica et Biophysica Acta, 773 (1984) 207–218
Elsevier

BBA 72102

KCl LOSS AND CELL SHRINKAGE IN THE EHRLICH ASCITES TUMOR CELL INDUCED BY HYPOTONIC MEDIA, 2-DEOXYGLUCOSE AND PROPRANOLOL

WILLIAM B. THORNHILL and PHILIP C. LARIS *

Department of Biological Sciences, University of California, Santa Barbara, CA 93106 (U.S.A.)

(Received June 14th, 1983)
(Revised manuscript received February 13th, 1984)

Key words: Cell shrinkage; Hypotonic medium; Cl^--Cl^- exchange; Deoxyglucose; Propranolol; (Ehrlich ascites cell)

Ehrlich ascites tumor cells lose KCl and shrink after swelling in hypotonic media and in response to the addition of 2-deoxyglucose, propranolol, or the Ca^{2+} ionophore, A23187, plus Ca^{2+} in isotonic media. All of these treatments activate cell shrinkage via a pathway with the following characteristics: (1) the KCl loss responsible for cell shrinkage does not alter the membrane potential; (2) NO_3^- does not substitute for Cl^-; (3) the net KCl movements are not inhibited by quinine or DIDS; and (4) early in this study furosemide was effective in inhibiting cell shrinkage but this sensitivity was subsequently lost. This evidence suggests that the KCl loss in these cells occurs via a cotransport mechanism. In addition, hypotonic media and the other agents used here stimulate a Cl^--Cl^- exchange, a net loss of K^+ and a net gain of Na^+ which are not responsible for cell shrinkage. The Ehrlich cell also appears to have a Ca^{2+}-activated, quinine-sensitive K^+ conductive pathway but this pathway is not part of the mechanism by which these cells regulate their volume following swelling or shrink in isotonic media in response to 2-deoxyglucose or propranolol. Shrinkage by the loss of K^+ through the Ca^{2+} stimulated pathway appears to be limited by Cl^- conductive movements; for when NO_3^-, an anion demonstrated here to have a higher conductive movement than Cl^-, is substituted for Cl^-, the cells will shrink when the Ca^{2+}-stimulated K^+ pathway is activated.

Introduction

Many animal cells, including the Ehrlich ascites tumor cell [1], are capable of regulating their volumes when exposed to hypotonic media (for reviews, see Refs. 2–4). Hypotonic shock initiates a rapid osmotic swelling of the cells and activates a K^+ and Cl^- loss which tends to restore the resting volume of the cells. Three mechanisms have been proposed to explain the ionic fluxes observed during the volume regulatory decrease: (1) an electrically neutral K^+ and Cl^- cotransport system [5,6], (2) a K^+/H^+ antiporter balanced by a HCO_3^- or OH^-/Cl^- antiporter [7], and (3) separate K^+ and Cl^- conductive pathways [8].

The trigger responsible for the activation of this K^+ and Cl^- loss in response to hypotonic media is poorly understood. Grinstein et al. [9] have proposed that a rise in intracellular free Ca^{2+} may be involved in initiating the regulatory decrease in lymphocytes in response to hypotonic media since these cells shrink in isotonic media with the addition of the calcium ionophore A23187 via a K^+ and Cl^- loss.

In this report and a previous one [10] evidence is presented demonstrating that in the Ehrlich cell a K^+ and Cl^- loss similar to that initiated by hypotonic media can also be activated in isotonic

* To whom correspondence should be addressed.
Abbreviation: DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid.

media by the addition of glucose, 2-deoxyglucose, propranolol or A23187 plus Ca^{2+}. These ion movements have been characterized so as to distinguish among the three possible modes of KCl loss proposed previously.

Methods

Ehrlich cells were prepared as described previously [11]. Packed cells were first diluted 1 : 300 with NaCl-Ringer for 60 min at 37°C to deplete endogenous amino acids [12]. NaCl-Ringer contained (in mM): NaCl 147, KCl 5.9, $MgSO_4$ 1.5 and sodium phosphate buffer 10 at pH 7.4. To deplete cells of chloride, cells were equilibrated in a $NaNO_3$-Ringer in which NaCl and KCl were replaced by 147 mM $NaNO_3$ and 5.9 mM KNO_3. They were then resuspended in the appropriate medium at a 1 : 20 dilution and exposed to different agents at room temperature for up to 60–90 min. Unless otherwise stated the following concentrations of the various agents used were: 10 mM D-glucose or 2-deoxy-D-glucose, 0.5 mM DL-propranolol, 1 μM A23187 in the presence of 0.5 mM Ca^{2+}, 0.35 mM quinine, 0.1 mM 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and 1 mM furosemide (pH was adjusted with NaOH). Hypotonic shock was initiated by centrifuging cells and resuspending them at 1 : 20 in a NaCl or $NaNO_3$ medium containing (in mM) NaCl or $NaNO_3$ 70.5, KCl or KNO_3 2.9, $MgSO_4$ 1.5 and sodium phosphate buffer 10 at pH 7.4 (final osmolarity 180 mosM).

Cellular volume was determined by calculating the cell water per milligram cell dry weight from measurements of the wet and dry weights and the [^{14}C]dextran content of centrifuged cell pellets as described previously [12].

The experimental procedures for Na^+ and K^+ determinations have been described previously [13]. Chloride was measured with an Aminco-Cotlove chloridimeter in cell pellets washed in ice-cold choline dihydrogen citrate [14].

The fluorescent dye, 3,3′-dipropylthiodicarbocyanine iodide (referred to as dye) used in these studies was obtained from either Dr. A. Waggoner of Amherst College or Dr. S. Parson of the University of California, Santa Barbara. The fluorescent intensity of this dye was measured in 1 : 300 cell suspensions at 37°C as described previously [12]. Although glucose was omitted from the cuvette under all conditions in the fluorescent studies, the results were the same in the presence or absence of glucose.

The steady-state distribution ratio of [^{3}H]tetraphenylphosphonium ion was used to estimate changes in the membrane potential as outlined by Cheng et al. [15]. In summary, [^{3}H]tetraphenylphosphonium (0.12 μCi/ml) was added along with 1 μM unlabeled tetraphenylphosphonium to a 1 : 20 cell suspension at 37°C together with the test compound. Samples were then taken at various times. No significant difference in the distribution ratio of [^{3}H]tetraphenylphosphonium was found if a permeant anion, tetraphenylboron (1 μM) was added or if unlabeled tetraphenylphosphonium (1 μM) was omitted.

