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ACTIVATION BY *N*-ETHYLMALEIMIDE OF A Cl^--DEPENDENT K^+ FLUX IN ISOLATED TROUT HEPATOCYTES

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Summary

Isolated trout hepatocytes when swollen in hypotonic medium undergo a regulatory volume decrease (RVD), which occurs *via* KCl loss. The system shows characteristics similar to those of the transporter described in red cells. This led us to investigate, in trout hepatocytes, the effect of another signal known to activate this flux in red cells, i.e. treatment with the sulphhydryl-group reagent *N*-ethylmaleimide (NEM). NEM treatment resulted in a striking increase in ouabain-resistant K^+ uptake measured by an isotope pulse uptake technique. The time course of the response to NEM was similar to that obtained with a hypotonic shock, indicating that the effect of NEM was immediate and transient. The NEM-stimulated K^+ influx demonstrated the same anion sensitivity as the volume-induced K^+ influx, i.e. a specific requirement for Br^- or Cl^-. Efflux experiments showed that NEM treatment produced a stimulation of both K^+ and Cl^- effluxes leading to a substantial net loss (10 %) of cellular KCl, as confirmed by analysis of ionic contents. This KCl loss is consistent with the rapid cell shrinkage observed after addition of NEM. The Cl^--dependent K^+ influx was found to be independent of external Na^+; in addition, NEM had no effect on Na^+ content, indicating that Na^+ is not implicated in this process. The effect of loop diuretics was tested on the NEM-stimulated K^+ influx. As observed for the volume-induced K^+ flux, a high concentration of furosemide (10^{-3} mol l^{-1}) is required for full inhibition of this flux; no effect was obtained with bumetanide (10^{-4} mol l^{-1}). Consequently, NEM appears to activate a KCl cotransport similar to the one induced in hypotonically swollen cells. Finally, the combination of the two treatments, NEM and hypotonic shock, was found to increase the K^+ fluxes even further, suggesting additivity of the two stimuli by mutual positive interaction.

Introduction

Fish hepatocytes have become a popular model for the study of various

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physiological functions or pathological states. We have recently proposed these cells as a model for volume regulation under anisosmotic conditions (Bianchini *et al.* 1988).

Hepatocytes of the rainbow trout respond to a hypo-osmotic challenge by an immediate cell swelling followed after a few minutes by a regulatory volume decrease (RVD) brought about by a simultaneous, ouabain-insensitive loss of K^+ and Cl^-. This RVD does not involve a net movement of Na^+ and has none of the characteristics of a $Na^+/K^+/Cl^-$ cotransport. The K^+ regulatory loss is dependent on the presence of Cl^- and does not possess most of the properties described in other systems for potassium channels. It is not affected by pharmacological compounds acting on the voltage- or calcium-dependent K^+ pathways. It is not sensitive to furosemide and bumetanide, which are extensively used to define Cl^--dependent transports of K^+ and Na^+. In addition, measurements of intracellular messengers show that the most common nucleotides, cyclic AMP and cyclic GMP, are not involved during this process.

These results led us to consider the responsiveness of trout hepatocytes to *N*-ethylmaleimide (NEM), the SH-reacting compound that activates Cl^--dependent K^+ fluxes under isosmotic conditions in red blood cells of many species (Lauf and Theg, 1980; Ellory *et al.* 1982; Bauer and Lauf, 1983; Wiater and Dunham, 1983; Lauf *et al.* 1984) and ascites tumour cells (Kramhoft *et al.* 1986). A comparison of volume- and NEM-induced K^+ loss should provide some insight into the mechanism by which KCl loss takes place across the cell membrane of trout hepatocytes. The present work is the first report related to this problem in fish liver cells.

Materials and methods

Isolated hepatocytes were obtained from rainbow trout *Salmo gairdneri* liver using the collagenase method described previously (Bianchini *et al.* 1988).

