Biochimica et Biophysica Acta, 688 (1982) 786–792
Elsevier Biomedical Press

BBA 71229

INHIBITION OF THE Na^+/K^+ COTRANSPORT SYSTEM BY CYCLIC AMP AND INTRACELLULAR Ca^{2+} IN HUMAN RED CELLS

RICARDO PABLO GARAY

INSERM U7/CNRS LA 318, Hopital Necker, 161 rue de Sèvres, 75015 Paris (France)

(Received January 21st, 1982)

Key words: Na^+ transport; cyclic AMP; Ca^{2+}; Na^+/K^+ cotransport; (Human erythrocyte)

Human erythrocytes are able to incorporate cyclic AMP (cAMP) in amounts larger than those required to saturate cAMP-dependent protein kinase. In contrast to previous observations in avian red blood cells in which cAMP stimulates the Na^+/K^+ cotransport system, we demonstrate that cAMP inhibits this system in human erythrocytes. The cotransport inhibition is enhanced by addition of phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine to the incubation medium. The cAMP concentration giving half-maximal cotransport inhibition showed a wide variation among different individuals (from 0.1 to 5 mM external cAMP concentration). In contrast to cAMP, cyclic GMP showed little effect on the cotransport system. Ca^{2+} introduced into the cell interior was an inhibitor of the Na^+/K^+ cotransport system. These results suggest that in human cells in which endogeneous levels of cAMP and Ca^{2+} are modulated by hormones, the Na^+/K^+ cotransport system may be under hormonal regulation.

Introduction

In avian red blood cells a furosemide-sensitive Na^+/K^+ cotransport system catalyzes the simultaneous efflux or influx of both Na^+ and K^+ [1]. This transport system is stimulated by a catecholamine-dependant adenylate cyclase system [1–4].

In human red cells the existence of the Na^+/K^+ cotransport system is well documented [5,6]. In spite of the functional absence of β-adrenergically stimulated adenylate cyclase [7–9], human red cell membranes contain cAMP-dependent protein kinases [10–12]. Thus, it was of interest to see whether the Na^+/K^+ cotransport might respond to cyclic AMP (cAMP) independently of catecholamine receptor interactions in these cells. In addition, the effect of other hormonal intermediates, intracellular Ca^{2+} and cyclic GMP, on the Na^+/K^+ cotransport system was examined.

Abbreviations: PCMBS, *p*-chloromercuribenzenesulphonate; Mops, 4-morpholinopropanesulphonic acid; cAMP, cyclic AMP; cGMP, cyclic GMP.

Methods

1. Preparation of fresh human red cells

Venous blood from normal subjects was collected in heparinized tubes and centrifuged at $1750 \times g$ for 10 min and plasma and buffy coat were removed. Red cells were washed twice with approx. 10 vol 150 mM NaCl and recentrifuged for 3 min at $1750 \times g$. All steps were carried out at 4°C.

2. Preparation of Na^+-loaded, K^+-depleted erythrocytes

Na^+-loaded, K^+-depleted erythrocytes were prepared as previously described [13]. 5 ml washed packed fresh cells were suspended in a

Na^+/choline-loading medium to a final hematocrit of 8%. The Na^+/choline-loading medium contained (mM): 50 NaCl, 4 KCl, 200 choline chloride, 2.5 sodium phosphate (pH 7.2 at 4°C), 1 $MgCl_2$ and 0.02 *p*-chloromercuribenzenesulphonate (PCMBS). Cells were incubated, with mild agitation, for 20 h at 4°C in Na^+/choline-loading medium which was renewed after the first 6 h of incubation. Cells were then centrifuged at 1750 × *g* at 4°C for 10 min, the supernatant was discarded and the cells were resuspended in a 'recovering medium' containing (mM) 150 NaCl, 1 $MgCl_2$, 5.4 sodium phosphate (pH at 37°C), 4 cysteine, 2 adenine, 3 inosine and 10 glucose to give a final hematocrit of 10%. The pH of the recovering solution was adjusted to 7.2 at 37°C with Tris-HCl. Cell suspensions were incubated at 37°C for 1 h. Cells were then centrifuged at 4°C for 3 min at 1750 × *g* and the supernant discarded.

After sodium loading, cells contained 20–30 mmol/litre cells intracellular Na^+ and 30–40 mmol/l cells intracellular K^+. The cells were maintained at a constant volume by the intracellular choline incorporated during the loading step.