The procedure for the uptake of [^{14}C]glycine has been described before [13]. In summary, [^{14}C]glycine (0.25 μCi/ml), 1 mM unlabeled glycine and the test compounds were added to a 1 : 20 cell suspension at 37°C and samples were taken with time.

^{86}Rb efflux. Cells were diluted with an appropriate Ringer containing ^{86}Rb (2 μCi/ml) and incubated for 60 min at 22°C. The cells were then washed twice in unlabeled Ringer and resuspended at 1 : 300 dilutions in flux media with the appropriate test conditions at 37°C. Efflux was followed for a 10-min period by measurements of the ^{86}Rb in the supernatant fluid and whole cell suspension as described previously [16]. Rate constants were determined directly from the slope of the plots $-\ln(Rb_\infty - Rb_t)/(Rb_\infty - Rb_0)$ versus time [17] where Rb_∞ is the counts per minute (cpm) ^{86}Rb in whole cell suspensions, Rb_0 is the supernatant cpm at time zero and Rb_t is the supernatant cpm at time t.

^{36}Cl efflux. Cells were diluted to 1 : 20 in NaCl-Ringer containing ^{36}Cl (0.5 μCi/ml) and equilibrated 60 min at 22°C. The cells were then centrifuged, washed twice in unlabeled NaCl-Ringer and resuspended at 1 : 300 dilution into NaCl or $NaNO_3$ flux media at 37°C. Periodically, over a 6 min time-course, 15 ml samples of flux suspension were centrifuged. Pellets were isolated and prepared along with supernatant samples for counting as described previously [11]. Rate con-

stants were determined from the slopes of $-\ln(Cl_t/Cl_0)$ versus time, where Cl_t is the cpm ^{36}Cl/mg cell dry weight at time t and Cl_0 is the cpm at time zero.

Liquid scintillation techniques described previously [16] were used to count [^{14}C]dextran, [^{3}H]TPP, [^{14}C]glycine, ^{36}Cl and ^{86}Rb. All values are the average of duplicate or triplicate determinations.

Furosemide was a gift from Hoechst-Roussel Pharmaceutical, Sommerville, N.J. D-Glucose, 2-deoxy-D-glucose, DL-propranolol, valinomycin and quinine were purchased from Sigma. The Ca^{2+} ionophore A23187 was purchased from Boehringer Mannheim, DIDS from The Biochemical Corporation and isotopes from New England Nuclear.

Results

When Ehrlich ascites tumor cells are exposed to hypotonic media, they swell rapidly and then decrease in size so that in 30 min the cells have lost approx. 50% of the water gained initially (Fig. 1). Glucose addition in isotonic media also results in cell shrinkage (Fig. 1) (see also Ref. 10). Since glucose appeared to initiate a response similar to that triggered by hypotonic shock, the relationship of glucose metabolism to cell shrinkage was examined. The addition of 10 mM 2-deoxyglucose, which is phosphorylated but not metabolized further, resulted in cell shrinkage similar in extent and time course to that reported earlier for 10 mM glucose (Fig. 1). On the other hand 10 mM 3-*O*-methylglucose, which is transported but not phosphorylated, had no effect on cell volume (data not shown). These studies indicate that some change in the Ehrlich cell resulting from the phosphorylation of glucose (or 2-deoxyglucose) can initiate cell shrinkage in an isotonic medium.

In lymphocytes, cell shrinkage is initiated by the addition of the Ca^{2+} ionophore A23187 plus Ca^{2+} in isotonic media [9]. The response to the ionophore in lymphocytes mimics the response of these cells to hypotonic shock in a number of ways including: (1) a net loss of K^+ and Cl^- from the cells, and (2) an inhibition by quinine. The possibility that the cell shrinkage in the Ehrlich cell might have a similar basis was examined. Two agents, A23187 and propranolol, which have been

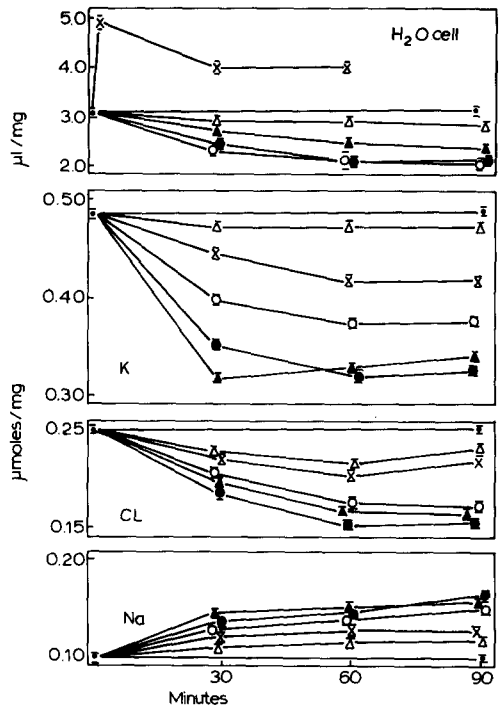

Fig. 1. Loss of cell water and ions under various conditions. Cells were incubated at 22°C in isotonic NaCl-Ringer with various agents and in hypotonic NaCl-Ringer and assayed for μl cell water per mg dry weight and μmol K^+, Cl^- and Na^+ per mg dry weight as a function of time. Control (•), 1 μM A23187 plus 0.5 mM Ca^{2+} (□), 0.5 mM propranolol (▲), 10 mM 2-deoxyglucose (●), 10 mM glucose (○) and hypotonic media (×). Each point represents the average of duplicate determinations. The bars represent the ranges of the samples.

employed to raise cell calcium levels in other preparations [18] were tested for their effect on cell volume. The data in Fig. 1 indicate that both 0.5 mM propranolol and 1 μM A23187 plus 0.5 mM Ca^{2+} could also initiate cell shrinkage in isotonic media in the Ehrlich cell. While the volume changes with propranolol were very reproducible, the changes with the ionophore were observed only 25% of the time. Hence most of the studies reported here employ propranolol as an agent to raise intracellular Ca^{2+}. In summary, 60 min following the addition of various agents, the percentage decrease in μl cell water/mg dry weight from the control is: 2-deoxyglucose or glucose 25%, propranolol 18% and A23187 plus Ca^{2+} 8%. Similar changes were observed when relative cell volume was measured using a Coulter counter (data not shown).

