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Hypotonic shock activated Cl^- and K^+ pathways in human fibroblasts

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The exposure of human fibroblasts to hypotonic medium (200 mosmolal) evoked the activation of both $^{36}Cl^-$ influx and efflux, which were insensitive to inhibitors of the anion exchanger and of the anion/cation cotransport, and conversely were inhibited by the Cl^--channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). $^{36}Cl^-$ efflux was linked to a parallel efflux of $^{86}Rb^+$; thus conductive K^+ and Cl^- pathways are activated during volume regulation in human fibroblasts. This conclusion is supported by evidence that, in hypotonic medium, $^{36}Cl^-$ influx and $^{86}Rb^+$ efflux were both enhanced by depolarization of the plasma membrane. Depletion of the intracellular K^+ content, obtained by preincubation with the ionophore gramicidin in Na^+-free medium, had no effect on Cl^- efflux in hypotonic medium. This result has been interpreted as evidence for independent activation of K^+ and Cl^- pathways. It is also concluded that the anion permeability is the rate-limiting factor in the response of human fibroblasts to hypotonic stress.

Introduction

Following osmotic perturbations, a variety of cell types display a regulatory response whereby the resting volume of the cell tends to be restored. It is widely accepted that cell volume regulation is achieved via dynamic and controlled changes of ion transport pathways (for reviews, see Refs. 1–4). The regulatory volume decrease (RVD), that occurs after hypotonic swelling of the cells, has been reported to be attained as a result of KCl loss from the cytosol, associated with osmotic water efflux. The mechanisms involved in KCl loss include different transport pathways. In red cells from low potassium sheep [5], duck [6], fish [7] and also in cultured HeLa cells [8], an electroneutral K^+/Cl^- cotransport has been reported. In *Amphiuma* red cells, the RVD involves electroneutral, functionally coupled exchange of K^+/H^+ and Cl^-/HCO_3^- [9]. In contrast, separate, conductive K^+ and Cl^- transport pathways have been shown to be activated during RVD in human lymphocytes [10] and Ehrlich ascites tumor cells [11].

We have recently reported that activation of Cl^- transport can be induced in human fibroblasts by hypotonic shock [12]. In the present paper we have analyzed in details the effect of various inhibitors of the different Cl^- transport pathways on $^{36}Cl^-$ fluxes in isotonic and hypotonic media. In the same conditions, we have also determined K^+ efflux (using $^{86}Rb^+$ as a K^+ analogue). The results are consistent with activation of separate conductive K^+ and Cl^- pathways during RVD in human fibroblasts.

Materials and Methods

Reagents

Hepes, DIDS, furosemide, oligomycin C, gramicidin D were from Sigma. $Na^{36}Cl$ and $^{86}RbCl$ were from Amersham, U.K.; NPPB was a generous gift of professor R. Greger, Albert Ludwigs Universität, Freiburg, Germany; bumetanide was kindly provided by Prodotti Roche, Milan.

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4′-diisotiocyanatostilbene-2,2′-disulfonic acid; NMG^+, *N*-methylglucamine$^+$; *k*, rate constant of efflux.

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Cell culture

Human skin fibroblasts derived from healthy individuals were maintained in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal calf serum and antibiotics, incubated at 37 °C in a Napco 5100 water jacket CO_2 incubator with humidified atmosphere of 5% CO_2 in 95% air. Cells were subcultured weekly by trypsinization. For the experiments, $(1-1.2) \cdot 10^5$ cells were seeded into each 9.6 cm^2 well of a Nunc 6-well culture plate and allowed to grow to confluence in 3 ml of growth medium for 5–7 days.

Incubation media

The standard incubation medium (300 mosmolal, corrected with mannitol) had the following composition: 135 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM D-glucose, 20 mM Na-Hepes (pH 7.4). In Na^+-free medium, Na^+ was substituted by equimolar NMG^+. The hypotonic media were prepared from the incubation medium with appropriate dilution with 7 mM Na-Hepes (pH 7.4).

In the experiments of $^{36}Cl^-$ influx, the isotonic medium was prepared by addition of 100 mM mannitol to the hypotonic medium (200 mosmolal); the concentration of Cl^- was 95 mM in both isotonic and hypotonic media. The osmolality of solutions was determined with a Osmotic Pressure Auto Start OM 6010 Menarini.

