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Na fluxes in human mononuclear leucocytes

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Summary. Unidirectional ^{22}Na fluxes were investigated in human peripheral mononuclear leucocytes. Ouabain inhibited about 60% of Na efflux and the addition of bumetanide further reduced Na efflux rate by about 45%, suggesting the presence of a transport pathway capable of extruding Na against its gradient. Prostaglandins E_1 and E_2 and exogenous cAMP were found to be potent inhibitors of the bumetanide-sensitive Na efflux without affecting the ouabain-sensitive or the ouabain and bumetanide resistant Na effluxes.
Key words. Na transport; lymphocytes; PGE.

The maintenance of cell Na and K levels is thought to be dependent on a balance between an active transport system that creates and maintains gradients and passive fluxes that dissipate these gradients. In most cells, the ouabain-sensitive Na, K pump is a major determinant of Na and K gradients across cell membranes. Recently, systematic study of ouabain-insensitive fluxes revealed the presence of a transport pathway sensitive to inhibition by derivatives of 2 sulfamoylbenzoic acid, such as furosemide, bumetanide, piretanide[1,2]. While various cell types exhibit furosemide-sensitive Na and K fluxes, human and avian erythrocytes have proved to be especially suitable for their study and various investigators showed it to be mediated by the Na, K, Cl cotransport system[3] and to be independent of other transport systems such as the Na, K-pump, Na-Na exchange or Na, Cl pathway[2,4,5]. Furthermore the Na, K, Cl cotransport has been shown to be regulated by various factors. This system appears to be sensitive to changes in metabolic status[6], as well as shifts in cAMP levels[7,8] or hypertonicity.

The erythrocyte has proven to be a very useful cell for studying the kinetics of the furosemide-sensitive transport system or its involvement in the regulation of cell volume. However, questions concerning the involvement of a cellular cation in cell growth, cell cycle or immunological response cannot be investigated using this cell. To date, analysis of cation transport in the human peripheral lymphocyte is far from complete. A Na, K pump has been identified in human lymphocytes[9,10], likewise a Na, H exchange and a K channel have been described[11]. These cation transport systems are less well characterized than their counterparts in the erythrocyte mainly because work with such cells is hampered by complications owing to a limited number of cells being available, and the need for delicate cell handling.

In the present study we have investigated Na transport in human peripheral blood mononuclear cells (PMC). We have described the presence of a ouabain-sensitive Na pump and a bumetanide-sensitive Na transport system. Furthermore, it has been shown that prostaglandins E_1 and E_2 and db cAMP regulate this latter system.

Materials and methods. Human peripheral blood mononuclear cells (PMC) were isolated by a Percoll gradient centrifugation technique[12]. PMC were obtained from plateletphoresis-residues following a method described by Segel et al.[13]. Cells were suspended at a cell concentration of 10^7 cells/ml in a PBS medium containing (mM): 137 NaCl, 3 KCl, 8.1 Na_2HPO_4, 1.2 KH_2PO_4, 10 glucose, 0.7 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, pH 7.4 at 37°C. Pyruvate 1 mM and albumin 1 mg/ml were added to the media and kept at 37°C for 60 min prior to assay. Addition of Trypan blue to the medium revealed that 95% of cells excluded the dye. Cell viability markedly decreased when suspensions with a cell number $> 3 \times 10^7$ cells/ml were kept for 12 h at 37°C in PBS medium. Cell counting was performed using a Malassez type

Table 1. The effect of ouabain and bumetanide on ^{22}Na efflux rate from peripheral mononuclear cells

Experiment No.	^{22}Na efflux rate (min^{-1}) PBS medium	PBS + ouabain (10^{-3}M)	PBS + ouabain (10^{-3} M) + bumetanide (10^{-4} M)
1	0.072	0.015	0.008
2	0.040	0.019	0.012
3	0.055	0.027	0.014
4	0.051	0.025	0.013
5	0.037	0.019	0.010
6	0.066	0.029	0.014
Mean ± SD	0.054 ± 0.014	0.022 ± 0.005*	0.012 ± 0.002**

^{22}Na loaded human peripheral mononuclear cells were incubated in phosphate buffered medium (PBS). Triplicate samples were taken at different time intervals and the rate constant k was calculated by linear regression analysis of values obtained using the equation $A_t = A_0 e^{-kt}$, where A_t: activity at time t, A_0: activity at time zero. Statistical significance was assessed using Student's t-test. *$p < 0.01$ when compared to values obtained in PBS medium; **$p < 0.001$ when compared to PBS+ouabain medium.