3. Net entry of [^{14}C]cAMP and dibutyryl [^{3}H]cAMP in human red cells

The net influx rates of [^{14}C]AMP and dibutyryl [^{3}H]cAMP were measured in fresh human red cells. 2 ml washed erythrocytes were resuspended in 20 ml of a Na^+-K^+ Ringer's medium containing (mM): 145 NaCl, 5 KCl, 2.5 phosphate buffer (pH 7.4 at 37°C) 10 glucose and 1 $MgCl_2$. 5 μCi [^{14}C]cAMP or 100 μCi of dibutyryl [^{3}H]cAMP were added to Ringer's medium. The concentration of unlabelled cAMP or dibutyryl cAMP was 1 mM. The resulting suspensions were distributed in 20 tubes (1 ml per tube) at 4°C. Tubes were incubated at 37°C. After 0, 1, 2, 4, 10, 20, 30, 40, 60, 90 min, two tubes were transferred to 0°C and the cells were washed three times with 150 mM NaCl at 4°C. The supernatant was discarded and cells were lysed with 1.5 ml of distilled water containing Acationox 0.02%. 0.5 ml of the hemolyzed cells was diluted with 2.5 ml distilled water in order to measure hemoglobin absorbance at 540 nm, 0.5 ml 20% trichloroacetic acid was added to 1 ml of the hemolyzed cells. The tubes were centrifuged for 10 min at 1750 × *g* and 1 ml of the supernatant was added to 9 ml Insta-gel solution. ^{14}C and ^{3}H were measured in a Packard Prias PL Tri-Carb liquid scintillation counter. The specific activity of [^{14}C]cAMP and dibutyryl [^{3}H]cAMP in the incubation medium was measured in a separate aliquot. The intracellular concentration of cAMP (or dibutyryl cAMP) was expressed in μmol/l cells. The influx rate constant was obtained by dividing the initial rate of cAMP influx by the external cAMP concentration. The efflux rate constant was obtained by the method described in Fig. 1 (inset).

4. Measurement of outward Na^+/K^+ cotransport fluxes

Outward Na^+/K^+ cotransport fluxes were measured in fresh and in Na^+-loaded, K^+-depleted erythrocytes using a previously published method [13]. Packed erythrocytes were washed five times with a cold solution of 110 mM $MgCl_2$ and resuspended in the efflux medium to obtain a hematocrit of approx. 25%. An aliquot of this suspension together with an aliquot of cells untreated with PCMBS was set aside to measure intracellular Na^+-K^+, hemoglobin and hematocrit.

0.5 ml of the cell suspension was added to two tubes each containing 2 ml of cold magnesium/sucrose medium with 0.1 mM bumetanide (or 1 mM furosemide) (neutralized with Tris) and to two other tubes containing 2 ml of magnesium/sucrose medium without bumetanide. The magnesium/sucrose medium contained (mM): 75 $MgCl_2$, 85 sucrose, 5 glucose, 0.1 ouabain and 10 Mops-Tris (pH 7.4 at 37°C). The resulting suspensions were incubated at 37°C with continuous agitation for 2 h. After incubation, the tubes were transferred to 0°C and then spun down at 4°C for 4 min at 1750 × *g*. The supernatant was carefully removed (avoiding pellet contamination) and Na^+ and K^+ contents were measured by flame photometry.

For the Ca^{2+} experiments cells were washed with 150 mM choline chloride instead of 110 mM $MgCl_2$ and a slightly different efflux medium was used (mM): 150 choline chloride, 10 Mops-Tris (pH 7.4 at 37°C), 1 $MgCl_2$, 5 glucose, 0.1 ouabain and 0.01 carbocyanin. The addition of carbocyanin prevented the 'Gardos effect' [15] and the replace-

ment of Mg^{2+} by choline prevented swelling of the cells in the presence of Ca^{2+} ionophore. Control experiments showed that 10 μM carbocyanin had no effect on outward cotransport fluxes.

5. Calculation of Na^+ and K^+ outward cotransport fluxes

Bumetanide- or furosemide-sensitive Na^+ and K^+ efflux rate in μmol/l cells per h was obtained according to the equation:

$$\text{flux} = \frac{D \times (1 - \text{hematocrit})}{2 \cdot \text{hematocrit}}$$

where D is the difference in external Na^+ or K^+ concentration induced by bumetanide and furosemide.