$^{36}Cl^-$ and $^{86}Rb^+$ fluxes

For the efflux experiments, cells were washed twice with 10 ml/well of 0.85% NaCl and 10 mM Na-Hepes (pH 7.4) (300 mosmolal) and incubated with the incubation medium containing 3 μCi/ml of $Na^{36}Cl$ or 0.1 μCi/ml of $^{86}RbCl$ for 90 min, in a water bath at 37°C. At the end of the incubation time, monolayers were washed three times with ice cold 0.3 M sucrose and 2.5 mM Na-Hepes (pH 7.4) and then incubated with the indicated media without the isotopes, in a water bath at 37 °C. Efflux was determined according to the procedure described in Ref. 13 with minor modifications. Briefly, after 15 s, the efflux medium was removed from a well and delivered in a scintillation vial for counting. An equal volume of the same medium was added to the well within 2–3 s. This process of removing efflux medium and adding fresh efflux medium was repeated at the indicated time points. At the end of the experiment, the monolayer was dissolved in 1 ml of 0.2 M NaOH (15 min at 55°C). Aliquots of the cell lysate were collected for determination of radioactivity and protein content [14]. The total radioactivity in the cells at zero time was calculated by summing the radioactivity in each aliquot of the efflux medium plus the radioactivity remaining in the cells. The rate constant (k) of Cl^- and K^+ efflux was determined from the experimental points fitted according to one- or two-compartment model by a computer program based on standard non-linear least-squares procedure [15]. The steady-state fluxes of Cl^- and K^+ were determined as described in Ref. 16, considering a total cell water of 4.5 μl/mg protein [17]. Fluxes were converted from nmol/mg protein to pmol/cm^2 per s, assuming that the cell membrane area was approximately twice the surface area of the monolayer [13].

The time course of $^{36}Cl^-$ influx into fibroblasts was determined in a Nunc 6-well culture plate. The cells were washed twice as previously described, and preincubated with the incubation medium in a water bath at 37 °C for 15 min. At zero time, the incubation medium was aspirated and the indicated (isotonic or hypotonic) medium (0.8 ml/well) containing 1 μCi/ml of $Na^{36}Cl$ was added. Cells were incubated at 37 °C for the indicated period of time and then the radioactive medium was aspirated and the cells were washed three times with ice cold 0.3 M sucrose and 2.5 mM Na-Hepes (pH 7.4). Cells were dissolved with 0.2 M NaOH and treated as described for the efflux.

Determination of cell viability

To test for fibroblast integrity in isotonic and hypotonic media, we have utilized two different approaches. Trypan blue exclusion was determined after 30 min incubation in isotonic and hypotonic media, by counting the amount of trypan blue-positive cells (not viable) in a hemocytometer, after trypsinization of monolayer and appropriate dilution. The release of the cytosolic enzyme lactate dehydrogenase (EC 1.1.1.27) into the efflux medium was monitored and compared with whole cell homogenate [17].

Statistical evaluation

In all the experiments, each measurement was done at least in duplicate. Representative data are presented in most cases. Where indicated, mean values are given together with standard deviation.

Results

Effect of decreased medium osmolality on $^{36}Cl^-$ efflux and cell viability

In human fibroblasts, the efflux of Cl^-, determined within two minutes, closely conforms the theoretical equation for a single-exponential decay [18]. In Fig. 1 the rate constant (k) of Cl^- efflux has been plotted as a function of the osmolality of the efflux media. The results are rather similar to those previously reported by us [12], where $^{36}Cl^-$ efflux was measured utilizing a 24-well dish, where each well corresponded to an efflux time. However, the experimental procedure utilized in the present study allowed both a significant increase in the experimental accuracy and a consistent reduction in the amount of radioactivity employed.

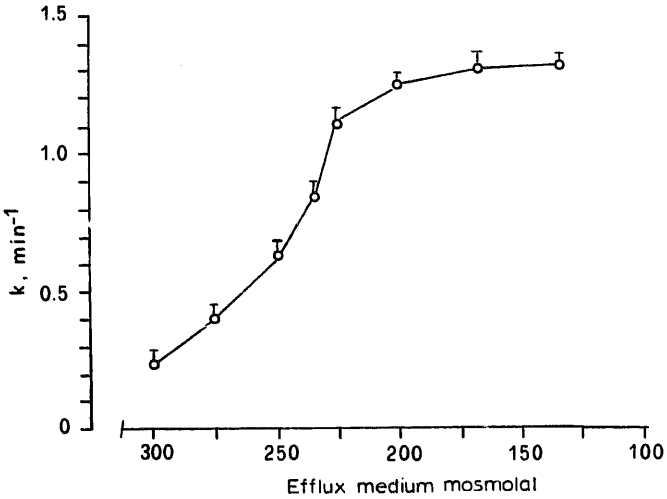

Fig. 1. Rate constants of $^{36}Cl^-$ efflux from human fibroblasts as a function of medium osmolality. Cells were loaded with $^{36}Cl^-$ and efflux was determined as described in Methods. The points are the mean values ± S.D. of four determinations.

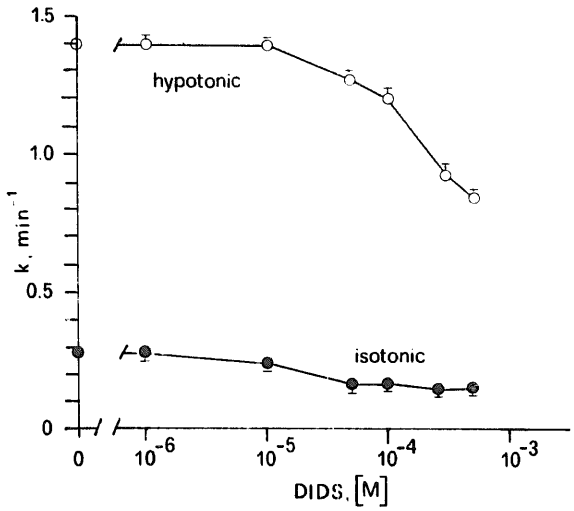

Fig. 2. Effect of DIDS on the rate constant of $^{36}Cl^-$ efflux from human fibroblasts incubated in isotonic and hypotonic media. Hypotonic medium was 200 mosmolal. DIDS was added to the efflux medium. The points are the mean values ± S.D. of three determinations.