The pattern of changes in cellular K^+, Na^+ and Cl^- content with hypotonic shock, glucose 2-deoxyglucose, propranolol and A23187 plus Ca^{2+} was similar. With each agent there was a net loss of K^+ and Cl^- and net gain of Na^+ in μmol/mg dry weight (Fig. 1). Since the loss of K^+ and Cl^- exceeds the gain of Na^+, the net loss of osmotic material in the form of KCl appears to be responsible for the volume change in each case. This conclusion is the same as that stated previously for hypotonic shock [1] and glucose addition [10]. In addition to the net KCl loss, all the agents employed activate a further loss of K^+ together with a gain of Na^+. If the volume change is accomplished through a 1 : 1 K^+ to Cl^- loss, then the ratio of the additional K^+ loss/Na^+ gain for 2-deoxyglucose and hypotonic shock calculated from the loss at 1 h is 1.35 K^+ : 1 Na^+ and 2.3 K^+ : 1 Na^+ for propranolol.

The fluxes leading to net KCl loss with hypotonic shock, 2-deoxyglucose, and propranolol were analyzed further by measuring the unidirectional effluxes of ^{86}Rb and ^{36}Cl. As shown in Table IA all of these agents increased both K^+ and Cl^- efflux. The additional Cl^- efflux with these treatments exceeded the additional K^+ efflux 2–3-fold. Since at 30 and 60 min the net K^+ loss was greater than the net Cl^- loss, it was suspected that a portion of the additional Cl^- efflux represented an exchange component. To test this possibility and also to determine whether the anion exchange pathway is required for volume regulation in these cells, the experiments were repeated in 0.1 mM DIDS, an inhibitor of the anion exchanger. With hypotonic shock, 2-deoxyglucose and propranolol the effluxes of both K^+ and Cl^- in the presence of DIDS were greater than the effluxes seen in control cells with DIDS, but now the additional Cl^- efflux exceeded the additional K^+ efflux by only 19 to 38% (Table IB). With hypotonic shock and 2-deoxyglucose the increase in K^+ efflux (above that of the control) was similar in the presence and absence of DIDS; with propranolol the additional K^+ efflux was larger with DIDS. The volume of control cells was not altered by 0.1 mM DIDS nor did this concentration of DIDS alter the net changes in ion content of the cells resulting from treatment with hypotonic shock, 2-deoxyglucose or propranolol for 60 min (Table II). These results indicate that the KCl loss leading to shrinkage in

TABLE I

THE INITIAL UNIDIRECTIONAL K^+ AND Cl^- EFFLUXES UNDER VARIOUS CONDITIONS IN THE ABSENCE AND PRESENCE OF DIDS

(A) and (B) are data from different experiments. Values are expressed in (A) as means ± S.D. ($n = 3$) and in (B) as the average of duplicate determinations. The numbers in the columns labelled Increase in (A) are the differences between each condition and the control. The value in the columns labelled DIDS-insensitive Increase in (B) are the differences between each condition and the control + DIDS.

Description	K^+ efflux (mmol/l cell water per h)		Cl^- efflux (mmol/l cell water per h)	
	Measured	Increase	Measured	Increase
(A) Control	156 ± 18	–	594 ± 56	–
Hypotonic	229 ± 25	73	821 ± 85	227
Deoxyglucose	325 ± 34	169	984 ± 95	390
Propranolol	291 ± 22	135	915 ± 89	321
	Measured	DIDS-insensitive Increase	Measured	DIDS-insensitive Increase
(B) Control	189	–	601	–
Control + DIDS	198	–	126	–
Hypotonic + DIDS	270	72	225	99
Deoxyglucose + DIDS	395	197	360	234
Propranolol + DIDS	442	244	440	314

TABLE II

μmol K^+, Cl^- AND Na^+ PER mg DRY WEIGHT OF CELLS INCUBATED FOR 60 MINUTES IN NaCl-RINGER WITH VARIOUS TREATMENTS

Values are expressed as means ± S.D. ($n = 3$) μmol/mg.

Description	μmol/mg dry weight		
	K^+	Cl^-	Na^+
Control	0.387 ± 0.019	0.270 ± 0.019	0.072 ± 0.011
Control + DIDS	0.363 ± 0.015	0.259 ± 0.011	0.091 ± 0.011
Hypotonic	0.323 ± 0.008	0.223 ± 0.019	0.118 ± 0.011
Hypotonic + DIDS	0.323 ± 0.009	0.201 ± 0.007	0.115 ± 0.027
Deoxyglucose	0.286 ± 0.011	0.176 ± 0.015	0.118 ± 0.019
Deoxyglucose + DIDS	0.280 ± 0.010	0.201 ± 0.027	0.134 ± 0.010
Propranolol	0.213 ± 0.011	0.161 ± 0.030	0.138 ± 0.019
Propranolol + DIDS	0.209 ± 0.004	0.179 ± 0.038	0.149 ± 0.027

these cells did not require the activity of the anion exchanger but that Cl^- efflux through the anion exchanger was increased with the various treatments.

We have previously reported that 1 mM furosemide inhibited the volume change initiated by glucose [10] or by either hypotonic shock, 2-deoxyglucose, propranolol or A23187 plus Ca^{2+} [19]. The inhibitory influence of furosemide on the initial increase in $^{86}Rb^+$ loss from the cells with these agents is seen in Table IIIA where the initial rate constants for ^{86}Rb efflux are listed for these agents in the presence and absence of furosemide. While furosemide inhibited the volume change and KCl loss induced by the agents, it did not inhibit the additional K^+ loss and Na^+ gain. Over a period of 1–2 years furosemide routinely blocked the increase in ^{86}Rb efflux induced by these agents 60–90%. At present, however, furosemide no longer has any significant inhibitory effect on the loss of ^{86}Rb from these cells (Table IIIB) induced by these agents nor on the changes in cell volume as measured by either weight or Coulter counter techniques. The loss of furosemide sensitivity was gradual; the inhibition decreased over a 4–6 month period until finally none was detectable. The changes in ionic composition of the cells induced by these agents remained the same as those seen earlier; only the sensitivity to furosemide changed. Different lot numbers of furosemide, and another loops diuretic, bumetanide, have been tested and these drugs are presently without significant effect on the shrinkage induced by any of the conditions reported here. Furosemide or bumetanide, however, continue to inhibit the (1 Na^+ : 1 K^+ : 2 Cl^-) cotransport system as first reported by Geck et al. [20]. All the data that follow in this report have been obtained with cells that have lost this furosemide sensitivity.

TABLE III

EFFECT OF FUROSEMIDE ON THE RATE CONSTANTS FOR ^{86}Rb EFFLUX UNDER VARIOUS CONDITIONS IN NaCl-RINGER

(A) Furosemide inhibits stimulated ^{86}Rb efflux. (B) Furosemide inhibitory effect is not detectable. Values are expressed as means ± S.D.