6. The effect of isoproterenol, cAMP, cGMP and Ca^{2+} on the outward Na^+/K^+ cotransport fluxes

The action of isoproterenol cAMP, cGMP and Ca^{2+} on the Na^+/K^+ cotransport system was studied in human erythrocytes. The bumetanide or furosemide-sensitive Na^+ and K^+ efflux was measured in media containing different concentrations of compounds. In order to study the effect of intracellular Ca^{2+} on the Na^+/K^+ cotransport system, the Ca^{2+}-ionophore A23187 was added to the Ca^{2+} medium.

Results

The effect of isoproterenol on the Na^+/K^+ cotransport system in human red cells

In contrast to avian red cells, no β-adrenergically stimulated adenylate cyclase activity has been observed in human erythrocytes [7–9]. Indeed, isoproterenol, a β-adrenergic agonist, showed no effect on the Na^+/K^+ cotransport system of these cells (Table I). In addition, isoproterenol has no effect on the erythrocyte Na^+,K^+-pump (Table I).

Net influx of [^{14}C]cAMP and dibutyryl [^{3}H]cAMP in human red cells

The net influx of [^{14}C]cAMP and dibutyryl [^{3}H]cAMP in human red cells is linear up to 4 min (Fig. 1). A quasi-stationary level representing only 3–7% of the external cAMP concentration and 1–2% of the external dibutyryl cAMP concentration is attained later. The overall net influx curve follows first-order kinetics (Fig. 1, inset). The rate constants obtained for unidirectional cAMP influx and efflux (Table II) are one order of magnitude lower than those previously reported for human erythrocyte ghosts [14] (indicating that human red cell membranes become leaky to cAMP during the ghost preparation). Surprisingly, the rate constant ratio between [^{14}C]cAMP efflux and influx is much higher than that predicted from thermodynamic equilibrium. This phenomenon is not consequent to cAMP degradation because the inhibition of

TABLE I

THE EFFECT OF ISOPROTERENOL ON THE Na^+/K^+ COTRANSPORT AND Na^+/K^+-PUMP IN FRESH HUMAN ERYTHROCYTES

Values are expressed as mean ± range. The same result was obtained in two other experiments. Ouabain-sensitive Na^+ efflux was measured according to Ref. 27.

Isoproterenol concentration (M)	Bumetanide-sensitive Na^+ efflux (μmol/l cells per h)	Ouabain-sensitive Na^+ efflux (μmol/l cells per h)
—	170 ± 3	1080 ± 50
10^{-8}	176 ± 5	1060 ± 30
10^{-7}	168 ± 6	1100 ± 80
10^{-6}	167 ± 6	1100 ± 40
10^{-5}	171 ± 3	1080 ± 50
10^{-4}	170 ± 2	1120 ± 70

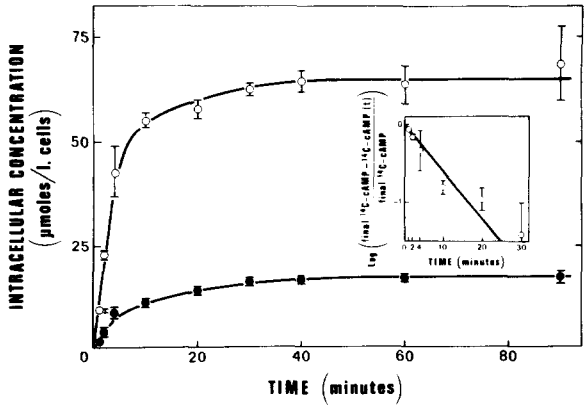

Fig. 1. Time-course of net [^{14}C]cAMP (○) and dibutyryl [^{3}H]cAMP (●) influx into human red cells. The external concentration of unlabelled cAMP and dibutyryl cAMP was 1 mM. Values are expressed as mean ± range of duplicates. Inset: first-order kinetics of net cAMP influx.

TABLE II

THE INCORPORATION OF CYCLIC [^{14}C]AMP INTO HUMAN RED CELLS

Values are expressed as mean±S.D. The number of experiments is given in brackets. See text for details on Methods. DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate, MIX, 1-methyl-3-isobutylxanthine.