As illustrated in Fig. 1, the increase in the k of Cl^- efflux was approximately proportional to the decrease in the tonicity of the medium from 300 to 250 mosmolal. At higher dilutions, a more rapid increase in the k was observed, suggesting that in the Cl^- response to hypotonic dilutions between 250 and 200 mosmolal a threshold phenomenon may be involved. At osmolality lower than 200 mosmolal, the k of Cl^- efflux was not further increased. These results indicate that the exposure to hypotonic media is a very efficient trigger for activation of Cl^- transport in human fibroblasts.

To exclude the possibility that the release of Cl^- induced by exposure to hypotonic solution was due to cellular damage, we have estimated the cell viability by both the Trypan blue exclusion and assaying the release of the cytoplasmic enzyme lactate dehydrogenase. Both methods indicated that exposure to hypotonic medium (200 mosmolal) had no deleterious effect on cell viability in comparison with incubation in isotonic medium (Table I). It is noteworthy that both assays were performed in the presence of isotonic and hypotonic medium after 30 min incubation, a time period considerably longer than that used in the experiments reported in the present study. It is therefore concluded that loss of cell viability was not appreciable following incubation in hypotonic medium.

TABLE I

Determination of cell viability in human fibroblasts incubated in isotonic and hypotonic media

Cells were incubated in isotonic and hypotonic (200 mosmolal) media for 30 min at 37 °C. At this time, the amount of Trypan blue positive cells was determined, as described in the Methods. In parallel experiments, the lactate dehydrogenase (LDH) activity released from the cells was assayed in the incubation medium. 100% activity was determined in cell homogenate. Values are the mean ± S.D. ($n = 3$).

	Trypan blue positive cells (% of total)	LDH release (% of total)
Isotonic medium	8 ± 3	11 ± 3
Hypotonic medium	10 ± 2	12 ± 3

Effect of some inhibitors of Cl^- transport

Fig. 2 shows the effect of DIDS, an inhibitor of the anion exchanger, on the k of efflux from fibroblasts exposed to isotonic and hypotonic media. In isotonic medium, a maximal 50% inhibition of the k of Cl^- efflux was obtained at the concentration $5 \cdot 10^{-5}$ M. This concentration of DIDS induced only a slight reduction (15%) of the k of Cl^- efflux in the hypotonic medium, whereas a 40% inhibition was obtained at $5 \cdot 10^{-4}$ M DIDS. As an alternative way to assess the contribution of the anion exchange, $^{36}Cl^-$ efflux was determined in Cl^--free media, containing gluconate instead of Cl^-. In isotonic gluconate medium, the k of efflux was approximately 40% inhibited in comparison with that measured in isotonic NaCl medium, whereas in hypotonic medium the k was only slightly modified (not shown). These results clearly indicate that in isotonic medium, a remarkable contribution to the overall Cl^- transport is due to the anion exchange. Conversely, in hypotonic medium, most of Cl^- efflux seems to occur through a transport mechanism different from the anion exchanger.

The possible contribution of the anion/cation cotransport to the activation of Cl^- transport induced by hypotonic shock, has been excluded from the results shown in Fig. 3, where the effect of bumetanide and furosemide, inhibitors of the anion/cation cotransport,

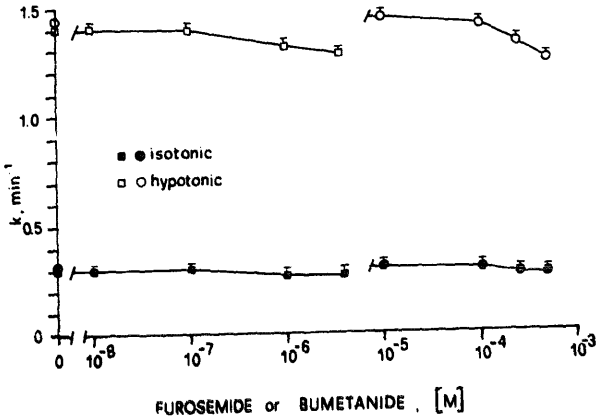

Fig. 3. Effect of the loop diuretics furosemide and bumetanide on the rate constant of $^{36}Cl^-$ efflux from fibroblasts incubated in isotonic and hypotonic media. Hypotonic medium was as in Fig. 2. Furosemide (●, ○) and bumetanide (■, □) were added to the efflux medium. The points are the mean values ± S.E. of three determinations.

is analyzed. Both inhibitors induced a maximal 20–25% reduction of the k of Cl^- efflux in isotonic and hypotonic media, bumetanide being effective at lower concentrations than furosemide, as expected from the well known rank order of potency of these diuretics [19]. The results of Figs. 2 and 3 clearly indicate that the efflux of Cl^- stimulated by hypotonic shock, does not seem to be mediated by the anion-exchanger and the anion/cation cotransport.