Media

The control medium, H-MEM, contained (mmol l^{-1}): NaCl 136.8, KCl 5.4, $CaCl_2$ 1.3, KH_2PO_4 0.44, $MgSO_4$ 0.81, Na_2HPO_4 0.34, Hepes 10 and (mg l^{-1}) vitamins 8.1, glucose 1000, phenol red 10, amino acids 566, glutamine 292; pH 7.4. For Cl^--free H-MEM, $Ca(NO_3)_2$ and K_2SO_4 were substituted, respectively, for $CaCl_2$ and KCl; NaCl was replaced by NaBr or $NaCH_3SO_4$. In Na^+-free medium, *N*-methyl-D-glucamine-H-MEM was prepared by isotonic replacement of NaCl by NMDG-Cl. The hypotonic medium was obtained by a one-third dilution of H-MEM, by adding a 6 mmol l^{-1} KCl solution buffered with 10 mmol l^{-1} Hepes, pH 7.4 (dilution solution). NEM (*N*-ethylmaleimide) was prepared fresh before each experiment and added to the experimental medium at the beginning of the experiment. All media contained 1 mmol l^{-1} ouabain (unless otherwise stated).

Chemicals

Ouabain (strophanthidin G) was purchased from Boehringer. NEM was purchased from Sigma. Bumetanide (Burinex) and furosemide (Lasilix) are pharmaceutical products obtained from Leo and Hoechst Laboratories, respectively. Silicone oils 550 (density 1.068 g cm^{-3}) and 556 (density 0.980 g cm^{-3}) were a gift from Dow Corning.

Radioisotopes

^{36}Cl (0.43 MBq mg^{-1}) was obtained from the Radiochemical Centre, Amersham, United Kingdom; ^{86}Rb (0.04–0.4 MBq mg^{-1}) and $^{3}H_2O$ (5.5 MBq ml^{-1}) from the Commissariat à l'Energie Atomique, Saclay, France; and [^{14}C]dextran (0.1 MBq mg^{-1}) from New England Nuclear, Boston, USA.

Unidirectional fluxes

Uptake

Unidirectional K^+ influx experiments were performed by using ^{86}Rb as tracer, according to the previously reported 'pulse' method (Bianchini *et al.* 1988). The experiment was started by the addition of cells to the incubation medium; ^{86}Rb uptake was then measured over 1 min at timed intervals. In ion substitution experiments, cells were washed twice with the substitution medium and resuspended in this medium just before the start of the experiment.

Efflux

In ^{86}Rb efflux experiments, hepatocytes were loaded with radioactive isotope during the 40 min equilibration period, then washed three times with H-MEM to remove external radioactivity and finally resuspended in the same medium. At set times, 200 μl samples were collected in triplicate and treated as described previously (Bianchini *et al.* 1988). The amount of radioactivity remaining in cells was determined and expressed per milligram of protein.

As chloride exchange was shown to be much faster than Na^+ and K^+ movements, we used a silicone oil technique for ^{36}Cl efflux measurements. Hepatocytes were loaded with radioactive isotope during the 40-min equilibration period, washed three times with Cl^--free H-MEM and resuspended in H-MEM. At various times, 200 μl samples were collected and each was added to 0.5 ml of silicone oil (appropriate mixture of oil 550 and oil 556) in conical microfuge Eppendorf tubes. The cells were sedimented by a 5 s centrifugation at 10 000 *g*, the supernatants were removed and the pellets were resuspended in 1 ml of distilled water for the counting of radioactivity.

Cell water measurements

Cell water content was determined by using a double labelling procedure as

previously described (Bianchini *et al.* 1988). ^3H_2O and [^{14}C]dextran were added to the medium to label the total water volume and extracellular space, respectively.

Radioactivity measurements

All isotopes were counted in a Kontron Betamatic scintillation spectrometer, ^{86}Rb by the Cerenkov radiation in aqueous samples, and ^{36}Cl and ^{3}H after addition of scintillant (Aqua Luma from Kontron).

Na^+, K^+ and Cl^- contents

Ionic cellular contents were determined on individual samples after cell sonication of the counted sample for ^{86}Rb flux measurements or of a duplicate for ^{36}Cl flux measurements. Na^+ and K^+ were measured with an Eppendorf flame photometer and Cl^- by the Technicon colorimetric method (after protein precipitation by perchloric acid, 2% final concentration) with a Shimadzu UV 2100 spectrophotometer.

Protein

Protein was assayed by the method of Lowry *et al.* (1951) adapted for a Technicon analyser, on part of each sample after counting and ultrasonic homogenization.

Measurements

The figures are representative examples of experiments repeated at least four times. In the other cases the number of experiments is indicated in the text.