Medium	Cyclic [^{14}C]AMP		Dibutyryl cyclic [^{3}H]AMP	
	influx rate constant (h^{-1})	efflux rate constant (h^{-1})	influx rate constant (h^{-1})	efflux rate constant (h^{-1})
Cl^-	0.37±0.12 (5)	8.5±3.0 (5)	0.08±0.03 (4)	3.6±1.7 (4)
Citrate	0.35±0.09 (5)	7.4±4.1 (5)	-	-
Citrate+0.2 mM DIDS	0.39±0.08 (3)	10.7±4.0 (3)	-	-
Cl^- +1 mM MIX	0.25±0.08 (3)	6.0±3.5 (3)	-	-

phosphodiesterase with 1-methyl-3-isobutylxanthine only slightly modifies the kinetics of net cAMP influx (Table II). In fact, these results suggest the existence of some cAMP transport mechanism able to catalyse a net cAMP extrusion from the cells. This mechanism is different from the very active erythrocyte anion carrier because net cAMP influx is not modified by replacement of Cl^- by citrate or by addition of 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) (Table II). Furthermore, the rate constant of cAMP influx is independent of the external cAMP concentration, suggesting that this transport mechanism is nonspecific for cAMP (Fig. 2).

Independently of the molecular nature of the transport mechanism mediating cAMP fluxes, human erythrocytes are able to incorporate cAMP in amounts larger than those required to saturate cAMP-dependent protein kinase [10,11]. This property allows the study of the effect of exogenous cAMP on the outward Na^+/K^+ cotransport fluxes in these cells.

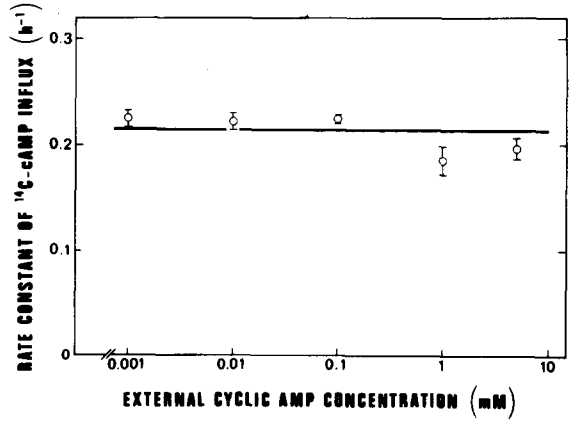

Fig. 2. The rate constant of [^{14}C]cAMP influx as a function of the external cAMP concentration. Values are expressed as mean±range of duplicates.

The effect of cAMP on the Na^+/K^+ cotransport system

Human erythrocytes incubated in an Mg^{2+}/sucrose/ouabain medium lose their internal Na^+ and K^+ content through the Na^+/K^+ cotransport system and by passive Na^+ and K^+ permeabilities [6]. Under these conditions, the outward cotransport fluxes can be specifically inhibited by loop diuretics such as furosemide and bumetanide. The addition of cAMP to fresh human erythrocytes inhibits the furosemide-sensitive outward Na^+/K^+ cotransport fluxes (Table III). This inhibition is increased by the addition of 1 mM methylisobutylxanthine, suggesting that it is due to cAMP and not to a degradation product (Table III). A dose-response curve in Na^+-loaded, K^+-depleted erythrocytes showed marked inter-individual variations between the different blood donors (Fig. 3a and b). Thus, the external cAMP concentration giving 50% cotransport inhibition varied from 0.1 to 5 mM. The kinetic constants of Table II indicate that this range corresponds to an upper limit of 3 to 150 μmol/l cells of intracellular cAMP. This seemed sufficient to saturate the cAMP-dependent protein kinase, which shows 50% activation for cAMP concentrations ranging from 0.1 to 1 μmol [12]. However, the actual cAMP concentration is probably much less than 3–150