The involvement of a conductive pathway for Cl^- transport in the response of human fibroblasts to hypotonic dilution was tested through the use of NPPB, a diphenylamine 2-carboxylate derivative, thought to be a specific inhibitor of the Cl^- channel in the thick ascending limb of the Henle loop [20]. In Fig. 4 it is

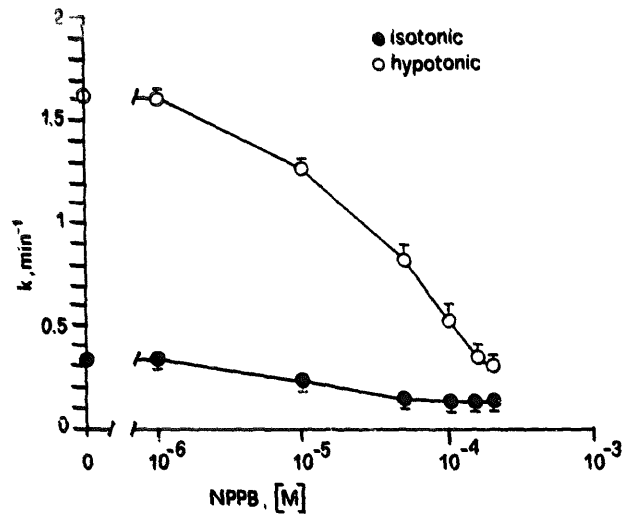

Fig. 4. Effect of the Cl^- channel blocker NPPB on the rate constant of $^{36}Cl^-$ efflux from fibroblasts incubated in isotonic and hypotonic media. Hypotonic medium was as in Fig. 2. NPPB was added to the efflux medium. The points are the mean values ± S.D. of four determinations.

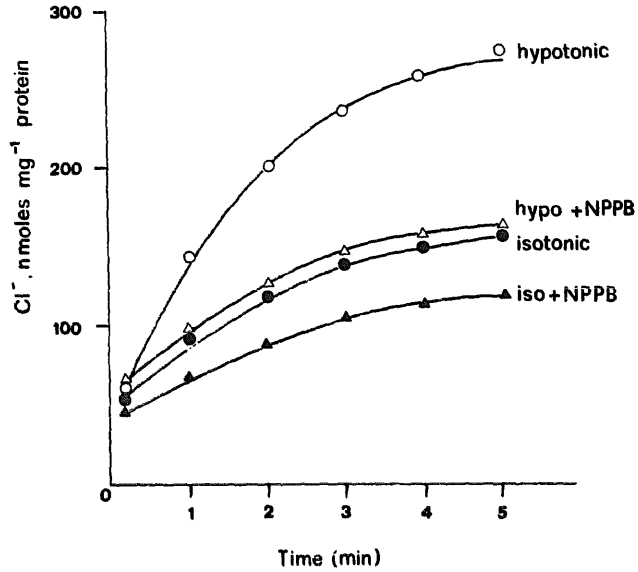

Fig. 5. Effect of NPPB on $^{36}Cl^-$ influx in fibroblasts incubated in isotonic and hypotonic media. $^{36}Cl^-$ influx was determined as described in Methods. $5 \cdot 10^{-5}$ M DIDS and 10^{-6} M bumetanide were added to both isotonic and hypotonic media. The composition of isotonic and hypotonic media is described in Methods. When present, NPPB was 10^{-4} M. The figure is representative of three independent experiments.

shown that the k of Cl^- efflux in hypotonic medium was significantly reduced at the NPPB concentration of $1.5 \cdot 10^{-4}$ M. At this concentration, the k of efflux from the isotonic medium was also inhibited (approx. 40%). Oligomycin C [3] and anthracene-9-carboxylic acid [13], Cl^--channel inhibitors in other cellular systems, did not significantly affect the efflux of Cl^- in both isotonic and hypotonic media (not shown). The inhibition by NPPB of the hypotonic medium-induced activation of Cl^- efflux can be therefore considered as an indirect indication of the involvement of a conductive pathway in volume regulation in human fibroblasts.

Activation and time-dependence of $^{36}Cl^-$ influx

It is well known that among the membrane transport proteins, channels can be distinguished from transporters by the fact that the binding sites for solutes to be transported are accessible from either sites of the membrane at the same time. It follows that if the exposure to hypotonic medium increases Cl^- efflux through the activation of a conductive pathway, also the influx of Cl^- ions is expected to be enhanced. In Fig. 5 it is shown that $^{36}Cl^-$ influx, determined in the presence of $5 \cdot 10^{-4}$ M DIDS and 10^{-6} M bumetanide, in order to abolish the contribution of the anion exchange and of the cotransport, respectively, was approximately linear from 10 s up to 2 min. In six different experiments, the initial rate of influx was determined within this time-period, and mean values of 51.8 ± 18 and 124.7 ± 57 nmol/mg protein per min

were determined in isotonic and hypotonic media, respectively. It must be noticed that, unlike $^{36}Cl^-$ efflux experiments, Cl^- influx assay was unfortunately characterized by a great degree of experimental variability. As also depicted in Fig. 5, a $60 \pm 15\%$ ($n = 3$) inhibition of the hypotonic medium stimulated Cl^- influx was observed in the presence of 10^{-4} M NPPB, which indeed inhibited ($30 \pm 10\%$, $n = 3$) also Cl^- influx in isotonic conditions. These data represent a clear evidence for the involvement of a conductive pathway during RVD in fibroblasts.

