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A volume-sensitive chloride conductance revealed in cultured human keratinocytes by $^{36}Cl^-$ efflux and whole-cell patch clamp recording

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The Cl^- transport mechanism responsible for the stimulation of $^{36}Cl^-$ efflux after exposure to hypotonic medium (210 mosmol/kg) was investigated in human keratinocytes. The involvement of the anion exchanger and of the Cl^-/cation cotransporters was ruled out by the finding that replacement of extracellular Cl^- by the poorly permeant anion gluconate, and the addition of bumetanide and furosemide, inhibitors of the $Na^+/K^+/Cl^-$ and K^+/Cl^- cotransporters, respectively, failed to significantly reduce the activation of Cl^- efflux by hypotonic medium. 'Whole cell' configuration of the patch clamp technique directly revealed the presence of a macroscopic Cl^- current, which was evoked by incubation with hypotonic medium and was reversed by elevation of the extracellular osmolality. Volume-sensitive current showed outward rectification of the current-voltage relationship and time-dependent inactivation at depolarizing voltages. This current was Cl^- selective, because the zero-current reversal potential approached the Cl^- equilibrium potential, when extracellular Cl^- was replaced by gluconate. 0.1 mM 1,9-dideoxyforskolin significantly reduced either $^{36}Cl^-$ efflux and the Cl^- current, suggesting that the Cl^- efflux and the macroscopic current activated after exposure to hypotonic medium are mediated by the same pathway. Electronic cell sizing showed that in keratinocytes hypotonic swelling was not followed by a significant regulatory volume decrease response.

Introduction

Keratinocytes are the main components of epidermis. Under proper culture conditions, these cells form colonies that rapidly proliferate in the presence of epidermal growth factor and cholera toxin. Colonies eventually fuse to form a stratified epithelium, which can also be used for grafting on burned patients [1,2].

By the use of isotopic fluxes, we have recently shown that Cl^- transport occurs in human keratinocytes by means of two mechanisms, an anion exchanger and a conductive pathway [3]. The anion exchanger accounts for 40% of total Cl^- efflux and exhibits a saturation behaviour over the range 10–135 mM external Cl^-, Cl^- being the most efficient among the transported anions [3]. The presence of a Cl^- conductive pathway has been revealed either by inhibition of Cl^- efflux by the Cl^- channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and by the sensitivity to manipulation of the plasma membrane potential [3]. Patch clamp experiments provided the biophysical characterization of a Cl^- selective channel, which showed an outward rectifying current–voltage relationship [4,5].

Here we describe a Cl^- pathway which is activated in keratinocytes as a response to cell swelling, apparently as a component of the so-called regulatory volume decrease (RVD). This general process occurs when cells from a variety of tissues are exposed to hypotonic solutions: they initially swell like more or less perfect osmometers, but then return to almost their original volume due to KCl loss from the cytosol, associated with osmotic water efflux (for a review see Ref. 6).

The Cl^- transport mechanism activated in human

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Abbreviations: k, rate constant of efflux; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; RVD, regulatory volume decrease; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; ddFSK, 1,9-dideoxyforskolin.

keratinocytes by cell swelling has been investigated by following $^{36}Cl^-$ efflux in the presence of various inhibitors of anion transport. Furthermore, direct evidence for activation of a Cl^- current was obtained with the 'whole-cell' configuration of the patch clamp technique.

Materials and Methods

Materials. $Na^{36}Cl$ was obtained from Amersham; bumetanide was a gift from Prodotti Roche, Milano; furosemide and 1,9-dideoxyforskolin were from Sigma.

Cell culture. Human epidermal keratinocytes were cultured as described in Ref. 2. For isotopic experiments, cells were seeded in 6-well dishes ($2 \cdot 10^5$ cells/well) on feeder layer of lethally irradiated 3T3-J2 fibroblasts. Experiments were performed using monolayers of keratinocytes immediately after confluence was reached. Contamination of culture by dermal fibroblasts was virtually absent.

