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INACTIVATION OF REGULATORY VOLUME DECREASE IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES BY N-ETHYLMALEIMIDE

J. Bauer and P. K. Lauf

Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710, USA

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The sulfhydryl group reagent N-ethylmaleimide was found to inhibit in a dose dependent manner regulatory volume decrease of human peripheral lymphocytes swollen in buffered hyposmotic NaCl media. In hyposmotic KCl media NEM treated lymphocytes prevented an additional secondary swelling seen in control lymphocytes. The data suggest that N-ethylmaleimide acts on ion transport mechanisms involved in volume regulatory changes. This effect contrasts with the stimulation by N-ethylmaleimide of apparently volume sensitive K/Cl fluxes in certain mamalian red cells.

A fraction of the passive, ouabain-insensitive K^+ movements across mammalian red cell membranes occurs not by electrodiffusion but apparently by Cl^--dependent transport systems exhibiting kinetically carrier characteristics. Exposure of low K^+ sheep (1), human (2) and pig (3) red cells to the sulfhydryl group (SH) reagent N-ethylmaleimide (NEM) has been shown to further activate Cl^- dependent K^+ fluxes suggesting the functional involvement of thiol groups which at least in the sheep appear to be metabolically dependent (4). At present it is not yet clear whether or not the thiol-stimulated K/Cl flux is related to K^+ transport induced by hyposmotic shock in red cells of sheep (5,6), human (7), birds (8,9) and certain fish (10), and to K^+ fluxes causing regulatory volume decrease (RVD,8) in Ehrlich ascites tumor cells (11) and lymphocytes (12). In contrast to red cells, the latter two cell types achieve RVD by probably Ca^{++} sensitive electrochemical translocation of

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K^+ and Cl^- via separate channels (12-14). On the other hand, amphiuma red cells seem to take up an intermediate position as during RVD a K^+/H^+ exchange is activated that is paralleled by Cl^-/HCO_3^- exchange (15). It is not known whether NEM exerts similar effects on passive K^+ movements in nucleated cells as compared to erythrocytes.

In the present study on human peripheral blood lymphocytes (PBL) we have chosen to study the effect of NEM on RVD rather than on K^+ flux as the latter technique requires large scale cell preparations. We monitored RVD of PBL with a flow cytometer (METRICELL), a technique well established for lymphocytes (12,13) and Enrlich ascites tumor cells (11). We found that NEM inhibited RVD in PBL in a dose dependent manner and at concentrations where K^+ flux stimulation was observed in red cells (16). As also inhibitory effects of NEM on K^+ fluxes in LK sheep red cells have been reported (17,18) it is possible that electroneutral K^+/Cl^- cotransport in these cells differs biochemically from independent K^+ and Cl^- movements effecting RVD in PBL and Enrlich ascites tumor cells by the presence of activating SH groups. A preliminary report of this work has been presented elsewhere (19).

MATERIAL AND METHODS

Twenty milliliters of heparinized blood from healthy adults were diluted threefold with washing medium (composition, see below), layered over 4 ml Lymphocytes Separation Medium (LM, Litton Bionetics, Kenington MD) in four 20 ml centrifuge tubes and centrifuged at 365 g for 20 minutes. The cell layers at the interfaces containing lymphocytes, monocytes and platelets were collected and washed once at 160 g for 10 minutes in 25 ml isosmotic (300 mOsm) washing medium containing (mM): 144.6 Na^+, 5.4K^+, 1.3 Ca^{++}, 0.9 Mg^{++}, 0.34 $HPO4^{--}$, 1.3 $H2PO4^-$, 14.3 $HCO3^-$, 135 Cl^- and 5.6 glucose, pH 7.4. The cells were resuspended in 50 µl washing medium and used within 2 hours.

The volume changes of PBL in hyposmotic media were induced by diluting 7.5 µl of the concentrated PBL suspen-

sion ($3x10^8$ cells/ml) into 750 µl of a 180 mOsmol buffer containing 5 mM phosphate pH 7.4 and 90 mM of either NaCl or KCl and either N-ethylmaleimide (NEM, Sigma, St.Louis, MO) or furosemide (5-sulfamoyl-N-furfuryl anthranilic acid, gift of Hoechst-Roussel, Sommerville, N.J.) at concentrations indicated in the figure legends. The hyposmotic dilution was carried out in 750 µl Eppendorf microcentrifuge tubes with a small hole at the bottom to be placed over the orifice of the Metricell in order to achieve a constant particle stream from the test tube through the sizing Coulter orifice (20).

Volume distribution curves of PBL were measured by electrical sizing of the cell volume pulses with a Metricell flow cytometer at 0.49 mA aperture current and a flow rate of 800-1200 cells/sec (21) at 25°C. The cylindrical orifice had a diameter of 80 µm and a length of 60 µm, and the cell flow was hydrodynamically focused through the center of the orifice. The pulse data were classified as a one dimensional histogram in a multichannel analyzer. The mean values of the curves were evaluated by computer programs described earlier (22).