Description	(A) k_e ($n = 4$) (h^{-1})	(B) k_e ($n = 3$) (h^{-1})
Control	1.25 ± 0.05	1.29 ± 0.09
Control + furosemide	1.06 ± 0.05	1.12 ± 0.05
Hypotonic	1.86 ± 0.09	1.82 ± 0.07
Hypotonic + furosemide	1.38 ± 0.04	1.74 ± 0.08
Deoxyglucose	2.60 ± 0.13	2.45 ± 0.11
Deoxyglucose + furosemide	1.33 ± 0.12	2.35 ± 0.06
Propranolol	2.23 ± 0.05	2.16 ± 0.05
Propranolol + furosemide	1.22 ± 0.10	2.10 ± 0.07

Since quinine (75 μM) inhibited the K^+ and Cl^- loss and cell shrinkage in lymphocytes in response to hypotonic shock or to A23187 plus Ca^{2+} in an isotonic medium [8], the effect of this drug on the agents which initiate changes in the ionic composition and volume of the Ehrlich ascites tumor cell was measured. Concentrations of quinine as high as 0.35 mM had little effect on the

TABLE IV

μmol K^+, Cl^- AND Na^+ PER mg DRY WEIGHT OF CELLS INCUBATED FOR 60 MINUTES IN NaCl-RINGER WITH VARIOUS TREATMENTS

Values are expressed as means ± S.D. ($n = 3$).

Description	μmol/mg dry weight		
	K^+	Cl^-	Na^+
Control	0.374 ± 0.011	0.282 ± 0.007	0.104 ± 0.011
Control + quinine	0.346 ± 0.022	0.281 ± 0.015	0.124 ± 0.011
Hypotonic	0.274 ± 0.015	0.207 ± 0.009	0.157 ± 0.007
Hypotonic + quinine	0.259 ± 0.011	0.229 ± 0.006	0.174 ± 0.015
Deoxyglucose	0.211 ± 0.022	0.207 ± 0.007	0.179 ± 0.007
Deoxyglucose + quinine	0.204 ± 0.019	0.200 ± 0.015	0.172 ± 0.007
Propranolol	0.193 ± 0.015	0.190 ± 0.019	0.168 ± 0.007
Propranolol + quinine	0.219 ± 0.011	0.198 ± 0.011	0.165 ± 0.011

levels of K^+, Na^+ and Cl^- per milligram dry weight in control cells after 60 min and did not inhibit the loss of K^+ and Cl^- or gain of Na^+ induced by hypotonic shock, deoxyglucose or propranolol (Table IV). Furthermore, the initial efflux of ^{86}Rb in response to hypotonic shock, deoxyglucose or propranolol was also not significantly inhibited by 0.35 mM quinine (data not shown). At concentrations of quinine higher than 0.35 mM the ionic composition of control cells was markedly influenced and hence studies with high concentrations of quinine were not pursued.

The specificity of the anion requirement for volume regulation in response to hypotonic shock varies in different cells. While a variety of anions including NO_3^- will substitute for Cl^- in the human lymphocyte [8] and the Amphiuma red cell [7], in other cells the anion required for net K^+ and water loss is rather specific and Br^- but not NO_3^-, for example, will substitute for Cl^- [6]. Since studies on anion specificity are important in distinguishing among possible mechanisms of shrinkage, we examined the influence of the substitution of NO_3^- for Cl^- on the volume changes and the alterations in K^+ and Na^+ content resulting from the treatments with the various agents described earlier. Cells (NO_3^- cells) that have been depleted of Cl^- during an equilibration in $NaNO_3$-Ringer have lost net K^+ without a concomitant gain of Na^+ and have shrunken 5–10% compared to cells maintained in NaCl-Ringer (Table V). The K^+ concentration in NO_3^- cells, 124 mM ± 12 K^+/l cell water, however, is approximately the same as that in Cl^- cells, 130 mM ± K^+/l cell water. The NO_3^- cells (unless stated otherwise, the behavior of these cells is tested in $NaNO_3$-Ringer) did not regulate their volume in response to hypotonic media or shrink upon the addition of 2-deoxyglucose in isotonic media (Table V). The same results were obtained when the $NaNO_3$-Ringer was made slightly hypotonic so as to prevent the cells from shrinking 5–10% initially. In contrast, the addition of propranolol to NO_3^- cells resulted in cell shrinkage (Table V) which followed a net loss of K^+ which was greater than the gain in cell Na^+ (Table VI).

The results of studies of the influence of hypotonic shock, 2-deoxyglucose, and propranolol on the initial ^{86}Rb efflux from NO_3^- cells were in

TABLE V

CELL WATER CONTENT OF CELLS EQUILIBRATED IN NaCl-RINGER (CL) OR $NaNO_3$-RINGER (NO_3) UNDER VARIOUS CONDITIONS FOR 60 MINUTES

Values are expressed as means ± S.D. ($n = 3$).

Description	μl cell H_2O per mg dry weight
CL, control	2.51 ± 0.15
NO_3, control	2.28 ± 0.08
NO_3, hypotonic, $t = 0$ min	3.63 ± 0.15
NO_3, hypotonic, $t = 60$ min	3.49 ± 0.14
NO_3, deoxyglucose	2.35 ± 0.09
NO_3, quinine	2.10 ± 0.07
NO_3, propranolol	1.91 ± 0.10
NO_3, propranolol + quinine	1.94 ± 0.13

TABLE VI

μmoles K^+, Cl^- AND Na^+ PER mg DRY WEIGHT IN CELLS INCUBATED IN NaCl-RINGER (CL) OR $NaNO_3$-RINGER (NO_3) FOR 60 MINUTES UNDER VARIOUS TREATMENTS IN THE ABSENCE AND PRESENCE OF 0.35 mM QUININE

Values are expressed as means ± S.D. ($n = 3$).

Description	μmol/mg dry weight			
	− quinine (a)		+ quinine (b)	
	K^+	Na^+	K^+	Na^+
CL, control	0.437 ± 0.020	0.085 ± 0.021	0.412 ± 0.021	0.105 ± 0.012
NO_3, control	0.355 ± 0.015	0.093 ± 0.006	0.220 ± 0.015	0.130 ± 0.017
NO_3, hypotonic, $t = 60$ min	0.315 ± 0.014	0.123 ± 0.005	0.198 ± 0.007	0.145 ± 0.015
NO_3, deoxyglucose	0.307 ± 0.012	0.131 ± 0.015	0.202 ± 0.012	0.137 ± 0.007
NO_3, propranolol	0.170 ± 0.005	0.125 ± 0.007	0.148 ± 0.007	0.122 ± 0.004

agreement with the observations on net volumes and ion content. As shown in Table VII, replacement of Cl^- by NO_3^- markedly reduced ^{86}Rb efflux as reported earlier [21]. With NO_3^- cells neither hypotonic shock nor 2-deoxyglucose influenced ^{86}Rb efflux (Table VII). In contrast, propranolol more than doubled the ^{86}Rb efflux.