In lymphocytes [3] and Ehrlich cells [11] the hypotonic shock-activated Cl^- channels close spontaneously after 10–15 min. Conversely, in human fibroblasts, the channels remain open for at least 18 min, as illustrated in Fig. 6. In this latter case, at time $t = 0$, the cells were exposed to isotonic and hypotonic media and $^{36}Cl^-$ influx was determined at various times. After 18 min incubation in hypotonic medium, the $^{36}Cl^-$ influx was even higher than at the zero time.

Effect of high [K^+] media on $^{36}Cl^-$ influx

The effect of incubation in media containing high K^+ concentration on $^{36}Cl^-$ influx is shown in Fig. 7. Under this condition, the electrochemical gradient for K^+ is virtually abolished and the plasma membrane potential is collapsed. Thus, the conductive influx of an anion should be affected. In the experiment reported in Fig. 7, isotonic high K^+ medium induced a significant enhancement of Cl^- influx, which was rather similar to that determined in hypotonic NaCl medium. Cl^- influx was greatly increased in hypotonic high K^+ medium. To compare the results obtained from 4 different experiments, if the initial rate of Cl^- influx in

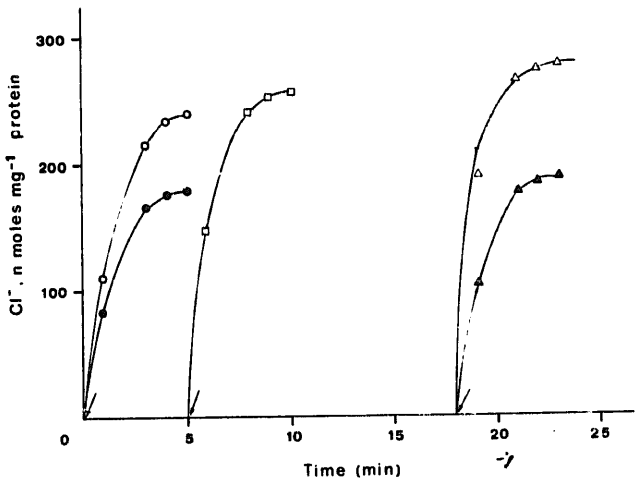

Fig. 6. Time-dependence of hypotonic medium-stimulated $^{36}Cl^-$ influx in fibroblasts. Experimental conditions as in Fig. 5. At time $t = 0$, cells were incubated in isotonic or hypotonic medium. At the times indicated by the arrows, $^{36}Cl^-$ influx was determined in (●, ▲) isotonic medium; (○, □, △) hypotonic medium. Both isotonic and hypotonic media contained $5 \cdot 10^{-5}$ M DIDS and 10^{-6} M bumetanide.

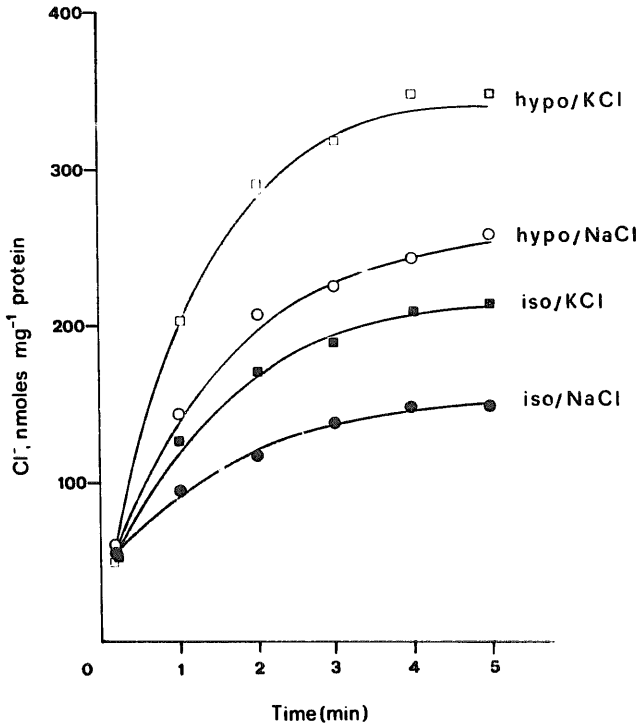

Fig. 7. Effect of high K^+ concentration on $^{36}Cl^-$ influx in fibroblasts exposed to isotonic and hypotonic media. In high K^+ media, 95 mM NaCl was replaced by 95 mM KCl. The figure is representative of four independent experiments.

isotonic NaCl medium is taken as 100%, values of $220\% \pm 50$, $225\% \pm 65$ and $358\% \pm 30$ were obtained in isotonic high K^+ medium, hypotonic NaCl medium and hypotonic high K^+ medium, respectively. The possibility that the enhanced Cl^- influx might be due to an increase in cell volume produced by high K^+, was ruled out by the data previously reported showing that within 5 min, cell volume was not changed appreciably by high K^+ either in isotonic [21] and in hypotonic [10] media. It can therefore be concluded that the collapse of membrane potential induces the activation of Cl^- influx in both isotonic and hypotonic media. Conversely, high K^+ concentration was without any effect on the efflux of Cl^- from fibroblasts incubated in isotonic and hypotonic media (not shown).