RESULTS AND DISCUSSION

The application of flow cytometric techniques to estimate relatively fast changes in cellular volume caused by movements of solute and water has been recently successfully demonstrated for PBLS (12,13) and Ehrlich ascites tumor cells (11), and for maturational volume changes of sheep reticulocytes (23). Fig.1 shows the volume changes of PBLS exposed to various hyposmotic media in the presence and absence of 2 mM NEM. Relative cell volumes are given as numbers of channels recorded in the multichannel analyzer representing the peaks of the volume distribution curves as plotted against time. The mean cell volume of PBLs freshly suspended in isosmotic NaCl media was usually equivalent to channel number 63 and had reached a stable value equivalent to channel 58 after 8 min of observation.

In contrast, and consistent with the observation of others (12-14), PBLs exposed to hyposmotic (180 mOsmol) salt media immediately swelled about 1.33 fold (channel 82) with subsequent volume changes dependent on the cations present.

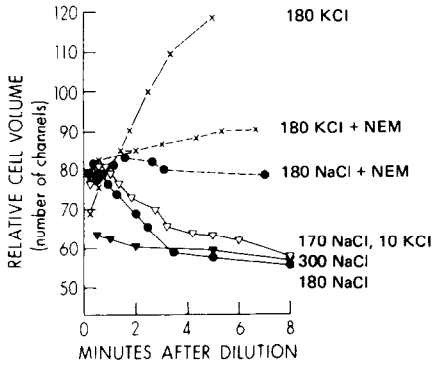

Fig. 1: RVD of human lymphocytes in hyposmotic media. Effect of K^+ and Na^+, and of N-ethylmaleimide (NEM). Controls were maintained in 300 mOsm NaCl (filled triangles) while experimental cells were diluted with 180 mOsm NaCl (filled circles, solid line), 180 mOsm KCl (x, solid line), 170 mOsm NaCl + 10 mOsm KCl (open triangles), or 180 mOsm NCl (filled circles, broken line) or KCl (x, broken line) with 2mM NEM.

Thus in hyposmotic NaCl media PBLS returned to their normal volume (channel 58) within less than 4 minutes, while in 180 mOsmol KCl medium further swelling occurred to a volume equivalent to channel number 120 after 5 min. It may be assumed that the second swelling in KCl media was due to entrance of KCl and water which is consistent with the reported hyperpolarization of the membrane potential upon hyposmotic shock (12,13).

Furosemide known to inhibit Cl^- dependent passive K^+ transport, did not have any significant effect on volume regulatory decrease of hyposmotically swollen lymphocytes (data not shown). Furthermore, replacement of extracellular Cl^- by NO_3^- also had no effect on the RVD indicating that the process of RVD is Cl^- independent, a finding confirming other observations (12,13).

Closer inspection of the early portion of the curves of fig.1 shows that the initial cell swelling was completed by

24 (± 3) seconds and that the cells maintained their new volume for about 15 seconds before shrinking. Assuming a lymphocyte volume of 260 μ^3 (Hempling et al. 1978) a mean water influx of .81 (±.09 n=2) μ^3/min was estimated for the primary swelling phase.

We have previously shown that NEM acts virtually immediately on the Cl^- dependent K^+ transport in LK sheep red cells (1-3). In the experiments involving NEM and shown in Figures 1 and 2, the chemical was added at t=0 (with the buffer) to the cell suspension. Under these conditions, 2mM NEM did not visibly interfere with cell swelling in hyposmotic media. However, both, volume reduction in 180 mOsmol NaCl as well as the further secondary volume increase in 180 mOsmol KCl were inhibited in the presence of 2 mM NEM (Fig.1). This finding suggests that NEM prevents K^+ movements known to be associated with RVD in these (12-14) and other cells (8,9,10,11). The concentration dependence of the NEM inhibition of RVD in hyposmotic NaCl media is shown in Fig.2. Apparently 20 µM NEM did not significantly affect

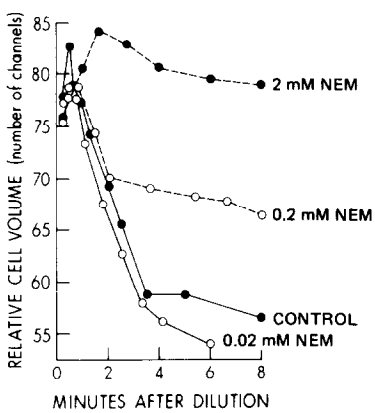

Fig. 2: RVD of human lymphocytes in 180 mOsm NaCl with varying NEM concentrations as indicated. The original volume of the lymphocytes in isotonic medium was close to channel 60 (see also Figure 1).

the rate and final level of RVD while 200 μM NEM produced about 50%, and 2 mM NEM full inhibition of RVD.

Based on previous work on red cells (1-4,17) we consider that NEM primarily reacts with membrane SH rather than with NH_2 groups. While in sheep red cells this irreversible SH-NEM adduct formation leads to a concentration (18) and pH (17) dependent small inhibition, but mainly stimulation of Cl^- dependent K^+ fluxes, the NEM effect on RVD of PBLs may involve separately K^+ and/or Cl^- channels. It is conceivable that NEM may reduce K^+ movements or, equally effective, prevent the swelling-induced opening of the Cl^- channel (12,13). Aside from these direct effects on membrane permeability it is not unlikely that NEM affects the latter indirectly via metabolic perturbations, a possibility underscored by the recent observation of metabolic dependence of the NEM induced K^+/Cl^- flux in LK sheep red cells (4). Whatever the mechanism of NEM action, the data suggest the crucial role of SH groups in regulation of cell volume and ion transport (25).

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