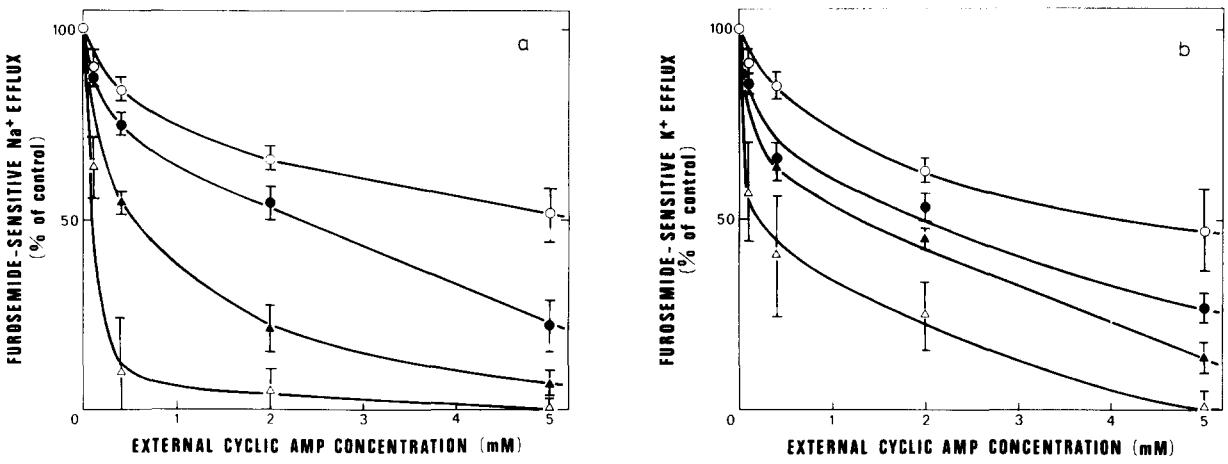

Fig. 3. Dose-response curve for the cotransport inhibition by cAMP. (a) Furosemide-sensitive Na^+ efflux. (b) Furosemide-sensitive K^+ efflux. In the four different donors tested (various symbols), the cells contained 20–30 mmol/l cells of intracellular Na^+ and 30 to 40 mmol/l cells of intracellular K^+. The cell volume was maintained constant with intracellular choline at a concentration of 40–50 mmol/l cells. Values are expressed as mean ± range of duplicates.

TABLE III

THE EFFECT OF CYCLIC AMP ON OUTWARD NA^+/K^+ COTRANSPORT FLUXES IN FRESH HUMAN RED CELLS

Values are expressed as mean ± range.

Donor	Furosemide-sensitive Na^+ efflux (μmol/l cells per h)					
	Mg^{2+}/sucrose medium	1 mM MIX	2 mM cAMP	% inhibition	cAMP+ MIX	% inhibition
1	353±8	350± 4	302±10	14.4	274±2	22.4
2	317±8	332±10	239± 4	24.6	219±4	30.9
3	33±5	38± 2	15± 1	55.5	12±2	65.4

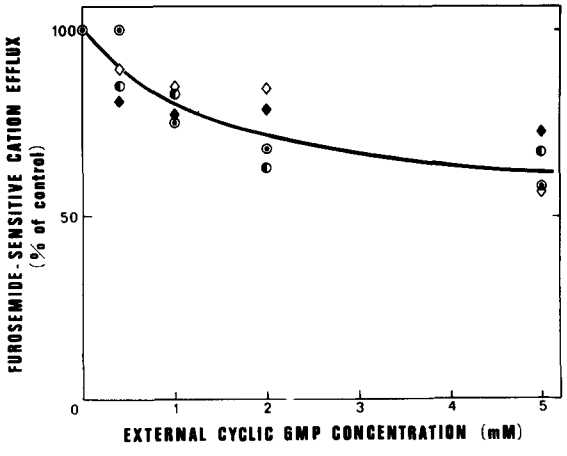

Fig. 4. The effect of cyclic GMP on the Na^+/K^+ cotransport system. Two donors were tested. Furosemide-sensitive Na^+ efflux is given in circles and furosemide-sensitive K^+ efflux is given in diamants.

μmol/l cells, because a portion of intracellular cAMP is degraded by internal phosphodiesterases. Fig. 4 shows that cGMP only minimally inhibits outward Na^+/K^+ cotransport.