For electrophysiological studies, cells were plated in 35 mm petri dishes (10^5 cells/dish) without feeder layer. The cells were used untill they were in a subconfluent condition, starting from 24 h after plating.

Chloride efflux. Cells were washed twice with 10 ml of 150 mM NaCl and 10 mM Na-Hepes (pH 7.4) and incubated for 90 min in a water bath at 37°C and ambient CO_2 with 1 ml of the standard incubation medium (300 mosmol/kg with mannitol) containing 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) and 1 μCi/ml of $Na^{36}Cl$. The efflux of Cl^- was determined as described in Ref. 3. The Cl^--free medium contained 135 mM Na-gluconate, 10 mM D-glucose, 1.3 mM $Ca(OH)_2$ and 20 mM Na-Hepes (pH 7.4). The hypotonic solutions (200–210 mosmol/kg) were prepared by dilution of isotonic solutions. Osmolality of solutions was measured with a Osmotic Pressure Auto Start OM 6010 Menarini.

Whole-cell recordings. The 'whole-cell' configuration of the patch clamp technique [7] was used to record membrane currents of cultured keratinocytes. The intracellular (pipette) solution contained 140 mM KCl (or NaCl), 0.18 mM $CaCl_2$, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM ATP, 10 mM Na-Hepes (pH 7.3; calculated free Ca^{2+} 10^{-8} M, assuming an apparent Ca-EGTA association constant of 10^{-7} M). The osmolality was adjusted to 300 mosmol/kg with mannitol. The hypotonic medium contained 90 mM NaCl (or Na-gluconate), 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose and 10 mM Na-Hepes (pH 7.3). Data acquisition and analysis have been described elsewhere [5]. Membrane potentials were indicated taking the extracellular cell side as ground. Positive currents represented anions flowing into the cell. Membrane conductance values were corrected for the series resistence. The voltage stimulation protocol consisted in 500 ms steps applied every 5 s to −100 mV from a holding potential of 0 mV. This allowed the simultaneous tracking of K^+ (at 0 mV) and Cl^- currents (at −100 mV). Occasionally, this protocol was interrupted to apply 300 ms long voltage steps to membrane potentials in the range from −80 to +80 mV. The experiments were carried out at room temperature (20–24°C).

Cell volume measurements. Cell volume distribution curves were obtained by electronic sizing using a Coulter Multisizer II. The tube orifice was 140 μm. For the measurements, cells were trypsinized, centrifuged and resuspended in the standard incubation medium; aliquots of the cell suspension were diluted 200-fold with the same medium to give a final cell density of about $3 \cdot 10^4$ cells/ml. The mean cell volume was calculated as the median of volume distribution curves. Calibration was carried out with polystyrene latex beads (13.3 μm diameter).

Statistical evaluations. All values are expressed as means ± S.E., with the number of experiments in brackets.

Results

Activation of $^{36}Cl^-$ efflux by hypotonic medium and effect of some anion transport inhibitors

A typical experiment of $^{36}Cl^-$ efflux from human keratinocytes exposed to isotonic and hypotonic medium is shown in Fig. 1. Under isotonic conditions, the efflux curve closely conformed the theoretical equation for a single-exponential decay, as previously discussed in Ref. 3. In the experiment shown in Fig. 1, the rate constant of Cl^- efflux (k) was increased from 0.166 min^{-1} to 0.354 min^{-1} in isotonic and hypotonic