An attempt was made to determine whether the K^+ loss and shrinkage of NO_3^- cells with propranolol was sensitive to quinine. These studies, however, were complicated by the observation that the addition of 0.35 mM quinine alone to NO_3^- cells resulted in shrinkage (Table V), the net loss of K^+ in excess of Na^+ gain (Table VI) and a doubling of the initial ^{86}Rb efflux (Table VIII). When both propranolol and quinine were added to NO_3^- cells simultaneously, net K^+ loss, water loss, and the ^{86}Rb efflux was only slightly larger than that recorded with either drug alone. Clearly the effects of propranolol on these parameters and quinine were not additive.

The studies with NO_3^- also suggested that the activity of the anion exchanger was not required for the shrinkage following either hypotonic shock, 2-deoxyglucose, or propranolol. Previous reports [22,23] have indicated that NO_3^- will be accepted by the anion exchanger. This observation was confirmed for our cells by demonstrating that a component of ^{36}Cl efflux into NO_3^- medium was inhibited by 0.1 mM DIDS. This component was comparable although somewhat smaller than the DIDS-sensitive fraction of ^{36}Cl efflux into Cl medium. The rate constants, k_e (h^{-1}), for $^{36}Cl^-$ efflux into NaCl and $NaNO_3$ were 7.08 ± 0.42 ($n = 3$) and 6.10 ± 0.51, respectively. With 0.1 mM DIDS these values fell to 4.33 ± 0.25 and 4.17 ± 0.24, respectively.

The ion movements responsible for the shrin-

TABLE VII

RATE CONSTANTS FOR ^{86}Rb EFFLUX IN NaCl-RINGER (CL) AND $NaNO_3$-RINGER (NO_3) UNDER VARIOUS CONDITIONS

Values are expressed as means ± S.D. ($n = 4$).

Description	k_e (h^{-1})
CL, control	1.27 ± 0.07
NO_3, control	0.56 ± 0.08
NO_3, hypotonic	0.53 ± 0.07
NO_3, A23187	0.58 ± 0.06
NO_3, deoxyglucose	0.50 ± 0.03
NO_3, propranolol	1.17 ± 0.10

TABLE VIII

RATE CONSTANTS FOR ^{86}Rb EFFLUX IN NaCl-RINGER (CL) AND $NaNO_3$-RINGER (NO_3) UNDER VARIOUS CONDITIONS

Values are expressed as means ± S.D. ($n = 3$).

Description	k_e (h^{-1})
CL, control	1.21 ± 0.04
CL, propranolol	2.25 ± 0.11
NO_3, control	0.54 ± 0.08
NO_3, quinine	1.14 ± 0.09
NO_3, propranolol	1.20 ± 0.04
NO_3, propranolol + quinine	1.27 ± 0.10

kage induced by hypotonic shock, 2-deoxyglucose, propranolol or A23187 plus Ca^{2+} were characterized further by monitoring the electrical potential across the plasma membrane following the addition of these agents. Previously, Laris and Henius [10] had reported that no change in the potential was observed with glucose as indicated by fluorescence measurements. Similar studies indicated that neither 2-deoxyglucose nor hypotonic shock changed the fluorescent intensity of a voltage-sensitive cyanine dye. In experiments with the sugar, 2-deoxyglucose was added directly to the cuvette after the mixture of dye, medium and cells had reached a steady level. No change in fluorescence was observed over a 10-min time period (changes in ^{86}Rb efflux are seen within this time). For the study with hypotonic shock a 200 μl aliquot of a 5% cell suspension (320 mosM) was added to 3 ml of 180 mosM NaCl-Ringer plus dye. The steady levels (approx. 4–5 min of mixing) of fluorescence were the same in the two media and, in fact, Ringers of different osmolarities (150–400 mosM) did not influence the steady level of fluorescence. In K^+ Ringer neither hypotonic shock nor addition of sugar influenced the fluorescence of the dye. In parallel experiments it was demonstrated that the dye had no effect on the volume

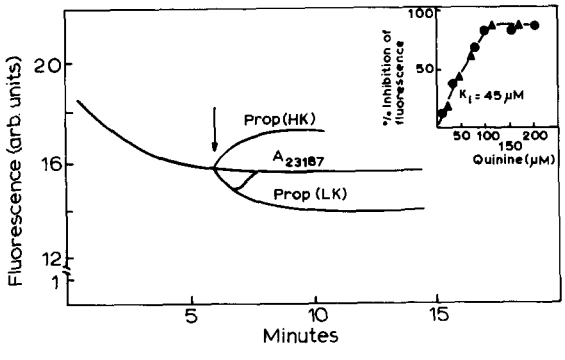

Fig. 2. The fluorescence intensity of dye ($3 \cdot 10^{-6}$ M) was assayed under various conditions as a function of time as described in Methods. The arrow indicates the addition of a test agent. Propranolol 0.05 mM (Prop) was added to the cells in low (5.9 mM) external K^+ (LK) or high (152.5 mM) external K^+ (HK). The addition of 1 μM A23187 plus 0.5 mM Ca^{2+} shows a transient hyperpolarization in low external K. Inset: dose-response curve of quinine inhibition of the changes in fluorescence induced by propranolol (●) and A23187 plus Ca^{2+} (▲).

responses to either hypotonic shock or 2-deoxyglucose (data not shown).

In contrast the addition of either 50 μM propranolol or 1 μM A23187 plus 0.5 mM Ca^{2+} did result in changes in the fluorescent intensity of the dye (Fig. 2). These changes varied with external K^+ indicating a hyperpolarization in low K^+ medium (decreased fluorescence) and an increase in high K^+ medium (increased fluorescence). These results are similar to those reported for propranolol earlier by Pershadsingh et al. [24]. While the changes with propranolol were observed for at least 10 min, those with the Ca^{2+} ionophore plus Ca^{2+} were invariably transient as shown in Fig. 2. Transient hyperpolarizations with A23187 have also been reported for human erythrocytes in the presence of 5 mM Mg^{2+} [25] and for macrophages [26].