Effect of Ca^{2+} on the Na^+/K^+ cotransport system

Fig. 5 shows that outward Na^+/K^+ cotransport fluxes are not modified by the increase in external Ca^{2+} concentration up to 1 mM. However, if the ionophore A23187 is added to a 1 mM Ca^{2+} solution, the cotransport system is strongly inhibited (Fig. 5). In five different experiments the ionophore concentration for 50% cotransport inhibition varied from 0.5 to 2 μM. The presence of 10 μM carbocyanin in the incubation medium completely blocked the 'Gardos effect' up to a ionophore concentration of 4–10 μM. For higher

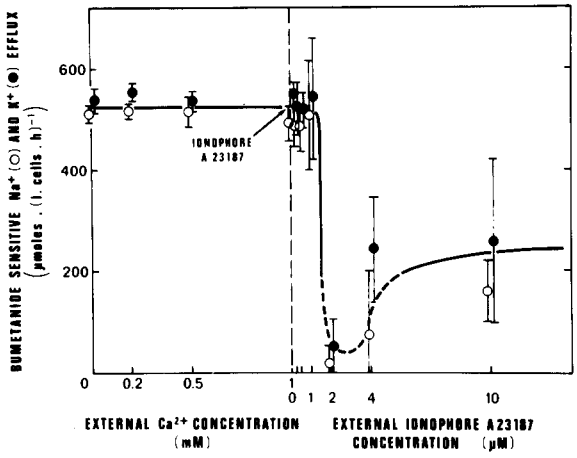

Fig. 5. The effect of extra- and intracellular Ca^{2+} on the outward Na^+/K^+ cotransport fluxes. Outward cotransport fluxes were performed in choline medium containing 10 μM carbocyanin in order to inhibit the 'Gardos effect' [15]. Values are expressed as mean ± range of triplicates. A similar result was obtained in four other experiments.

ionophore concentrations a small Gardos effect appears.

Discussion

Na^+ and K^+ transport across cell membranes is modified by environmental constraints and particularly by changes in Na^+ and K^+ intake and excretion [16]. The homeostasis of these disturbances in cation transport seems to involve a hormonal control of the turnover rate of transmembrane cation translocation [17,18] and a genetic regulation of the density of membrane transport units [19].

At the molecular level, a number of hormones, including catecholamines, glucagon, insulin and thyroxine, may modify the activity of the Na^+,K^+ pump [20–22]. Some of these effects seems to be mediated by cAMP, which activates [20] or inhibits [23] the pump and by intracellular Ca^{2+} which inhibits the pump [24] (Fig. 6).

On the other hand, the Na^+/K^+ cotransport system [1,5,6] seems to be a more likely candidate for hormonal regulation. In human red cells under basal conditions, this system is near equilibrium, i.e., no net Na^+ or K^+ cotransport movements are performed. Any deviation of the cotransport equilibrium by a variation in the intracellular Na^+

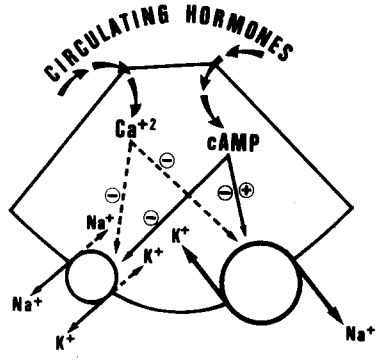

Fig. 6. The hormonal network model for the regulation of Na^+ and K^+ electrochemical gradients across cell membranes.

concentration (or in the level of plasma K^+) activates a net flux via this system in the opposite direction of the ionic perturbation, thus returning the internal Na^+ (or external K^+) levels to basal conditions. This indicates that the physiological role of the Na^+/K^+ cotransport system is the regulation of the Na^+ and K^+ gradients across cell membranes. Further evidence for a regulator role of this system comes from experiments in avian red cells. In these cells, the Na^+/K^+ cotransport system is stimulated by β-adrenergic agents through the intermediate cAMP [1–4]. The interaction of cAMP (and Ca^{2+}) with a protein kinase phosphorylating an intermediary protein, globin of 230 kDa, suggests a complex chain of chemical reactions between the hormone-receptor and the Na^+/K^+ cotransport system [4]. Thus, the regulatory role of the Na^+/K^+ cotransport system may be submitted to a network of interactions involving circulating hormones and second messengers (Fig. 6).