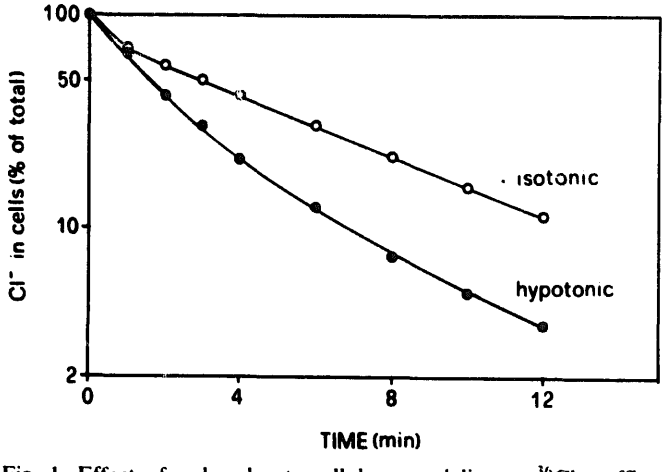

Fig. 1. Effect of reduced extracellular osmolality on $^{36}Cl^-$ efflux from human keratinocytes under steady-state conditions. Cells were loaded as described in Methods and $^{36}Cl^-$ efflux in isotonic (300 mosmol/kg) and hypotonic (210 mosmol/kg) media was determined. Total Cl^- content (100%) was 382 ± 21 nmol/mg protein ($n = 2$). The curves are representative of five different experiments.

TABLE I

Cl^- efflux from human keratinocytes incubated in isotonic and hypotonic media: effect of Cl^- substitution by gluconate

Cells were loaded with $^{36}Cl^-$ and efflux was determined in the indicated media. The increase of the mean value of k of efflux in hypotonic medium is expressed as % over the mean value of the k in isotonic medium, considered as 100%.

	Rate constant of Cl^- efflux (min^{-1})		
	isotonic medium	hypotonic medium	% increase over control
NaCl medium	0.175 ± 0.009 ($n = 5$)	0.306 ± 0.025 ($n = 5$)	74.8
Na-gluconate medium	0.126 ± 0.005 ($n = 18$)	0.215 ± 0.007 ($n = 15$)	70.6

medium, respectively; the mean values of the k obtained in five experiments are reported in Table I. Table I also shows that omission of Cl^- in the efflux medium and replacement by the poorly permeant anion gluconate inhibited (40%) the k of Cl^- efflux in isotonic medium, whereas only slightly reduced the percent of activation of the k of Cl^- efflux by hypotonic medium. From this result it can be concluded that the anion exchanger, which is responsible for a remarkable portion of Cl^- efflux under isotonic condition [3], is not involved in the response of human keratinocytes to hypotonic shock.

The experiment reported in Fig. 2 shows that after 10 and 20 min exposure to hypotonic medium, Cl^- efflux was still enhanced, the k of efflux being 0.214 ± 0.002, 0.212 ± 0.003 and 0.204 ± 0.003 min^{-1} ($n = 3$), at $t = 0$, $t = 10$ min and $t = 20$ min, respectively. When cells were incubated 20 min in hypotonic medium and

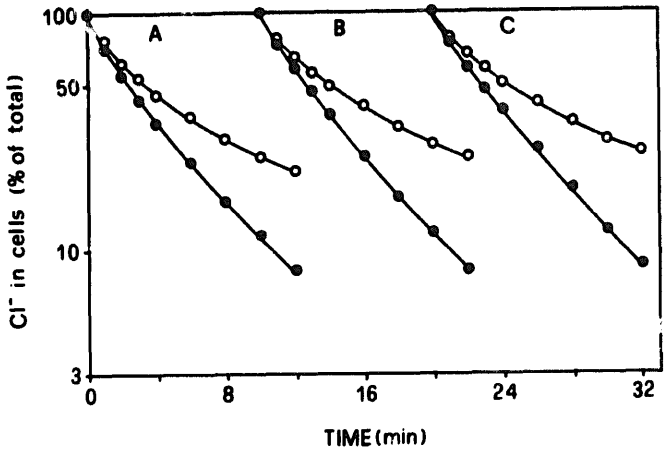

Fig. 2. Time-course of hypotonic-shock stimulated Cl^- efflux in human keratinocytes. Experimental conditions as in Fig. 1. (A) at time $t = 0$, Cl^- efflux in isotonic (○) and hypotonic (●) Na-gluconate medium was determined. (B) Cells were kept for 10 min or (C) 20 min in hypotonic medium containing $Na^{36}Cl$ before efflux. Total Cl^- content (100%) was 345 ± 18 nmol/mg protein ($n = 6$). The figure is representative of three different experiments.