$^{86}Rb^+$ efflux from human fibroblasts exposed to isotonic and hypotonic media

The activation of a conductive Cl^- pathway during RVD requires the compensating flux of a cation to preserve the electroneutrality. The possibility that the hypotonic stress-activated Cl^- transport is dependent on the movements of Na^+ ions, through operation of the Na^+/K^+-ATPase or of the Na^+/H^+ exchange was ruled out from evidence that ouabain (0.2 mM) and amiloride (1 mM) were unable to inhibit the hypotonic medium stimulated Cl^- efflux (not shown).

K^+ constitutes a large fraction of the cellular osmotic content and therefore this cation might play a

role in volume regulation. The involvement of K^+ movements during RVD in fibroblasts has been determined following $^{86}Rb^+$ efflux under steady-state conditions. It has been previously shown that the use of $^{42}K^+$ and $^{86}Rb^+$ gave identical results [13], providing evidence that Rb^+ is a good analogue for K^+ transport in fibroblasts. In the semilog plot shown in Fig. 8A, the time course of $^{86}Rb^+$ efflux in isotonic medium is reported. The experimental points nicely conform to the theoretical equation for a two-compartment model. The slow K^+ compartment contains more than 90% of total K^+ (910 ± 30 nmol/mg protein), with a k of 0.013 min^{-1}. These results are in agreement with those previously reported by Lin and Gruenstein [13], who showed that the small, short-lived, fast phase of K^+ efflux in isotonic medium is due to cells removed during the first 15–20 s of efflux.

The addition of gramicidin (0.8 μM) to isotonic medium with Na^+ replaced by NMG^+ (to avoid ionophore-induced Na^+ influx), imposes a high K^+ permeability, so that almost 50% of K^+ was released within 2 min, as illustrated in Fig. 8A.

In hypotonic medium, Rb^+ efflux was increased substantially but transiently, the effect being apparent within the first 4–5 min (Fig. 8A), whereas at longer times the k was not significantly modified (0.016 min^{-1}). To quantify the extent of activation of K^+ efflux immediately after hypotonic dilution, the k of K^+ efflux has been calculated within the time period 15 s–3 min, according to equation for a single-exponential decay. From the k of K^+ and Cl^- efflux, the fluxes of K^+ and Cl^-, in pmol/ cm^2 per s, have been determined. From these data, (see Table II), it is possible to compare the extent of K^+ and Cl^- movements immediately after the exposition to hypotonic medium. It can be observed that K^+ flux was almost twice as high in hypotonic cells, whereas Cl^- flux was

TABLE II

K^+ and Cl^- fluxes determined in human fibroblasts exposed to isotonic and hypotonic (200 mosmolal) media

The steady-state tracer exchange fluxes of K^+ and Cl^- were determined from the k of efflux, determined within 15 s–3 min, as described in Methods. Values are the mean ± S.D. ($n = 3$).

	pmol/cm^2 per s	
	K^+	Cl^-
Isotonic medium	72.5 ± 3	211.5 ± 26
Hypotonic medium	145.4 ± 15	882.0 ± 33

increased approximately four times. The high Cl^- flux in isotonic medium can be explained by the contribution of the anion exchange and of the anion/cation cotransport, which accounted for approximately 40–50% and 25% of the total Cl^- efflux, respectively (see also the results of Figs. 2 and 3). Thus, the conductive component constitutes only a small fraction of total efflux in isotonic medium. During RVD, the Cl^- flux is greatly enhanced, without any significant activation of the anion exchange and of the anion-cation cotransport (see also Figs. 2 and 3), and therefore it is likely to conclude that Cl^- conductance becomes predominant during volume regulation, exceeding that of K^+.