Results

Activation of K^+ influx by NEM

Hepatocytes suspended in isosmotic H-MEM containing ouabain were exposed to 1 mmol l^{-1} NEM. Although RVD results in a net loss of K^+, hypotonic shock enhances ouabain-insensitive ^{86}Rb uptake and efflux, indicating an increase in K^+ permeability. So the initial rate of K^+ uptake was then measured over 1 min at 2-min intervals ('pulse' technique). Fig. 1 shows the time course of the response to NEM treatment in comparison to the response to a hypotonic shock (in one-third dilution of the isotonic medium). NEM treatment resulted in a striking (nearly twofold) increase in basal, ouabain-resistant K^+ influx, similar to that obtained in hypotonic medium. The maximal effect was achieved within 4–6 min.

Changes in cellular ionic contents induced by NEM

Time-course analysis of changes in cellular ionic contents indicates that after NEM exposure (1 mmol l^{-1}, 20 min) cell [K^+] fell to about 70% of its initial value, compared with 80% in cells submitted to a hypotonic shock (Fig. 2). A similar decrease was observed for intracellular Cl^- in NEM-treated cells. After 60 min of

NEM exposure, cellular K^+ and Cl^- contents decreased by similar amounts, 88 ± 27 nequiv μg^{-1} protein for K^+ and 70 ± 12 nequiv mg^{-1} protein for Cl^- ($N=5$), in comparison to control values measured under the same conditions in the absence of NEM. It is worth noting that under the same conditions neither the NEM treatment nor the hypotonic shock had any effect on Na^+ content.

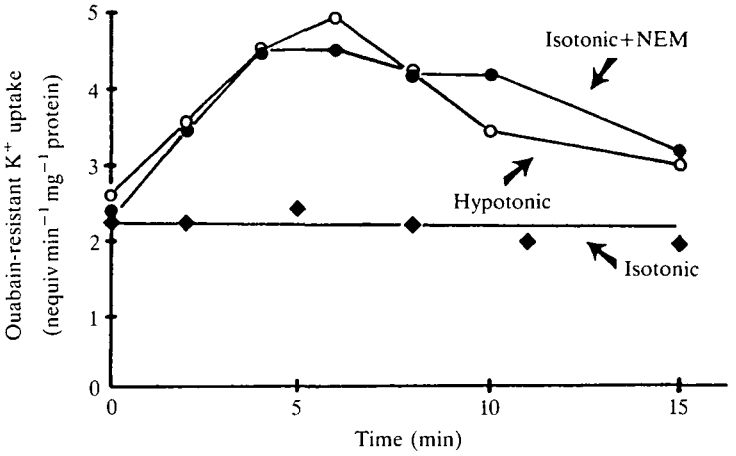

Fig. 1. Comparison of the effect of 1 mmol l^{-1} NEM (●) or hypotonic shock (○) on ouabain-resistant K^+ uptake measured by 1 min pulse experiments; (◆) control values in isotonic medium. One experiment. The data in this and subsequent figures are typical examples of experiments that we repeated at least four times.

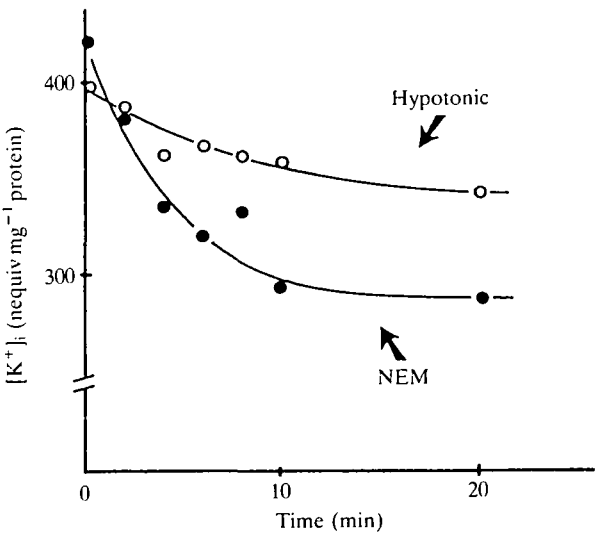

Fig. 2. Time course of changes in K^+ cellular content: effect of 1 mmol l^{-1} NEM (●) or hypotonic shock (○). *One experiment.*