TABLE II

Effect of some inhibitors of Cl^- transport on the rate constant of Cl^- efflux from human keratinocytes incubated in gluconate medium

The increase of the mean value of the k of efflux in hypotonic medium is expressed as % over the mean value of the k in isotonic medium, considered as 100%.

Additions	Rate constant of Cl^- efflux (min^{-1})		
	isotonic medium	hypotonic medium	% increase over control
None	0.121 ± 0.008 ($n = 7$)	0.204 ± 0.012 ($n = 5$)	68.6 *
Bumet. 10 μM	0.109 ± 0.009 ($n = 3$)	0.191 ± 0.004 ($n = 4$)	75.2
Furos. 0.5 mM	0.090 ± 0.008 ($n = 3$)	0.153 ± 0.008 ($n = 5$)	70.0
ddFSK 0.1 mM	0.098 ± 0.005 ($n = 3$)	0.132 ± 0.003 ($n = 3$)	34.7 *

* $P < 0.01$.

then transferred to isotonic medium, the k of efflux decreased to 0.113 ± 0.002 min^{-1} ($n = 4$), a value even lower than that estimated in cells maintained in isotonic medium (0.128 ± 0.005 min^{-1}, $n = 4$). It is therefore concluded that the activation of Cl^- efflux persists for several minutes and can be completely reversed by restoration of isotonic osmolality.

To identify the mechanism responsible for the activation of Cl^- efflux under hypotonic conditions, the effect of some inhibitors of Cl^- transport was investigated. It is well known that the RVD response is mainly due to KCl loss from the cells. Table II shows that the addition of 10^{-5} M bumetanide, an inhibitor of the $Na^+/K^+/Cl^-$ cotransport [8], slightly inhibited (10%) the k of efflux in isotonic medium, whereas the percent of activation of the k of efflux by hypotonic medium was even increased. A similar effect was observed by the addition of a high concentration ($5 \cdot 10^{-4}$ M) of furosemide, an inhibitor of the K^+/Cl^- cotransport [9]. The percent of stimulation of Cl^- efflux by hypotonic medium was unimpaired by furosemide, which conversely 25% inhibited the k of efflux under isotonic conditions. The results obtained with bumetanide and furosemide rule out a major role for Cl^-/cation cotransporters on cell swelling-activated Cl^- efflux.

Recently, 1,9-dideoxyforskolin has been reported to be a very effective inhibitor of volume-regulated Cl^- currents [10]. In our experiments, 0.1 mM dideoxyforskolin induced 50% inhibition of the activation of the k of efflux by hypotonic medium (Table II). This result suggests that a conductive Cl^- transport pathway is activated by osmotic cell swelling.

Whole-cell Cl^- currents

Whole cell recordings on cultured keratinocytes revealed the presence of a Cl^- current, which was sensi-