Table 2. The effect of PGEs, dbAMPc and IBMX on ouabain-sensitive, bumetanide-sensitive and ouabain- and bumetanide-resistant Na efflux rate constants

	Ouabain-sensitive Na efflux (min^{-1})	Ouabain-resistant, bumetanide-sensitive Na efflux (min^{-1})	Ouabain- and bumetanide-resistant Na efflux (min^{-1})
Control	0.027 ± 0.006 (4)	0.011 ± 0.004 (6)	0.012 ± 0.003 (6)
PGE_1 (10^{-6}M)	0.028 ± 0.015 (2)	0.005 ± 0.002 (3)*	0.013 ± 0.003 (3)
PGE_1 (10^{-5}M)	–	0.001 ± 0.003**	0.007 ± 0.002
PGE_2 (10^{-6}M)	0.028 ± 0.013 (2)	0.002 ± 0.003 (2)**	0.013 ± 0.002 (2)
dbAMPc (2 mM)	0.031 ± 0.009	0.005 ± 0.001 (2)*	0.009 ± 0.005 (2)
IBMX (0.5 mM)	0.025 ± 0.011	0.017 ± 0.009 (2)	0.012 ± 0.003 (2)
dbAMPc+IBMX	0.031 ± 0.007	0.003 ± 0.004 (2)**	0.013 ± 0.006 (2)

^{22}Na efflux rate was assessed in PBS medium as described in table 1. *$p < 0.01$; **$p < 0.001$.

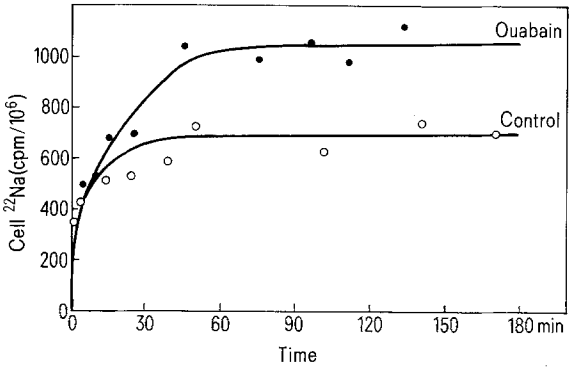

Figure 1. Time-course of ^{22}Na influx in peripheral mononuclear cells. Cells were incubated in phosphate buffered saline (PBS) media with and without ouabain 10^{-3} M. ^{22}Na (1.5×10^5 Bq/ml) was added to the medium. At various time periods, samples were taken to assess cellular ^{22}Na content. Specific activity was 79 cpm/nmole. Influx corrected for zero time uptake could be fitted by equation $A_t = A_s\ (1 - e^{-kt})$ where A_t: activity at time t, A_s: activity at steady state, k: rate constant. Initial influx rate ± SD calculated by linear regression was 0.047 ± 0.009 min^{-1} for control medium and 0.042 ± 0.017 min^{-1} in presence of ouabain.