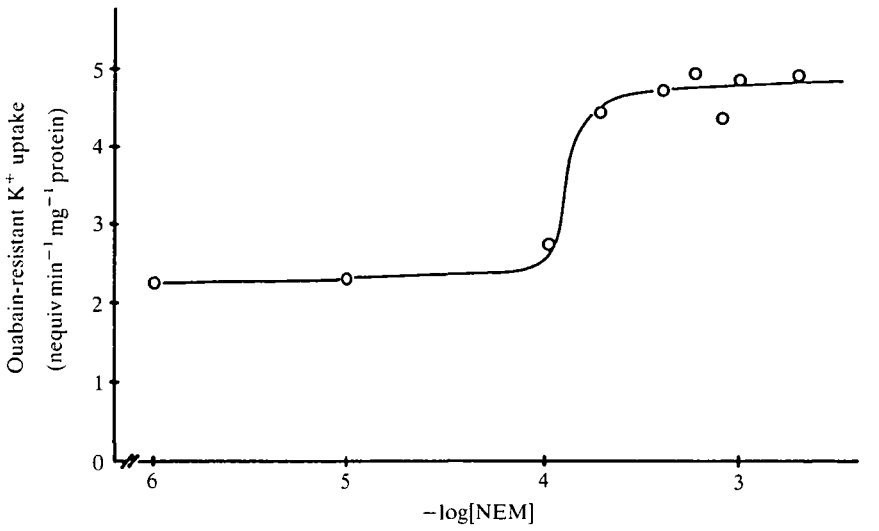

Fig. 3. Effect of increasing concentrations of NEM (in mol l^{-1}) on ouabain-resistant K^+ uptake measured by 1 min pulse experiments after 6 min of exposure. One experiment.

Dose–response relationship of NEM on K^+ influx

Fig. 3 shows the effect of increasing concentrations of NEM on ouabain-resistant K^+ uptake. The flux was measured over 1 min after 6 min of exposure to the drug (the time required for maximal effect). No significant effect was seen until 0.2 mmol l^{-1} NEM was added to the medium. The maximum flux occurred at a concentration a little higher than this. Increasing the NEM concentration to 2 mmol l^{-1} failed to stimulate the ouabain-resistant K^+ uptake further.

Effect of NEM on K^+ efflux

To extend the investigation of the influence of NEM on K^+ transport, ouabain-resistant ^{86}Rb efflux was measured in the presence of NEM. On untreated cells, 1 mmol l^{-1} NEM produced a strong stimulation of ^{86}Rb efflux with a $T_{1/2}$ of 38±1 min (N=3). Moreover, the half-time of this effect was similar to that measured in hypotonic medium (34 min, Bianchini *et al.* 1988), compared to 128 min in H-MEM conditions (Bianchini *et al.* 1990).

Effect of NEM on cell water content

Stimulation of K^+ efflux is consistent with the net K^+ loss (30 %) shown in Fig. 2. Since a movement of 'osmotically coupled water' must accompany the cation loss, NEM treatment was expected to result in cell shrinkage. We therefore investigated the effects of NEM on cell water content. The values in Fig. 4 are given relative to those measured at the same time under control conditions. Addition of NEM to hepatocytes suspended in isotonic medium induced cell

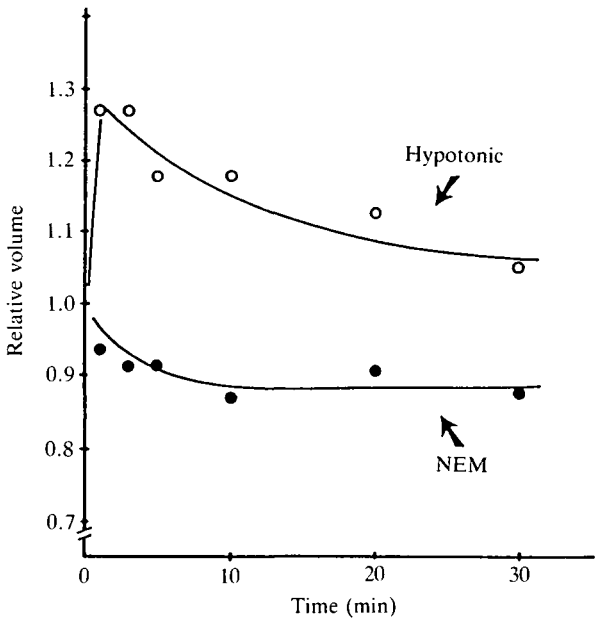

Fig. 4. Comparison of effect of 1 mmol l^{-1} NEM (●) or hypotonic shock (○) on relative changes in cell water content. Lines fitted by eye. One experiment.

shrinkage; cell water content was reduced by 10%, leading to a new steady-state volume 10 min after addition of NEM. In comparison, cells submitted to a hypotonic shock displayed the response already observed (Bianchini *et al.* 1988), i.e. a rapid increase in cell water content that reached a maximum value after 3 min, followed by a shrinkage phase allowing a return to the control value within 30 min.