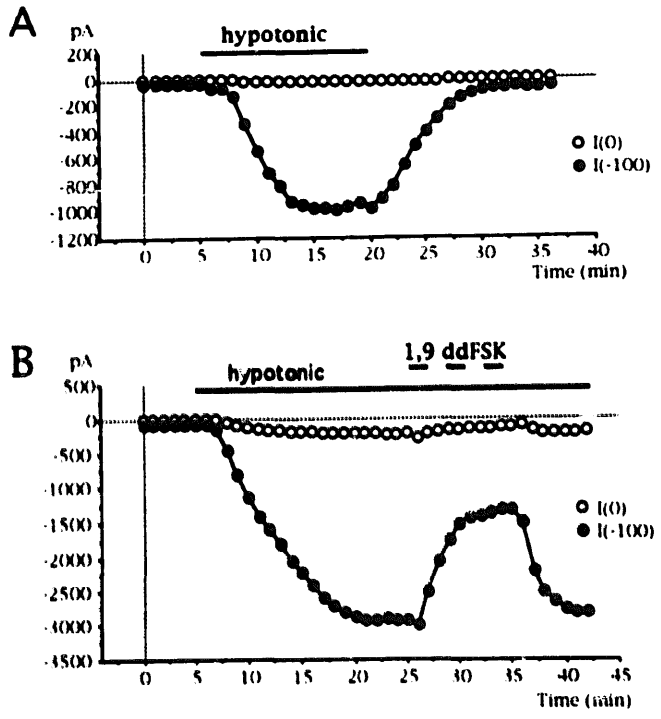

Fig. 3. Membrane currents activated by hypotonic shock. Data represent the time-course of the current elicited at 0 and −100 mV. The horizontal bars indicate the duration of application of the hypotonic medium (210 mosmol/kg) and of 0.1 mM 1,9-dideoxyforskolin. The pipette was filled with 140 mM KCl (see Methods).

tive to changes of extracellular osmolality. In fact, cell perfusion with the hypotonic medium caused the activation of a large membrane current which was reversed by termination of hypotonic shock (Fig. 3A). When cells were kept for 15–20 min in hypotonic medium, the membrane conductance remained high. In nine experiments performed with K^+-rich pipette solution, the membrane current recorded at −100 mV increased in 8–15 min from resting values of 20–100 pA to 700–4300 pA, under hypotonic conditions. Normalizing for the membrane capacitance, this corresponded to a change of membrane conductance from 0.018 ± 0.006 nS/pF to 0.719 ± 0.087 nS/pF ($n = 9$). It is noteworthy that there was no increase of the current at 0 mV during hypotonic shock, indicating that, at least in our experimental conditions, K^+ currents were not activated. Fig. 3B shows that the application of 0.1 mM 1,9-dideoxyforskolin reduced the currents at −100 mV. In six experiments, this forskolin analogue inhibited the cell swelling-stimulated currents by 62.6 ± 7%. The recovery of the current from the block induced by 1,9-dideoxyforskolin was complete (Fig. 3B) in 4/6 experiments.

The characteristics of the current activated by the hypotonic shock were assessed with the Na^+-rich pipette solution, to exclude any contribution by the K^+ currents. As shown in Fig. 4A, volume-sensitive currents were characterized by a peculiar time-dependent inactivation, which occurred at membrane potentials as large as +80 mV (Fig. 4A). Recovery from inactivation was achieved by returning the membrane potential at negative values.

The Cl^- selectivity of hypotonically stimulated currents was assessed by partially replacing extracellular Cl^- with gluconate. Since Cl^- channels are poorly permeable to this inorganic anion, this intervention produced a shift of the zero-current reversal potential (E_{REV}) towards positive values, as reported in Fig. 4B. In five experiments, membrane currents in hypotonic medium (100 mM Cl^-) reverted at +6.3 ± 1.4 mV. After replacement of 90 mM Cl^- with gluconate, E_{REV} shifted to 48.6 ± 3.6 mV. The Nernst potentials under these ionic conditions are +8 and +63 mV, respectively.

Keratinocytes volume

In the experiment reported in Fig. 5A, the changes in cell volume of keratinocytes after exposure to hypotonic medium are shown. The mean cell volume of keratinocytes incubated in isotonic medium was 2541