The effect of high K^+ concentration on $^{86}Rb^+$ efflux from fibroblasts exposed to isotonic and hypotonic media is reported in Fig. 8B. The effect is apparent in the slow phase of Rb^+ efflux; in fact, in isotonic high K^+ medium, the k of Rb^+ efflux was increased from 0.013 to 0.028 min^{-1}. As shown in Fig. 8B, Rb^+ efflux was further enhanced in hypotonic high K^+ medium, the k of efflux being 0.036 min^{-1}. Although the effect of high K^+ at short times is less apparent, a 30–40% increase was observed in both isotonic and hypotonic

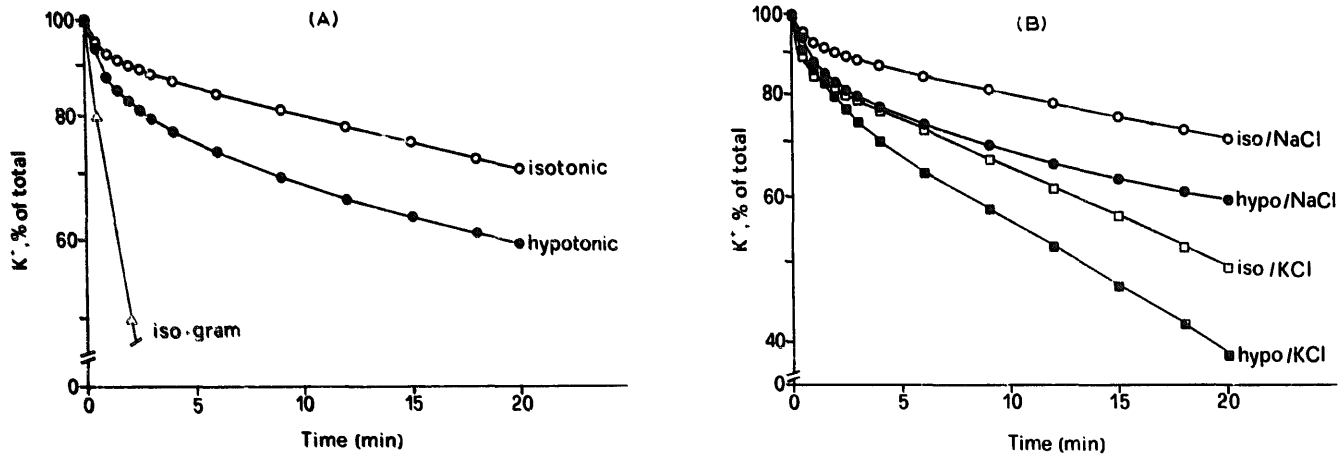

Fig. 8. $^{86}Rb^+$ efflux from fibroblasts exposed to isotonic and hypotonic media. (A) 0.8 μM gramicidin was added to Na^+-free isotonic medium containing NMG^+ instead of Na^+ (△). (B) High K^+ media contained KCl instead of NaCl. K^+ content of fibroblasts (100%) was 1010 ± 30 nmol/mg protein. The figure is representative of three independent experiments.

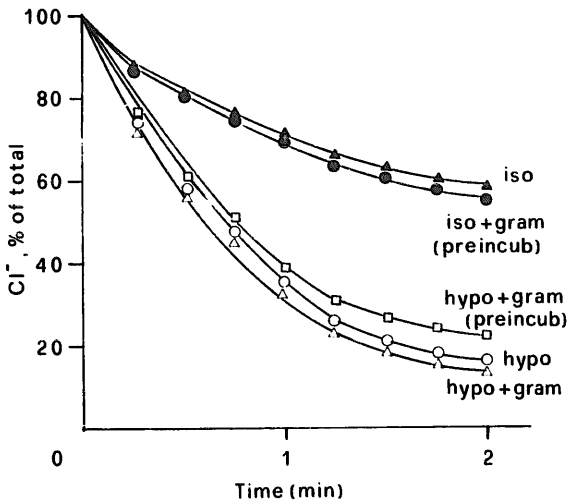

Fig. 9. Effect of gramicidin on $^{36}Cl^-$ efflux from fibroblasts exposed to isotonic and hypotonic media. At the end of the $^{36}Cl^-$ loading period, the cells were incubated for 4 min in Na^+-free incubation medium containing $^{36}Cl^-$ (▲, △, ○), plus 1 μM gramicidin (●, □). Cl^- efflux was determined in Na^+-free media: (●, ▲) isotonic medium; (○, □) hypotonic medium; (△) hypotonic medium plus 1 μM gramicidin. Cl^- content of fibroblasts (100%) was 325 ± 28 nmol/mg protein.

media. It is therefore concluded that the abolition of the plasma membrane potential significantly increases K^+ efflux in both isotonic and hypotonic media.

Effect of gramicidin on Cl^- efflux

To assess the interdependence of anion and cation fluxes during volume regulation, we have determined whether volume-induced anion efflux could be observed in the absence of K^+. To this purpose, at the end of the $^{36}Cl^-$-load period, the cells have been incubated for 4 min with Na^+-free isotonic medium containing $^{36}Cl^-$, plus 1 μM gramicidin, to rapidly deplete the intracellular K^+ content. As shown in Fig. 9, Cl^- efflux was measured in isotonic as well hypotonic conditions and compared with the efflux from fibroblasts with normal K^+ content. The presence of the ionophore did not significantly affect Cl^- efflux in both isotonic and hypotonic media. This result supports the notion that anions and cations move independently during RVD.