Although no β-adrenergically stimulated adenylate cyclase activity could be observed in human red cells [7–9], these cells are rich in cAMP-dependent protein kinases of unknown physiological function [10–12]. This suggests that the distal limb of the β-adrenergic-dependent cotransport loop may be preserved in human red cells, allowing the study of the effect of exogenous cAMP on erythrocyte Na^+/K^+ cotransport. In contrast to previous observations in avian red cells, cAMP inhibits the cotransport system in human erythrocytes. This result suggests that the Na^+/K^+ cotransport system may be modulated by circulat-

ing hormones in other non-erythrocyte cells (Fig. 6). For example, the adenylate cyclase stimulation in the cells of the loop of Henle may induce natriuresis by inhibition of apical Na^+ reabsorption, which seems mediated by a Na^+, K^+, Cl^- cotransport system [25,26]. Further evidence for hormonal modulation of the cotransport system in man is given by the inhibition of this system by intracellular Ca^{2+} (Fig. 5).

In conclusion, the results presented in this paper further suggest the existence in man of an hormonal network for the regulation of Na^+ and K^+ electrochemical gradients through such intermediates as cAMP and Ca^{2+} (Fig. 6).

Acknowledgments

I am indebted to C. Nazaret for technical help and to M. Price (Baylor College of Medicine, Houston) who helped with the English.

References

1 McManus, T.J. and Schmitt, W.F. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), vol. 1, pp. 79–106, Raven Press, New York
2 Gardner, J.D., Aurbach, G.D., Spiegel, A.M. and Brown, E.M. (1976) Recent Prog. Hormone Res. 32, 567–595
3 Kregenow, F.M. (1977) in Osmotic and Volume Regulation (Jorgensen, C.B. and Skadhauge, E., eds.), pp. 379–391, Academic Press, New York
4 Alper, S.L., Palfrey, H.C., De Riemer, S.A. and Greengard, P. (1980) J. Biol. Chem. 255, 11029–11039
5 Wiley, S.J. and Cooper, R.A. (1974), J. Clin. Invest. 53, 745–755
6 Garay, R.P., Adragna, N., Canessa, M. and Tosteson, D. (1981) J. Membrane Biol. 62, 169–174
7 Sheppard, H. and Burghardt, C. (1969) Biochem. Pharmacol. 18, 2576–2578
8 Rasmussen, H., Lake, W. and Allen, J.E. (1975) Biochim. Biophys. Acta 411, 63–73
9 Kaiser, G., Quiring, K., Gauger, D., Palm, D., Becker, H. and Schoeppe, W. (1974) Blut 29, 115–122
10 Kubin, C.S., Erlichman, J. and Rasmussen, H. (1972) J. Biol. Chem. 247, 6135–6139
11 Hosey, M.M. and Tao, M. (1977) J. Biol. Chem. 252, 102–109
12 Grant, B.F., Breithaupt, T. and Cunningham, E.B. (1979) J. Biol. Chem. 254, 5726–5733
13 Dagher, G. and Garay, R. (1980) Can. J. Biochem. 58, 1069–1074
14 Holman, G.D. (1978) Biochim. Biophys. Acta 508, 174–183
15 Simons, T. (1979) J. Physiol. (London) 288, 481–507
16 Garay, R.P. and Meyer, P. (1979) Lancet i, 349–353
17 Sweadner, K.J. and Goldin, S.M. (1980) N. England J. Med. 302, 777–783
18 Grantham, J.J. (1980) Am. J. Physiol. 239, F97–F106
19 Erdmann, E., Krawietz, W. and Koch, M. (1979) in ($Na^+ + K^+$)-ATPase, Structure and Kinetics (Skov, J. and Nørby, J., eds.), pp. 517–524, Academic Press, New York
20 Clausen, T. and Flatman, J. (1977) J. Physiol. 270, 383–414
21 Clausen, T. and Hansen, O. (1977) J. Physiol. 270, 415–430
22 Biron, R., Burger, A., Chinet, A., Clausen, T. and Dubois-Ferriere (1979) J. Physiol. 297, 47–60
23 Luly, P., Barnabei, O. and Tria, E. (1972) Biochim. Biophys. Acta 282, 447–452
24 Sarkadi, B., Szasz, I., Gerloczy, A. and Gardos, G. (1977) Biochim. Biophys. Acta 464, 93
25 Palfrey, H.C., Feit, P.W. and Greengard, P. (1980) Am. J. Physiol. 238 (Cell Physiology), C139–C148
26 Geck, P. (1979) Nieren Hochdruckkr. 5, 226–227
27 Garay, R.P. and Garrahan, P. (1973) J. Physiol. 231, 297–325