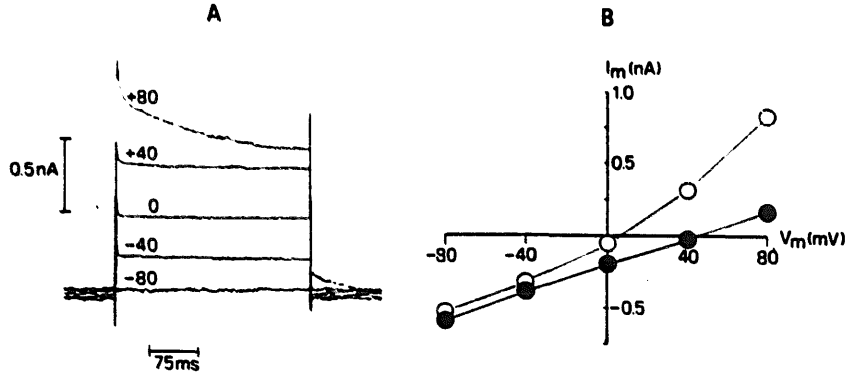

Fig. 4. (A) Representative currents activated by hypotonic shock. The graphic shows superimposed membrane currents elicited by 300-ms long voltage steps to the potentials indicated for each trace. The holding potential was −80 mV. (B) Current-voltage relationship from the same experiment. Current values were taken 10ms after the beginning of the pulse. Pipette solution contained 140 mM NaCl (see Methods). The extracellular hypotonic medium contained 90 mM Cl^- (○) or 10 mM Cl^- plus 90 mM Na-gluconate (●).

± 30 fl ($n = 4$). Following exposure to hypotonic medium, keratinocyte volume increased of approx. 13 ± 2% ($n = 3$), achieving the maximal value within 1–1.5 min. As shown in Fig. 5A, keratinocytes were unable to return to their normal (isotonic) volume within 27 min. This behaviour is different from that of human fibroblasts, which was determined for comparison and is illustrated in Fig. 5B. The mean cell volume of fibroblasts under isotonic condition was 4406 ± 120 fl ($n = 4$) and after hypotonic dilution increased of 12 ± 3% ($n = 3$) over the isotonic value. Thereafter, fibroblasts shrank gradually and after 6–8 min an almost complete volume recovery was achieved.

Discussion

We used both $^{36}Cl^-$ efflux measurements and 'whole cell' patch-clamp recordings to identify and characterize a Cl^- transport in human keratinocytes that becomes activated by cell swelling.

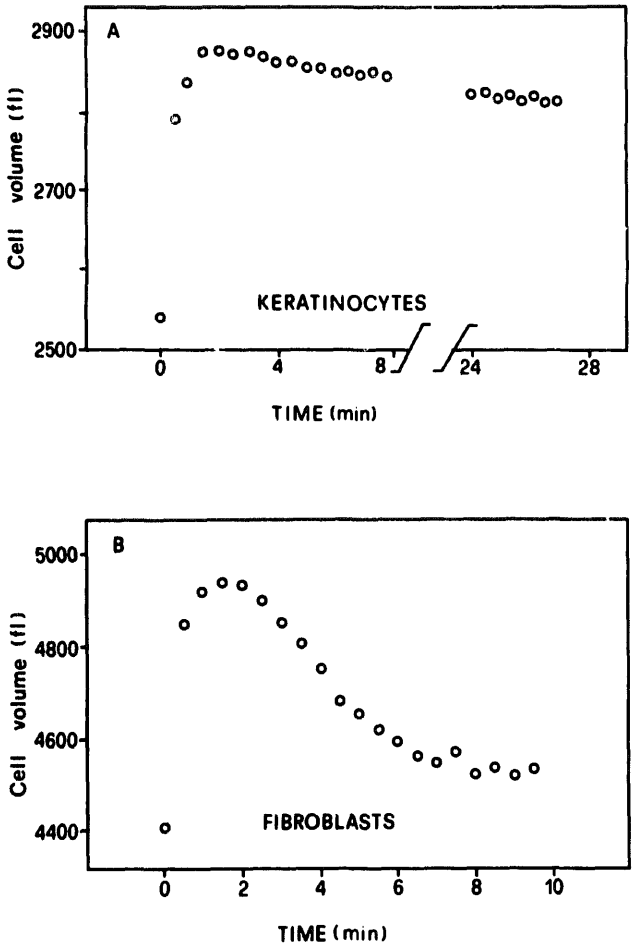

Fig. 5. Changes of the cell volume of human keratinocytes (A) and fibroblasts (B) exposed to hypotonic medium. Cell volume is expressed in femtoliters (10^{-15} liter). Cells were kept in isotonic medium. At zero time, a sample of the cell suspension was 200-fold diluted in hypotonic medium (200 mosmol/kg) and the cell volume was followed with time, as described in Methods. The initial (zero time) cell volume under isotonic conditions (mean value of four determinations) was measured by dilution of a parallel sample of the cell suspension in standard incubation medium (300 mosmol/kg). The curves are representative of three determinations with keratinocytes and three with fibroblasts.