Discussion

The present study shows that the exposure to hypotonic medium has a profound effect upon the activity of Cl^- and K^+ transport pathways in human fibroblasts. The results, mainly focused on the Cl^- pathway, indicate that a conductive mechanism is operative. Several evidences support this conclusion, namely: (i) both $^{36}Cl^-$ influx and efflux are enhanced after exposure to hypotonic medium; (ii) the Cl^- channel blocker NPPB was the only effective inhibitor of the Cl^- fluxes induced by hypotonic medium; (iii) the efflux of Cl^- is linked to a parallel, independent efflux of K^+; (iv) the fluxes of Cl^- and K^+ are affected by plasma membrane depolarization.

The finding that the inhibitors of the anion exchange and of the anion/cation cotransport were poorly effective on the hypotonic medium-activated Cl^- efflux excludes the possible involvement in fibroblasts of the electroneutral K^+/Cl^- cotransport [5–8] and of the functionally coupled exchange of K^+/H^+ and Cl^-/HCO_3^-, operating in other cell systems [9]. Conversely, the inhibition by NPPB of the hypotonic shock-stimulated influx and efflux of Cl^- ions confirms the conductive nature of the Cl^- pathway involved in RVD.

NPPB is a lipophilic compounds, which has a high affinity for the Cl^- channel of the basolateral membrane of the thick ascending limb of the loop of Henle [20]. The concentration of NPPB which is effective in human fibroblasts ($1.5 \cdot 10^{-4}$ M) is one order of magnitude higher than that required for complete inhibition in kidney cells, and therefore the inhibitory effect of this compound has to be considered rather unspecific. In this respect, it has been reported that, in rat mesenchimal cells, NPPB inhibits prostaglandin E_2 biosynthesis, as a consequence of its structural similarity to some cycloxygenase inhibitors [22]. Indomethacin, one of these inhibitors, is without any effect on the hypotonic shock-activated Cl^- channel of human fibroblasts (Mastrocola, T. and Rugolo, M. in preparation). It is therefore possible that the inhibitory effect of NPPB can vary depending on the channel subtype and the assay method.

Our finding that NPPB inhibits Cl^- fluxes in both isotonic and hypotonic media suggests that a conductive pathway for Cl^- transport is operative also in isotonic conditions, in agreement with the previous data showing that in human fibroblasts a conductive Cl^- pathway accounts for approx. 20% of total Cl^- efflux [13].

Recently, a preliminary electrophysiological evidence has been presented by Rothstein and Bear [23] indicating that Cl^- mini channels are activated by hypotonic medium in human fibroblasts. A unitary conductance of 3 pS was estimated in excised inside-out patches. Conversely, in Ehrlich cells [24] and in opossum kidney epithelial cells [25], the unitary conductance of volume-activated Cl^- channels has been reported to be moderately large, about 25 pS. Given the large size of the volume-activated fluxes, in fibroblasts the number of these channels must be quite large. In fibroblasts, in cell-attached configuration, the Cl^- current steps were substained for several minutes [23], with no evidence of spontaneous closing. The same behaviour can be seen in the experiment of Cl^- influx illustrated in Fig. 6. The reason for this prolonged

opening of the channel is at present unknown. Also in Madin-Darby canine kidney cells, the Cl^- channels opened by osmotic stress do not spontaneously close for at least 20 min [26]. To summarize, in each of the cell types studied, the volume-activated Cl^- channels were observed, but their properties were quite variable.

$^{36}Cl^-$ influx as well as $^{86}Rb^+$ efflux in hypotonic medium are increased by plasma membrane depolarization (Figs. 7 and 8B). However, it can be noticed that membrane depolarization affects Cl^- and K^+ fluxes also under isotonic conditions. Our finding that the effect of high K^+ and of reduced tonicity are additive (see also Figs. 7 and 8B) might suggest that more than one type of Cl^- and K^+ channels are indeed present in the plasma membrane of fibroblasts. Nevertheless, it cannot be ruled out the possibility that the same conductance mechanism might be activated by different intracellular regulators.

The increase in anion permeability during RVD is coupled to a concomitant enhancement in the K^+ permeability (Fig. 8A). However, K^+ permeability does not limit the rate of Cl^- efflux in volume-static fibroblasts, as supported by the fact that the ionophore gramicidin failed to induce any increase in anion permeability, despite the fact that it produces an increase in cation permeability greater than that resulting from hypotonic stress. This finding suggests that an increase in K^+ permeability is not sufficient to account for cell volume alterations. It follows that the anion permeability is the rate limiting factor in the RVD response, as it has been reported to occur in human lymphocytes [27] and Ehrlich cells [11].

Finally, our finding that the k of Cl^- efflux is twice as high after exposure to a medium in which the osmolality was decreased only 8% (Fig. 1) suggests that the mechanism underlying the response to cell swelling must be very sensitive to changes in cell volume. Hudson and Schultz [24] have reported that in isotonic conditions, the intracellular accumulation of glycine by a Na^+-dependent system, results in a slow increase (5%) of cell volume, which stimulates the activity of Cl^- channels, whose properties closely resemble those activated by rapid swelling after exposure to hypotonic medium. Therefore, changes in the concentration of osmotically active metabolites, which can be induced by various stimuli, can affect the activity of membrane transport systems, probably through mechanical tension developed in the membrane itself. This observation gives prominence to the general relevance of these ion transport systems and of the mechanisms involved in their regulation.

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