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Effects of ATP and cyclic AMP on the ($Na^+ + K^+ + 2Cl^-$)-cotransport system in turkey erythrocytes

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(1) As turkey erythrocytes were progressively depleted of ATP by preincubation with dinitrophenol, the ($Na^+ + K^+ + 2Cl^-$)-cotransport system (assayed by the bumetanide-sensitive fraction of $^{86}Rb^+$ influx) became less responsive to activation. The dependence upon intracellular ATP concentration was significantly steeper for transport activated by hypertonic shock (halfmaximal activity at 0.7 mM ATP) than for that activated by either epinephrine or cyclic AMP (halfmaximal activity at 1.7 mM ATP). (2) Upon removal of epinephrine or cyclic AMP from cells that had been preincubated with those substances, bumetanide-sensitive transport activity declined sharply, even though the intracellular cyclic AMP concentration was still over 10-fold that required to maximally activate the transport system. (3) These data are in agreement with the notion that the ($Na^+ + K^+ + 2Cl^-$)-cotransport system in turkey erythrocytes is activated by cyclic AMP, presumably through the 'classical' pathway involving a protein kinase. They do however indicate that some other, as yet undefined aspect of cyclic AMP metabolism is important for the maintenance of transport activity.

Introduction

In the past few years, bumetanide- or furosemide-sensitive transport systems that cotransport Na^+, K^+ plus Cl^- or any two of these ions have been found in at least half of the two dozen or so cell types that have been investigated on their existence (see, for instance, Refs. 1–9). Generally, these systems are driven by the sum of the chemical gradients of the ions involved rather than by ATP [1,13]. Even so, in several cell types ATP seems to be required in some regulatory capacity [1,4,9,10].

The ($Na^+ + K^+ + 2Cl^-$)-cotransport systems are activated by cell shrinkage and/or inactivated by cell swelling. In their active state they catalyze a net salt uptake with concomitant cell swelling, and thereby appear to play a role in cellular volume regulation [2,6]. In 'leaky' epithelia like that of the thick ascending limb of Henle's loop [7] or of the shark rectal gland [3] they presumably cooperate with the ($Na^+ + K^+$)-ATPase in the generation of a transcellular salt flux.

By contrast, the ($Na^+ + K^+ + 2Cl^-$)-cotransport system in bird erythrocytes (for reviews, see Refs. 2 and 8), though one of the oldest to have been studied, has no clear function and may just be an evolutionary remnant. In its basal state it is inactive. Like its relatives in other cell types it can be activated by cell shrinkage (experimentally induced by hypertonic shock), but also by β-adrenergic hormones. The latter probably act through the 'classical' pathway involving cyclic AMP and

Abbreviation: MIX, 1-methyl-3-isobutylxanthine.

at least one protein kinase [8]. The mode of action of hypertonic shock is unknown; since it does not affect the basal cyclic AMP level to any significant degree, it apparently does not involve this nucleotide [2].

The experiments presented here center around the roles of ATP and cyclic AMP in the activation of $(Na^+ + K^+ + 2Cl^-)$-cotransport in turkey red cells. Our results confirm similar observations by Palfrey [10] that in these cells, as in ascites cells [1], MDCK cells [4] and squid axons [9] ATP is needed for optimal transport activity, and that this requirement surpasses the need for ATP posed by the 'classical' cyclic-AMP-dependent activation pathway. We also present evidence that transport activity induced by the 'classical' pathway is modulated by an as yet undefined secondary factor tied to cyclic AMP turnover.

Materials and Methods

Erythrocytes were obtained from the wing veins of two female turkeys and washed twice in a saline solution (standard saline) containing (mM): NaCl, 120; KCl, 2.5; $MgCl_2$, 1; Hepes, 20, neutralized with NaOH, 10. pH was 7.4 at 41°C. The same medium, supplemented with 10 mM glucose, was used for the incubations, unless indicated otherwise. Cells were diluted to a haematocrit of approx. 20% and preincubated for at least 60 min at 41°C.

For $^{86}Rb^+$ flux measurements and cyclic AMP determinations cells were further diluted to a haematocrit of 10–15%. Epinephrine, sorbitol, cyclic AMP and 1-methyl-3-isobutylxanthine (MIX) were added in this dilution step. Incubations were started either by the addition of the activating substance or by warming the cells from 0°C to 41°C. For the measurement of $^{86}Rb^+$ influx, 1 ml cell suspension was withdrawn and added to standard medium containing $^{86}Rb^+$ (30 nCi/ml, Amersham) and 0.2 mM ouabain to inhibit influx through the $(Na^+ + K^+)$-ATPase. Final haematocrit was 4–5%. Tracer uptake was allowed to proceed for 10 min and stopped by a 1-min spin in a microcentrifuge. Usually, the cell pellet was washed twice with an ice-cold washing saline, containing 100 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.4). Alternatively, cells were spun through a silicon oil layer and the supernatant was aspirated; those two procedures yielded comparable results. Cells were extracted with trichloroacetic acid and radioactivity was counted as in Ref. 11. K^+ influx was calculated from the difference in uptake of $^{86}Rb^+$ in the presence and in the absence of 10 μM bumetanide. The bumetanide- and ouabain-insensitive component of K^+ influx ranged between 10 and 60 nmol · $min^{-1} \cdot ml^{-1}$.

For the measurement of cyclic AMP content, duplicate samples of 25–500 μl were withdrawn at the indicated time points and mixed with at least two volumes of ice-cold standard medium containing 4 mM EDTA. Cells were washed up to three times and resuspended with EDTA-containing medium to a final volume of 200 μl. The cell suspension was lysed in a boiling water bath for 5 min, diluted with 200 μl 4 mM EDTA and frozen. For determination of cyclic AMP the samples were thawed and spun, and 50 μl of the supernatant was analyzed with a cyclic AMP test kit (Amersham).