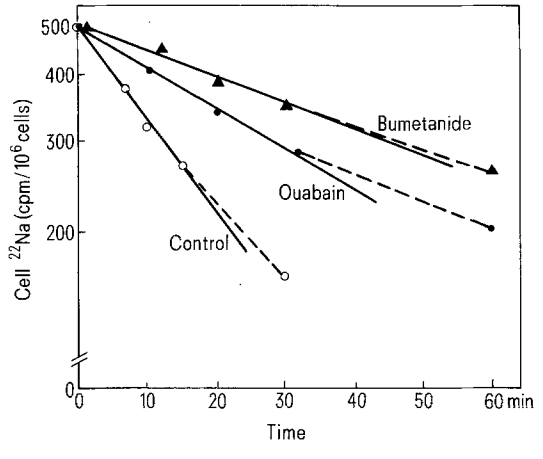

Figure 2. Effect of ouabain and bumetanide on ^{22}Na efflux from peripheral mononuclear cells. Cells were incubated in PBS medium with ^{22}Na (10^5 Bq/ml) for 2 hrs, after which the cells were rapidly washed with unlabelled medium. The cells were then incubated at 37°C in PBS medium. At various time periods triplicate samples were taken and assayed for radioactivity. Na efflux in the intitial phase followed a single exponential function and could be fitted by equation $A_t = A_o\ (e^{-kt})$ where A_t: activity at time t, A_0: activity at time zero, k: rate constant. The semilog plot represents the mean radioactivity left in the cell as a function of time. Open circles: efflux in control medium, the rate coefficient for the data obtained by linear regression analysis was 0.04 min^{-1}. Middle curve, closed circles: efflux into medium with 10^{-3} M ouabain; the rate coefficient was 0.019 min^{-1}. Upper curve, closed triangles: efflux into medium with 10^{-3} M ouabain and 10^{-4} M bumetanide, the rate coefficient was 0.012 min^{-1}. 5 separate experiments gave similar results.

counting chamber. Cell sizing was performed with a Coulter Counter/channelizer combination (Coultronics, France). The median cell volume was 150 μm^3–170 μm^3 and was not affected by the addition of ouabain (10^{-3} M) or bumetanide (10^{-4} M).

Na flux measurement. ^{22}Na efflux was assessed following previously described techniques[6,9]. ^{22}Na preloaded cells were suspended at 10^7 cells/ml in PBS medium at 37°C, ouabain or bumetanide was added as indicated in results section. At appropriate time intervals, 1 ml triplicate samples were layered on top of 0.4 ml silicon oil d = 1.05 and centrifuged immediately. The pellet was then lyzed and counted.

^{22}Na influx measurement was started by adding 1.5×10^5 Bq (4 μCi/ml) ^{22}Na to PBS media. Samples were then layered on top of Whatman GFC filters under moderate vacuum suction and washed 3 times with a K-free PBS medium.

Results and discussion. Na influx into PMC from PBS medium is shown in figure 1. A steady state distribution of ^{22}Na is reached after 1 h of incubation at 37°C with a total extrapolated exchangeable Na of 5.02 ± 0.78 nmoles/10^6 cells (mean ± SD, n = 6). This value is similar to that observed after 12 h incubation at 37°C and amounted to about 75% of total Na content of the cell(6.9 ± 1.1 nmoles/10^6 cells). The average Na influx value was of 0.21 ± 0.04 nmoles/10^6 cells × min with a rate constant of 0.047 ± 0.009 min^{-1} (n = 6).

Na efflux was measured in ^{22}Na preloaded cells incubated in PBS media. The time course of cell ^{22}Na content is shown in figure 2. An initial phase with a half time constant of 13.1 ± 3.4 min can be fitted by a single exponential form with an average rate constant of 0.054 ± 0.014 min^{-1} (n = 6) (table 1). This corresponds to a Na efflux of 0.27 ± 0.08 nmoles/10^6 cells × min. The similarity of Na influx and Na efflux is in accord with the steady state distribution observed in these conditions. The addition of increasing concentrations of ouabain gradually reduces Na efflux with a maximal inhibition being attained for concentrations $> 5 \times 10^{-6}$ M (fig. 3). At 10^{-3} M ouabain, the Na efflux rate constant was 0.022 ± 0.006 min^{-1} (table 1) and the ouabain-sensitive Na efflux had a value of 0.16 ± 0.07 nmoles/10^6 cells × min (n = 6). This value is similar to that observed by different authors and can be safely assumed to take place through the Na,K pump[9,14].

^{22}Na uptake by PMC was studied in the presence of 10^{-4} M ouabain. Interestingly, Na distribution between cell and medium reached a steady state after 1 h of incubation, with an extrapolated exchangeable Na pool of 9 nmoles/10^6 cells. This observation rules out net diffusion of the Na ion along its electrochemical gradient and strongly suggests the presence of a ouabain-insensitive transport pathway capable of extruding Na against its gradient. Indeed, measuring Na efflux in the presence of 10^{-3} M ouabain from ^{22}Na preloaded cells revealed net Na extrusion against its gradient with a magnitude of 0.11 ± 0.03 nmoles/10^6 cells × min (rate constant 0.022 ± 0.006 min^{-1}) (table 1). The addition of increasing concentrations of bumetanide gradually reduces the ouabain-insensitive Na efflux rate, with a maximal inhibition being reached at concentrations $> 6 \times 10^{-6}$ M (fig. 3). Ouabain-resistant bumetanide-sensitive Na efflux represents