Anion dependence of the NEM-induced flux

The anion dependence of the NEM-induced K^+ influx was measured in Cl^--free media. Fig. 5 shows the effect of replacing external Cl^- with methyl sulphate. For this experiment, K^+ influx was measured over 1 min in a Cl^- or methyl sulphate medium, in the absence of NEM or 0, 6, 10 and 15 min after addition of NEM. These data demonstrate that the substitution of Cl^- abolishes the stimulation of K^+ uptake induced by NEM treatment. The same result was obtained when Cl^- was replaced with gluconate. In contrast, the NEM-induced K^+ pathway remained unaffected when Br^- was substituted for Cl^- (data not shown). The NEM-stimulated K^+ influx also revealed the same anion sensitivity as the volume-induced K^+ influx, i.e. a specific requirement for Br^- or Cl^-.

Effect of loop diuretics

This requirement for Cl^- raised the possibility that the response of hepatocytes

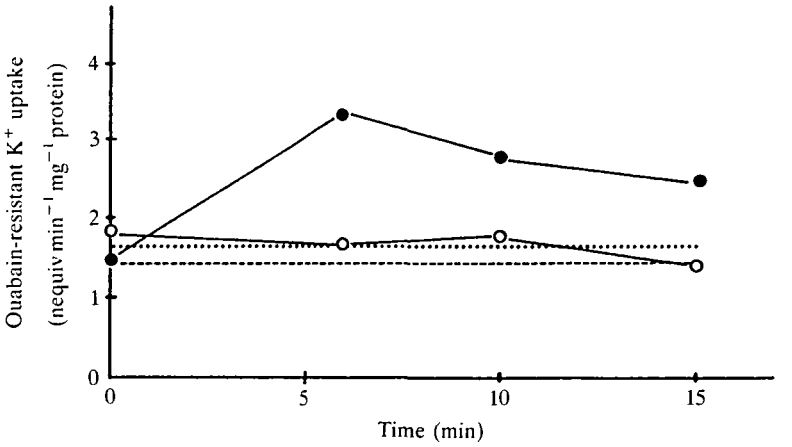

Fig. 5. Ouabain-resistant K^+ uptake measured by 1 min pulse experiments in H-MEM+NEM (●) or methyl sulphate-H-MEM+NEM (○). Dotted lines, control values in H-MEM; dotted line, control values in methyl sulphate-H-MEM. One experiment.

to NEM treatment could be mediated by KCl cotranport. We therefore tested the sensitivity of the pathway to the loop diuretics bumetanide and furosemide, which are known to be potent inhibitors of coupled transport pathways in various cell types. The effect of the two loop diuretics on both volume- and NEM-induced K^+ influx measured over 1 min after 6 min of exposure to NEM or to hypotonic medium was similar in both cases: 0.1 mmol l^{-1} bumetanide was totally ineffective, whereas 0.1 mmol l^{-1} furosemide caused a slight inhibition of ^{86}Rb uptake (17%) and a tenfold higher concentration of furosemide (1 mmol l^{-1}) was required for full inhibition.

Effect of NEM on Cl^- efflux

We therefore considered the effects of NEM on ^{36}Cl efflux. In Fig. 6 we compared the efflux rates of ^{36}Cl in the absence and presence of NEM as a function of time. NEM treatment caused a rapid increase of Cl^- efflux, as indicated by the half-times measured under both conditions: $T_{1/2}=6.7\pm0.2$ min in NEM medium, compared with 9.4 ± 0.4 min under control conditions ($N=4$).

Effect of replacing external Na^+

We investigated a possible dependence of NEM-stimulated K^+ flux on external Na^+. As shown in Fig. 7, replacement of Na^+ with the organic univalent cation *N*-methyl-D-glucamine (NMDG) produced no inhibition of the NEM-induced K^+ influx, indicating that this K^+ pathway is not dependent on external sodium.

Are the stimuli additive?