The finding that replacement of extracellular Cl^- by gluconate and the addition of bumetanide and furosemide had no effect on the hypotonic medium-stimulated $^{36}Cl^-$ efflux ruled out the possible involvement of the electroneutral $Na^+/K^+/Cl^-$ and of the K^+/Cl^- cotransporters [11–14] and of the functionally coupled exchanges of K^+/H^+ and Cl^-/HCO_3^- [15], which have been shown to be activated in other cellular systems (for a review, see Ref. 6).

The macroscopic currents measured during 'whole cell' recordings showed inactivation at positive membrane potentials and outward rectification of the current–voltage relationship (shown in Figs. 4A, B). Volume-sensitive whole cell currents with similar properties have been reported to develop across the plasma membrane of colonic tumor cell line T_{84} [16] and of airway epithelial cells [17,18].

The similar inhibitory effect caused by 1,9-dideoxyforskolin provides evidence that $^{36}Cl^-$ efflux (Table II) and Cl^- current (Fig. 3B) are mediated by the same pathway. Furthermore, both isotopic and electrophysiological measurements indicate that the volume-sensitive Cl^- transport remains activated in keratinocytes as long as the hypotonic shock is applied. A persistent (up to 20 min) activation of Cl^- efflux has also been observed by us in human fibroblasts incubated in hypotonic medium [19]. However, fibroblasts almost completely recovered their volume within 6–8 min, even if they are maintained in hypotonic medium (Fig. 5B and also Mastrocola et al., submitted). Conversely, keratinocytes remained swollen and regulatory volume decrease did not take place (Fig. 5A). It is likely that the lack of any K^+ current activation, accompanying the increase of Cl^- conductance, could explain this behaviour. In this respect, it is noteworthy that a significant increase of both K^+ and Cl^- effluxes was determined in fibroblasts exposed to hypotonic medium [19].

Failure to undergo RVD has also been reported to occur in lymphocytes obtained from human tonsil, whereas peripheral blood lymphocytes exhibited a normal RVD response [20]. The difference in RVD capacity was shown to be due to the very low K^+ permeability in tonsil lymphocytes after hypotonic shock, whereas the Cl^- permeability was similar to that observed in peripheral blood lymphocytes [20].

In a previous single-channel study, we have identified in human keratinocytes an outwardly rectifying Cl^- channel, characterized by an inactivation process at large positive membrane potentials [5]. Such properties suggest that this type of Cl^- channel accounts for the volume-sensitive currents described here by the use of whole-cell recordings. Similar conclusions have been reported also for other epithelial cells [16,18].

Up to recent years, it was believed that ubiquitous

rectifying Cl^- channels had a role in cAMP- and Ca^{2+}-activated Cl^- secretion in epithelial cells [21]. It is now clear that at least three distinct Cl^- conductances exist, each with different conductive and kinetic properties, and each of which responds to cAMP, Ca^{2+} and cell volume, respectively [22]. Under isotonic conditions, elevation of cytosolic cAMP and Ca^{2+} failed to induce any stimulation of Cl^- efflux in human keratinocytes [3]. It can therefore be concluded that these cells, at least under our culture conditions, express only volume-sensitive Cl^- channels.

It is well established that keratinocyte is a cell type whose stage of terminal differentiation is closely related to its size [23]. The keratinocyte enlarges progressively moving from the basal to the granular layer. Enlargement of keratinocyte may be important for its differentiation function and may even be a source of signals controlling the differentation program [23]. In this respect, it might be of interest to verify in a future study whether the lack of RVD described in this paper is related to the need of keratinocytes to attain a large size during terminal differentiation.

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