Quinine inhibited the fluorescent changes with propranolol or A23187 plus Ca^{2+} in a dose-dependent manner as shown in the insert of Figure 2. A quinine concentration of 0.1 mM maximally inhibited (approx. 90%) the change in fluorescence. Earlier in our studies when furosemide inhibited the cell shrinkage elicited by propranolol or A23187 plus Ca^{2+}, furosemide (as high as 8 mM) did not block the change in fluorescence with these agents.

In the previous studies reported here 0.5 mM propranolol was required to induce net ion movements and cell shrinkage consistently. Since concentrations of propranolol higher than 0.05 mM produced large artifacts with the fluorescent dye, our various studies with propranolol and quinine are not directly comparable. Hence other methods to monitor electrical potential differences were also employed. The results of two indirect measurements of membrane potential, the distribution ratio of [^{3}H]tetraphenylphosphonium, a lipid soluble cation, and the rate of entrance of glycine, a Na^+-dependent amino acid, also indicated that a hyperpolarization occurs when 0.5 mM propranolol was added to cells in NaCl-Ringer. At 5 min the distribution ratio of tetraphenylphosphonium (cell/medium) was 50% greater with 0.5 mM propranolol than in controls (see also Ref. 27) and the rate of entrance of glycine entrance was 83% greater with 0.5 mM propranolol (see also Ref. 24). Quinine (0.35 mM) abolished both of the increases

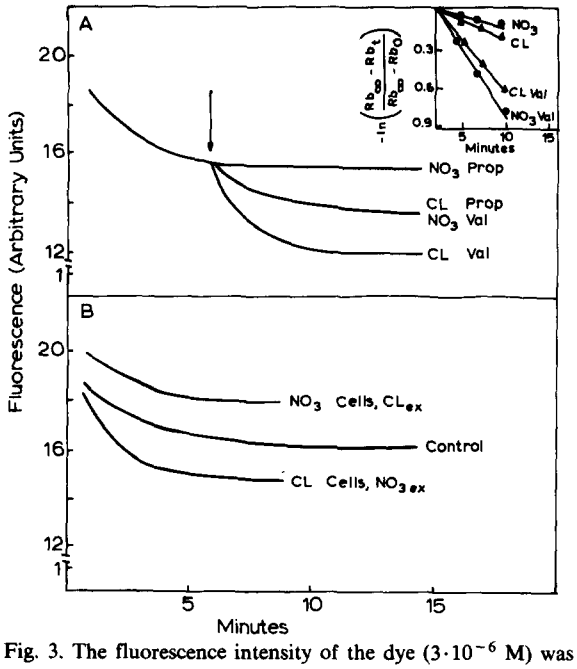

Fig. 3. The fluorescence intensity of the dye ($3 \cdot 10^{-6}$ M) was assayed under various conditions as a function of time. The arrow indicates the addition of a test agent. (A) Cells were equilibrated in NaCl-Ringer (CL) or $NaNO_3$-Ringer (NO_3) and assayed for fluorescence with the addition of 0.05 mM propranolol or 1 μM valinomycin. NaCl-Ringer cells plus valinomycin (Cl Val) or propranolol (Cl Prop), $NaNO_3$-Ringer cells plus valinomycin (NO_3 Val) or propranolol (NO_3 Prop). Inset: ^{86}Rb efflux in cells equilibrated in NaCl-Ringer or $NaNO_3$-Ringer with the addition of 1 μM valinomycin. (B) A 200 μl aliquot of cells equilibrated in $NaNO_3$-Ringer was added to a 3 ml cuvette containing NaCl-Ringer (NO_3 cells, CL_{ex}), or NaCl-Ringer equilibrated cells were added to $NaNO_3$-Ringer (CL cells, NO_{3ex}). The control trace represents NaCl-Ringer or $NaNO_3$-Ringer equilibrated cells assayed in the same Ringer.

seem with propranolol. The distributions of tetraphenylphosphonium and the rate of entrance of glycine were identical in control cells, in cells with 0.35 mM quinine and in cells with 0.5 mM propranolol plus 0.35 mM quinine. Qualitatively similar results have been reported by Valdeolmillos et al. [27] for the Ehrlich cell with 1 mM quinine.

A quinine concentration (0.35 mM) which completely inhibits the change in membrane potential elicited by propranolol, had no significant inhibitory effect on the increase in ^{86}Rb efflux induced by propranolol. The rate constants (k_e (h^{-1})) for ^{86}Rb efflux ($n = 4$) into NaCl-Ringer for the control and for 0.5 mM propranolol were 1.41 ± 0.10 and 2.39 ± 0.11, respectively. With 0.35 mM quinine the rate constant was for the control 1.55 ± 0.09 and for propranolol, 2.37 ± 0.12. This observation suggests that the greater part of the increase in ^{86}Rb efflux induced by propranolol is quinine insensitive and probably non-conductive.

The changes in membrane potential seen in NaCl-Ringer with propranolol or A23187 plus Ca^{2+} were not seen with $NaNO_3$-Ringer (Fig. 3A). Two explanations for the 'apparent' lack of a K^+-conductive flux with propranolol in NO_3^- cells were examined. (1) This K^+-conductive flux may have a specific anion requirement and NO_3^- does not substitute for Cl^-, and (2) NO_3^- conductance exceeds Cl^- conductance and hence with lower membrane resistance in NO_3^-, the increase in K^+ conductance has essentially no influence on the membrane potential. Since the K^+ efflux is larger in NO_3^- media in the presence of propranolol (Table VIII), the former possibility is unlikely. In addition, two kinds of experiments favor the latter explanation. In the first series of experiments the change in membrane potential when cells equilibrated NaCl-Ringer were placed in $NaNO_3$-Ringer was compared to the change when cells equilibrated in $NaNO_3$-Ringer were placed in NaCl-Ringer. As shown in Fig. 3B, Cl^- cells are hyperpolarized (decrease in fluorescence) in NO_3^- medium and NO_3^- cells are depolarized (increase in fluorescence) in Cl^- medium. These observations are consistent with the hypothesis that P_{NO_3} exceeds P_{Cl}. This view is also supported by the results of studies in which the membrane potential and ^{86}Rb efflux of cells treated with the K^+ ionophore, valinomycin, were compared in Cl^- and NO_3^- media. If $P_{NO_3} > P_{Cl}$ then one would predict that valinomycin would have a smaller influence on the membrane potential in NO_3^- medium but that ^{86}Rb efflux with valinomycin should be larger in NO_3^- medium since ^{86}Rb movement would be limited by the movement of ions to balance the charge. These predictions are confirmed in Fig. 3A where the fluorescence and ^{86}Rb efflux is shown in $NaNO_3$ and NaCl media with the addition of valinomycin.