The above results show that both hypotonic shock and NEM activate a Cl^--

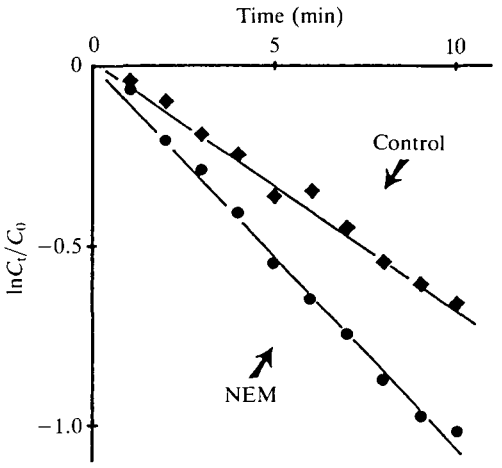

Fig. 6. Release of intracellular ^{36}Cl in H-MEM (●) or H-MEM+NEM (◆). C_t/C_0, $[Cl^-]_i$ at time $t/[Cl^-]_i$ at time zero. One experiment.

dependent K^+ flux. To determine whether both stimuli activate the same transport mechanism, we measured K^+ influx in hypotonic medium in the presence of NEM and compared it to K^+ influx stimulated either by NEM or by a hypotonic shock. As shown in Fig. 8, both stimuli given together produced a stimulation much greater than that caused by either of them separately, indicating additivity of the two stimuli. K^+ influx measured after 6 min (time required for maximal effect) was 5.63 ± 0.54 nequiv mg^{-1} protein ($N=12$) in the presence of NEM in hypotonic medium, significantly different from K^+ influx stimulated by NEM alone (3.45 ± 0.34 nequiv mg^{-1} protein, $N=12$, $P<0.01$) or by hypotonic shock

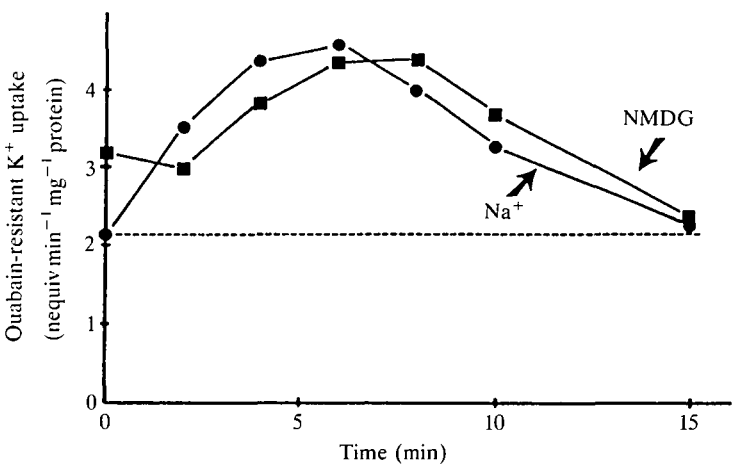

Fig. 7. Ouabain-resistant K^+ uptake measured by 1 min pulse experiments in H-MEM+NEM (●) or NMDG-H-MEM+NEM (■). Dashed line, control values in H-MEM. One experiment.

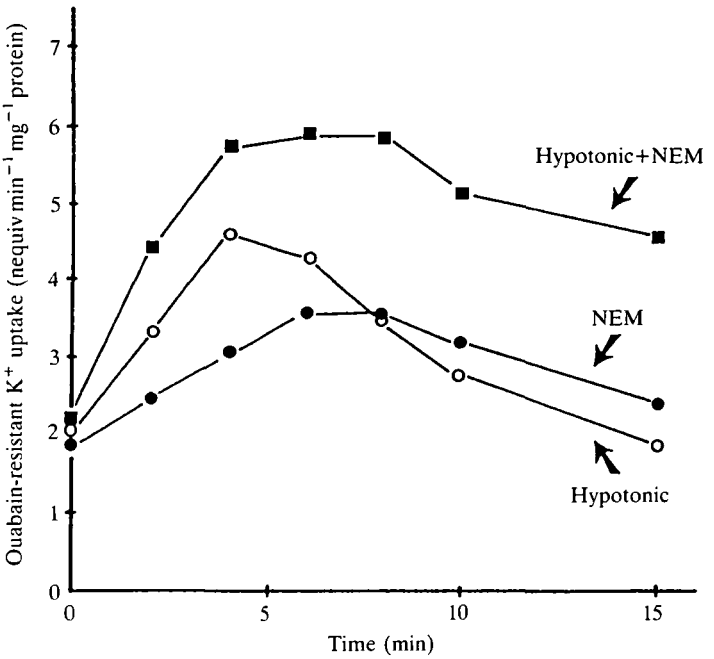

Fig. 8. Effect of 0.2 mmol l^{-1} NEM (●), hypotonic shock (○) or 0.2 mmol l^{-1} NEM+hypotonic shock (■) on ouabain-resistant K^+ uptake measured by 1 min pulse experiments. One experiment.