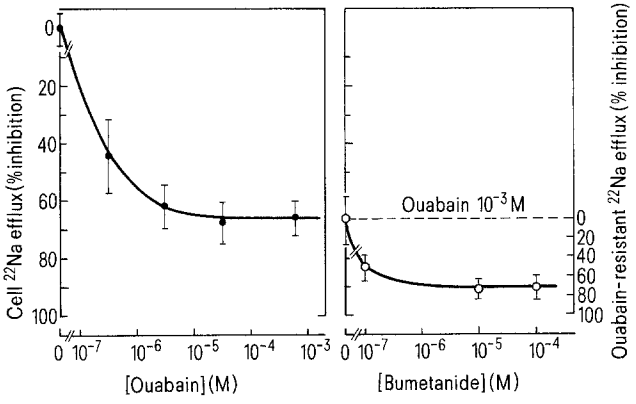

Figure 3. The effect of different concentrations of ouabain and bumetanide on ^{22}Na efflux rate from peripheral mononuclear cells. Cells were incubated at 37 °C for 2 h in PBS medium containing ^{22}Na (10^5 Bq/ml) after which the cells were rapidly washed with unlabeled medium. Cells were further incubated in PBS medium and duplicate samples were taken at two different time intervals and assayed for cell radioactivity. Ouabain was added at different concentrations and the effect of increasing bumetanide concentrations was assayed in the presence of 10^{-3} M ouabain. Rate constants ± SD were calculated from least square fits of single exponentials, the data are represented as % inhibition of ^{22}Na efflux rates in the absence of ouabain and bumetanide.

20% of total Na efflux and had a value of 0.05 ± 0.02 nmoles/10^6 cells × min.

Bumetanide- or furosemide-sensitive Na fluxes have been described in various cell types[15-17]. Extensive studies done in red cells[15,18] and cultured fibroblasts[16] showed it to be mediated by a Na, K, Cl-cotransport system. It is tempting to postulate that bumetanide-sensitive Na efflux in human lymphocytes is mediated by such a system; however, the thorough investigations and kinetic analysis required are hampered in lymphocytes by the limited amount of cells which could be obtained from human blood, and will have to await the development of new techniques.

The role of PGE in lymphocytes is not fully understood. They are known to be involved in immunological and blastogenic responses[19,20]. In these cases variations of Na transport properties have also been reported to occur. We have therefore tested the effect of PGE on Na transport pathways in PMC. PGE_1 and PGE_2 at concentrations $> 10^{-6}$ M markedly reduce (80%) the bumetanide-sensitive Na efflux rate without altering ouabain-sensitive or ouabain- and bumetanide-resistant Na efflux (table 2). The action of PGEs is known to be mediated by variations in cAMP level[21]. In our hands, the addition of dibutyryl cAMP (2 mM), similarly to PGE_1 and PGE_2, reduced the bumetanide-sensitive Na efflux without affecting the ouabain-sensitive or the ouabain- and bumetanide-resistant fluxes. It is interesting to note that isobutyl-methylxanthine (0.5 mM), a phosphodiesterase inhibitor, has no effect on Na transport pathways (table 2). These results are similar to those observed in mouse lymphocytes and human fibroblasts[16,22]. It is clear that regulatory mechanisms controlling cotransport fluxes are tissue and cell specific, as the cotransport system in avian erythrocytes is stimulated by agents which elevate cAMP[1]. On the other hand, the effect of PGE on Na transport might not be mediated solely by cyclic nucleotide variations; a role for calcium and phospholipid metabolism should be considered, and further analysis is required to determine their implication.

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NADH-methemoglobin reductase activity in the erythrocytes of newborn and adult mammals

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Summary. NADH-MR activity was measured in the erythrocytes of newborn and adult horses, pigs, cattle, sheep, goats, red kangaroos, fruit bats, rats, mice, rabbits and humans. Our results fail to support an earlier hypothesis that higher NADH-MR activity may be an adaptation to increased ruminal nitrite production leading to accelerated oxidation of fetal hemoglobin.
Key words. Erythrocytes; NADH-methemoglobin reductase; mammals; newborn; adults.

The enzyme primarily responsible for reducing methemoglobin in mammalian erythrocytes is NADH-methemoglobin reductase (NADH-MR)[2], (also called cytochrome b_5 reductase[3] and NADH-ferricyanide reductase[4,5]). Several cases of methemo-