Discussion

In this report and an earlier one by one of us [10] we demonstrated in the Ehrlich cell that a

shrinkage, similar to that observed following hypotonic shock, occurs when a number of agents are added to the cells in isotonic media. In summary, hypotonic stress or the addition of either glucose, 2-deoxyglucose, propranolol or A23187 plus Ca^{2+} all lead to a loss of KCl through a pathway with the following characteristics: (1) the KCl losses responsible for the volume change do not alter the membrane potential (while a K^+-dependent change in the membrane potential is seen with either propranolol or A23187 plus Ca^{2+}, the ion movements leading to the alteration of the membrane potential are not the cause of the volume change); (2) NO_3^- does not substitute for Cl^-; (3) the KCl movements are not inhibited by quinine or DIDS; (4) early in our study the shrinkage with these agents was sensitive to 1 mM furosemide. Loss of sensitivity to furosemide in our cells occurred simultaneously for all of these agents without a simultaneous change in the pattern of ion loss, cell shrinkage, or NO_3^- sensitivity.

It is unlikely that the KCl loss and shrinkage in our cells with hypotonic stress and various agents occurs through the combined K^+-H^+ and Cl^--OH^- exchange system described by Cala [7] in the Amphiuma erythrocyte. DIDS, an inhibitor of anion exchange, for example, did not inhibit the loss of KCl elicited by the various agents which induce shrinkage. Furthermore, while NO_3^- can substitute for Cl^- on the anion exchanger, NO_3^- cannot substitute for Cl^- in the shrinkage mechanism.

The data presented here also suggest that the regulatory mechanism initiated by hypotonic stress in the Ehrlich cell is different from that in the human lymphocyte [9]. While the mechanism in the Ehrlich cell is electrically neutral and quinine insensitive and does not accept NO_3^- as a substitute for Cl^-, the system in the lymphocyte uses Ca^{2+}-stimulated K^+ and Cl^- conductive pathways, is sensitive to quinine, and accepts a variety of anions including NO_3^-. The Ehrlich cell, however, appears to have a Ca^{2+}-activated K^+ conductive mechanism similar to that described in the lymphocyte and the erythrocyte [18]; for in the presence of agents (propranolol and A23187 plus Ca^{2+}) which are reported to raise cytoplasmic Ca^{2+}, the membrane potential of the Ehrlich cell becomes more sensitive to extracellular K^+. Furthermore, quinine which blocks K^+ movements through the Ca^{2+}-dependent pathway [18] was shown to prevent changes in the membrane potential in the Ehrlich cell seen with propranolol and A23187. Significantly, quinine does not inhibit the shrinkage which follows hypotonic stress or the addition of the other agents reported here in NaCl Ringer. The Ca^{2+}-stimulated K conductive pathway in the Ehrlich cell, therefore, does not appear to be a part of the mechanism responding to hypotonic stress. Shrinkage in the Ehrlich cell with propranolol, however, does occur in cells in which NO_3^- has been substituted for Cl^- probably because, as the evidence presented above indicates, the conductive movement of NO_3^- exceeds that of Cl^-. Hence with propranolol there can be a rapid net loss of KNO_3 but not of KCl through conductive channels. The net conductive K^+ movement through the Ca^{2+}-sensitive channel in the Ehrlich cell appears to be limited by the conductive Cl^- movement. In the lymphocyte an increase in cytoplasmic Ca^{2+} also increases anion conductance allowing for rapid net KCl loss [8].

It was expected that the K^+ loss with propranolol in NO_3^- medium would be sensitive to quinine. Experiments which attempted to test for this sensitivity, however, were complicated by the observation that quinine alone in a NO_3^- medium stimulated the initial unidirectional ^{86}Rb efflux, and had net K and water loss. The addition of quinine or propranolol together to the NO_3^- cells did not result in an additive stimulation of these parameters. Either quinine and propranolol now activate the same mechanism which is operating maximally with propranolol, or their actions differ and quinine partially inhibits the action of propranolol. Either explanation requires that the anionic composition of the medium influences the action of quinine. To our knowledge, the only other report that a NO_3^- medium influences the inhibition of the Ca^{2+}-stimulated K^+ efflux by quinine is that of Reichstein and Rothstein [28]. These investigators demonstrated that the $K_{50\%}$ for quinine inhibition of this channel was reduced in NO_3^- as compared to Cl^- media.

Since the loss of KCl responsible for the volume decrease in our Ehrlich cells is electrically silent and anion specific, we suggest that this net KCl loss following hypotonic stress, and the addition of 2-deoxyglucose and propranolol occurs through a

neutral cotransport system. The stoichiometry of the KCl loss could not be established because other ion fluxes also appeared to be activated by all of these treatments. These ion fluxes included a net K^+ loss and gain of Na^+ that was not dependent on the presence of Cl^- and an increase in anion exchange activity. Since the Cl^--independent K^+ loss and Na^+ gain at 1 h were not equivalent, some other ions must also move to balance the charge. The nature of these ion movements was not studied. Although the cause of the Cl^--independent K^+ loss and Na^+ gain were not investigated, they could be the result of a partial inhibition or alteration of the mode of the ($Na^+ + K^+$)-ATPase activity resulting from a reduction in cellular ATP. Glucose or 2-deoxyglucose [29] and propranolol [24] have been shown to lower ATP levels in the Ehrlich cell. In addition, an initial lowering of the ATP concentration would also be expected with hypotonic shock.

In an earlier report and as shown in these experiments the KCl loss was inhibited by 1 mM furosemide. The effect of this drug on KCl loss in response to hypotonic stress and the other agents studied was gradually reduced over a period of 6 months until, at present, the KCl loss is insensitive to the drug. The (1 Na:1 K:2 Cl) cotransport system described by Geck et al. [20], however, has continued to be sensitive to furosemide over the same period. The difference in furosemide sensitivity of the (1 K:1 Cl) and (1 Na:1 K:2 Cl) pathways in these cells suggests that the two pathways are separate structures. Hence we have seen our line of cells change in a specific way. We have also noticed a number of differences between the properties of our cells and those reported for Ehrlich cells by a number of other investigators. For example, according to Heinz et al. [30], glucose addition to Ehrlich cells results in an immediate hyperpolarization (-70 mV) of the plasma membrane due to H^+ extrusion while our cells do not hyperpolarize with glucose until Na^+ levels rise. We have recently tested the strain of cells used by this group and have confirmed their results. Similarly we have no evidence that ouabain stimulates a KCl exchange as reported by Bakker-Grunwald et al. [31]. These variations should not be surprising since the differences among established malignant cell populations propagated for many years in a variety of immunologically different host mice are probably considerable as pointed out by Levinson [32]. In a recent review Hoffman [3] reported that in her strain of Ehrlich cells the volume regulatory decrease is inhibited by quinine but not by bumetanide. She suggested that the Ca^{2+}-sensitive conductive K^+ pathway is involved in the volume regulatory decrease. Hence it appears that different strains of Ehrlich ascites tumor cells may exhibit different mechanisms of volume regulation.