(4.22±0.33 nequiv mg^{-1} protein, $N=12$, $0.05<P<0.02$). Moreover, whereas the effect of NEM is transitory, the two treatments combined produce a persistent stimulation for periods of up to 15 min. Return to the control value was observed within 30 min (data not shown).

Discussion

The present report extends our previous findings, which showed that trout hepatocytes exhibit volume regulation in response to a hypo-osmotic shock by a simultaneous and equivalent release of potassium and chloride ions (Bianchini *et al.* 1988). This KCl loss, which induces a return of cell volume to its original level within 20 min, has been characterized in our previous work by means of ion kinetics in association with ion replacement and pharmacological tests. The K^+ fluxes measured during RVD were found to be Cl^--dependent and insensitive to current modulators of Ca^{2+}-dependent and voltage-dependent K^+ channel inhibitors. They were not Na^+-dependent, were not inhibited by bumetanide and were sensitive only to high concentrations of furosemide (approx. 10^{-3} mol l^{-1}). These observations, obtained in buffered medium in the absence of added bicarbonate ions, led us to conclude that K^+ and Cl^- were extruded in the form of a KCl electroneutral cotransport.

This mechanism is found in other systems, such as fish red cells (Cala, 1977; Lauf, 1982; Bourne and Cossins, 1984), duck red cells (Kregenow and Caryk, 1979) and across the basolateral membrane of *Necturus* gallbladder cells (Larson and Spring, 1984). It is sometimes described as a slow process, since the regulatory volume decrease (RVD) associated with this cotransport in duck red cells requires about 90 min to be completed. In *Amphiuma* red cells, RVD occurs within minutes but is achieved by a K^+/H^+ antiport operating in parallel with a Cl^-/HCO_3^- exchanger (Cala, 1983). In lymphocytes, several lines of evidence indicate that cell swelling in hypotonic media is associated with independent conductive movements of K^+ and Cl^- (Grinstein *et al.* 1982, 1984). In trout hepatocytes, RVD is fast and there is no evidence for the presence of conductive pathways in our experimental conditions.

A feature common to cells losing K^+ in response to cell swelling is that treatment with NEM produces a similar effect on potassium in cells maintained under isosmotic conditions. The original observation was made on duck red cells (Tosteson and Johnson, 1957). Subsequently, it was also found in red cells of sheep (Ellory *et al.* 1982; Bauer and Lauf, 1983; Lauf, 1983*a*,*b*, 1985), goat (Lauf and Theg, 1980) and man (Wiater and Dunham, 1983; Ellory *et al.* 1985; Lauf *et al.* 1984) as well as in Ehrlich ascites tumour cells (Kramhoft *et al.* 1986).

In ghosts of human red cells, NEM does not increase the K^+ flux (O'Neill, 1989), although it stimulates it fourfold in intact cells. However, Smith and Lauf (1985) observed that treatment of ghosts with NEM may activate a K^+ flux that is different from the flux stimulated by this drug in intact cells.

NEM is a sulphhydryl-alkylating agent which is believed to react with the sulphhydryl groups of the membrane proteins rather than with cell metabolism. In all cases, the agent induces a ouabain-resistant, Cl^--dependent K^+ flux. In other words, the effect of NEM is to create or to reveal a KCl electroneutral cotransport, e.g. in Ehrlich cells. In trout hepatocytes, since this cotransport takes place in cells submitted to hypotonic shock, it was interesting to determine whether the transport pathway is the same under the two conditions (chemically induced and volume-induced).

A striking characteristic is the time course of the NEM effect on potassium flux, which is essentially similar to that of hypotonic shock (Fig. 1). Therefore, the drug acts immediately on trout hepatocytes. This also holds for cell volume (Fig. 4). This is at variance with most other systems in which the cells are usually preincubated for periods ranging between 15 min and 4 h prior to flux measurements (Berkowitz *et al.* 1987; O'Neill, 1989).

In human young red cells (Berkowitz *et al.* 1987), at the end of 4 h of preincubation with NEM, cell $[K^+]$ dropped by 50 % of its initial value and cell water content by about 20 %. In *Amphiuma* red cells, after 1 h of exposure, cell water was reduced by about 20 % in the presence of 8 mmol l^{-1} NEM (Adorante and Cala, 1987). For comparison, in our experiments, the NEM-induced potassium flux had a maximum rate after 4 min and the cell volume dropped immediately to reach a stable plateau after 10 min. Nevertheless, in Ehrlich cells

(Kramhoft *et al.* 1986) and in human red cells (Berkowitz *et al.* 1987), short-term effects of NEM have been shown. In both cases, a significant change in K^+ efflux or in volume was obtained after a 1-min exposure only. These results indicate that long-term exposure to NEM is not a pre-requisite for the production of KCl loss and cell shrinkage.

NEM is generally used at a concentration of $1\,mmol\,l^{-1}$ or more. However, in trout hepatocytes (Fig. 2) and in human red cells (Berkowitz *et al.* 1987), a clear effect is obtained at $0.2\,mmol\,l^{-1}$, and the dose–response relationship described by a sigmoid curve gives a maximum a little above this value. The sigmoid dose–response curve may be interpreted as indicating an interaction of NEM at multiple sites of heterogeneous affinity. In principle, it should be interesting to study the kinetics of NEM binding to cell membranes by using a radiolabelled compound. However, this may prove difficult to interpret because the sulphhydryl groups on the cell membrane are likely to be present in large excess in comparison to the KCl cotransport sites, and there would be no correspondence between the two reactions.

The results of ion substitutions show that trout hepatocytes exhibit a specificity similar to that demonstrated in other systems: Cl^- may be replaced by Br^- without affecting the rate of K^+ influx. Similarly, removal of Na^+ and its replacement with the impermeant cation NMDG (Fig. 7) does not alter this flux. The effect of NEM is therefore confined to the ouabain-resistant cotransport of KCl. The NEM-induced decreases in cellular K^+ and Cl^- contents suggests that the KCl transporter has a stoichiometry of 1:1.

The essential questions remain whether the NEM-induced and the volume-induced pathways of KCl exit are identical and what mechanism triggers their activation. There seems to be a general agreement that the KCl pathway pre-exists in the cell membrane and is not created *de novo* in either situation. Our observations that the NEM, as well as the volume, stimulatory effect takes place in a very short time (3-min exposure) is in favour of this interpretation.

From his observations on low-potassium (LK) sheep red cells, Lauf (1984) provided a two-domain model to explain the functional heterogeneity and immunological homogeneity of KCl fluxes while hypothesizing that the KCl pathway is common to both cases. In addition, Lauf (1985) proposed that all three stimuli of KCl flux, i.e. cell swelling, NEM and calcium removal (by A23187 associated with EGTA), affect the same transport system through which the ion flux is activated. An important difference in the kinetics of their respective effects is that NEM-induced stimulation of KCl transport is irreversible. For example, in human red blood cells, K^+ efflux in Cl^--containing medium was several times the control value after 1 h of incubation in the presence of NEM (Lauf *et al.* 1984). Similarly, in LK sheep red cells, the time course of K^+ influx in the presence of low concentrations of NEM indicates prolonged stimulation (up to 120 min at least) (Logue *et al.* 1983). In our experiments on trout hepatocytes, stimulation of K^+ influx by NEM and by hypotonic shock follow the same pattern. Thus, the effect of NEM appears to be transitory (Figs 1, 5, 7, 8). When the two treatments are

combined, the stimuli are obviously additive (Fig. 8). In addition, the effect of NEM largely persists after the initial period of 15 min. The concentration of NEM used in this experiment ($0.2\,mmol\,l^{-1}$) is sufficient to produce a maximal action (see Fig. 3). This result therefore indicates either that the target pathways are different for NEM and for volume increase or that there is a mutual positive interaction between them at the same pathway. The first hypothesis is unlikely because trout hepatocytes do not have K^+ conductive pathway(s) other than the KCl cotransport described above. The second hypothesis may be considered as follows. Following hypotonic swelling, physical constraints on the membrane affect the NEM-sensitive sites located at the inner face during shrinkage. Conformational change, or better access to NEM, or relationships with some intermediate component or messenger may result in a sustained effect on the KCl cotransport site located nearby.

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