The mechanism controlling the KCl loss has not yet been identified in our cells. It is very unlikely that an increase in cytoplasmic Ca^{2+} in the Ehrlich cell triggers the shrinkage response as Ca^{2+} was proposed to do in the human lymphocyte [9]. In a recent report [33], however, no changes in free Ca^{2+} were recorded in human lymphocytes undergoing a volume regulatory decrease. With propranolol changes in the membrane potential of the Ehrlich cell can be detected at lower concentrations than those required to produce changes in cell volume. If intracellular Ca^{2+} concentration is a function of propranolol concentration, then one would have to postulate that the conductive mechanism is more sensitive to Ca^{2+} than is the mechanism controlling cell volume. Yet changes in volume following hypotonic stress and 2-deoxyglucose addition occur without a change in membrane potential.

The results presented here demonstrate that hypotonic shock or the additions of 2-deoxyglucose or propranolol to cells in isotonic media are all followed by similar changes in ion movements. This similarity suggests that each of these three agents may activate the KCl loss and other ion movements through a common mechanism. Of the three types of treatment, the one most readily defined is 2-deoxyglucose which is metabolized:

2-deoxyglucose + ATP → 2-deoxyglucose 6-phosphate + ADP + H^+

Since the addition of the non-metabolizable sugar 3-*O*-methylglucose does not lead to shrinkage, we suspect that some factor (or factors) whose concentration changes during the phosphorylation reaction plays a role in the triggering mechanism. We are currently examining the possi-

bility that changes in cellular ATP, ADP, H^+ or P_i have a role in the triggering mechanism leading to shrinkage through the loss of KCl in these cells.

References

1 Hendil, K.B. and Hoffmann, E.K. (1974) J. Cell Physiol. 84, 115–126
2 Kregenow, F.M. (1981) Annu. Rev. Physiol. 43, 493–505
3 Hoffman, E.K. (1983) in Cellular Acclimatization to Environmental Change (Cossins, A.R. and Shetterlin, P.G., eds.), pp. 55–79, Soc. Exp. Biol. Publ., University Press, Cambridge
4 MacKnight, A.C. and Leaf, A. (1977) Physiol. Rev. 57, 510–561
5 Dunham, P.B. and Ellory, T.C. (1981) J. Physiol. (London) 318, 511–530
6 Lauf, P.K. (1982) J. Comp. Physiol. 146, 9–16
7 Cala, P.M. (1980) J. Gen. Physiol. 76, 683–708
8 Grinstein, S., Clarke, C.A., DuPre, A. and Rothstein, A. (1982) J. Gen. Physiol. 80, 801–823
9 Grinstein, S., DuPre, A. and Rothstein, A. (1982) J. Gen. Physiol. 79, 849–868
10 Laris, P.C. and Henius, G.V. (1982) Am. J. Physiol. 242, C326–C332
11 Johnstone, R.M. and Scholefield, P.G. (1961) J. Biol. Chem. 236, 1419–1427
12 Laris, P.C., Pershadsingh, H.A. and Johnstone, R.M. (1976) Biochim. Biophys. Acta 436, 475–488
13 Potashner, S.J. and Johnstone, R.M. (1971) Biochim. Biophys. Acta 233, 91–103
14 Levinson, C. (1978) J. Cell Physiol. 95, 23–32
15 Cheng, K., Haspel, H.C., Vallano, M.L., Osotinehin, B. and Sunenberg, M. (1980) J. Membrane Biol. 56, 191–201
16 Laris, P.C., Bootman, M., Pershadsingh, H.A. and Johnstone, R.M. (1978) Biochim. Biophys. Acta 512, 397–414
17 Gardos, G., Hoffman, J.H. and Passow, H. (1969) in Laboratory Techniques in Membrane Biophysics (Passow, H. and Stampfli, R., eds.), pp. 9–20, Springer, New York
18 Lew, V.L. and Ferreira, H.G. (1978) in Current Topics Membranes and Transport (Bronner, F. and Kleinfeller, A., eds.), Vol. 10, pp. 217–277
19 Thornhill, W.B., Paxman, D., Zarka, M., Henius, G.V. and Laris, P.C. (1982) Fed. Proc. 41, 1371
20 Geck, P., Pietrzyk, C., Burckhardt, B.C., Pfeiffer, B. and Heinz, E. (1980) Biochim. Biophys. Acta 600, 432–447
21 Bakker-Grunwald, T. (1978) Biochim. Biophys. Acta 513, 292–295
22 Aull, F. (1972) J. Physiol. 221, 751–771
23 Hoffmann, E.K., Simonsen, L.O. and Sjøholm, C. (1979) J. Physiol. 296, 61–84
24 Pershadsingh, H.A., Johnstone, R.M. and Laris, P.C. (1978) Biochim. Biophys. Acta 509, 360–373
25 Freedman, J.C. and Novak, T.S. (1983) J. Membrane Biol. 72, 59–74
26 Oliveira-Castro, G.M. and Dos Reis, G.A. (1980) Biochim. Biophys. Acta 650, 500–512
27 Valdeolmillos, M., Garcia-Sancho, J. and Herreros, B. (1982) Biochim. Biophys. Acta 689, 177–179
28 Reichstein, E. and Rothstein, A. (1981) J. Membrane Biol. 59, 57–63
29 Wu, R. and Racker, E. (1958) J. Biol. Chem. 234, 1029–1041
30 Heinz, A., Sachs, G. and Schafer, J.A. (1981) J. Membrane Biol. 61, 143–153
31 Bakker-Grunwald, T., Andrew, J.S. and Neville, M.C. (1980) J. Membrane Biol. 52, 141–146
32 Levinson, C. (1982) in Chloride Transport in Biological Membranes (Zadunaisky, J.A., ed.), pp. 383–387, Academic Press, New York
33 Rink, T.J., Sanchez, A., Grinstein, S. and Rothstein, A. (1983) Biochim. Biophys. Acta 762, 593–596