To determine extracellular cyclic-AMP concentrations, cells were removed by centrifugation in a microfuge. Supernatants were adjusted to 4 mM EDTA, boiled, diluted as necessary, and frozen. Further treatment was identical to that of the cell samples.

Cellular ATP was depleted by incubating the cells for various times (up till 120 min) in the presence of 1 mM dinitrophenol and 0.2 mM ouabain, without glucose. ATP depletion was stopped by washing the cells three times with ice-cold standard medium. Cellular ATP concentrations were determined with the luciferin-luciferase method according to [12].

For the determination of cell water, triplicate samples of 20% cell suspensions were spun for 1 min in preweighed 0.4 ml microfuge tubes. The supernatant and the top layer of the pellet were removed, and the internal volume calculated from the wet and dry weights of the pellets, assuming an extracellular space of 15% [11]. The value thus obtained was used to calculate intracellular concentrations. In none of the experiments except that of Table I did cell volume or cellular K^+- and Na^+-content (as measured by flame photometry)

change to any significant degree during the experimental incubations.

Results are expressed per ml packed cells. Differences between duplicates and standard errors of triplicates were less than 5%, unless indicated by an error bar in the drawings.

Results

It has been well established [13] that in turkey erytrhocytes like in other cells [6], the driving force for the $(Na^+ + K^+ + 2Cl^-)$-cotransport system is supplied by the sum of the chemical gradients of the ions involved: apparently, no additional stoichiometric input of metabolic energy is necessary. Nevertheless, in turkey erythrocytes [10] as in several other cell types [1,4,9] ATP appears to be required for $(Na^+ + K^+ + 2Cl^-)$-cotransport activity, presumably in some regulatory function. To quantitatively investigate this requirement we determined the ATP dependence of $(Na^+ + K^+ + 2Cl^-)$-cotransport as activated either through the hormonal pathway or by hypertonic shock.

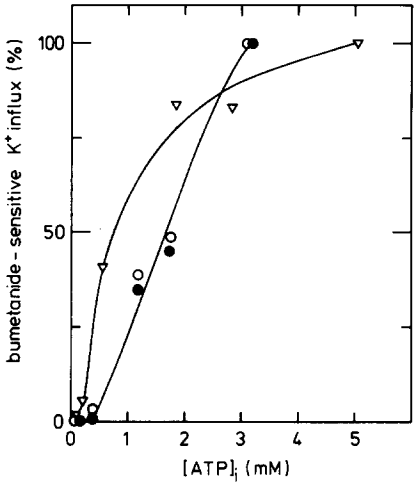

Fig. 1. Bumetanide-sensitive K^+ influx after activation by hypertonic shock, epinephrine or cyclic AMP as a function of intracellular ATP concentration. Cells were progressively depleted of ATP by preincubation with dinitrophenol, as described in Materials and Methods. $^{86}Rb^+$ influx was allowed to proceed from 2 to 17 min after rewarming. Influx was stopped by spinning the cells through silicone oil. Bumetanide-sensitive transport was activated by: ▽, 200 mM sorbitol; ●, 2 mM cyclic AMP plus 0.5 mM MIX; ○, 10 μM epinephrine. 100% values correspond to 264, 226 and 301 nmol $K^+ \cdot min^{-1} \cdot ml^{-1}$, respectively.

As before [11], we took for $(Na^+ + K^+ + 2Cl^-)$-cotransport activity the bumetanide-sensitive fraction of unidirectional $^{86}Rb^+$-influx, $^{86}Rb^+$ serving as tracer for K^+. This influx was determined in cells that had been depleted to different degrees of their ATP by preincubation with the uncoupler dinitrophenol. Bumetanide-sensitive transport was activated either by epinephrine (10 μM) or by cyclic AMP (2 mM) or by sorbitol (200 mM). Each of those treatments causes maximal activation under normal conditions [11]. As shown in Fig. 1, in no case was bumetanide-sensitive activity observed below 0.1 mM ATP. But, strikingly, the dependence upon intracellular ATP concentration was much steeper for transport activated by hypertonic shock than for that activated by either epinephrine or cyclic AMP (Fig. 1; halfmaximal transport rates at concentrations of ATP of 0.7 and 1.7 mM, respectively. In another experiment these concentrations were 0.4 and 1.2 mM, respectively).

TABLE I

BUMETANIDE-SENSITIVE VOLUME CHANGES IN NORMAL AND ATP-DEPLETED CELLS

Control cells and cells to which dinitrophenol (1 mM) had been added were preincubated for 90 min at 38°C. After washing, both control and dinitrophenol-treated cells were resuspended in (a) saline containing 2 mM cyclic AMP and an elevated K^+ concentration of 22.5 mM, or (b) saline containing 200 mM sorbitol and a decreased K^+ concentration of 0.2 mM. Cell water was determined as indicated in Materials and Methods, after a 60-min incubation at 38°C. At the end of this incubation, the cellular ATP concentration was around 2 mM (a) and 3 mM (b) in the control cells, and approximately 0.1 mM in the dinitrophenol-treated cells.

	Cell water (g/g dry wt.)	Bumetanide-sensitive volume change (%)
Control cells		
(a) cyclic AMP, high-K^+	1.37 ± 0.02	+7
+ bumetanide	1.28 ± 0.01	
(b) hypertonic, low-K^+	0.81 ± 0.01	−11
+ bumetanide	0.91 ± 0.02	
ATP-depleted cells		
(a) cyclic AMP, high-K^+	1.22 ± 0.02	0
+ bumetanide	1.22 ± 0.02	
(b) hypertonic, low-K^+	0.83 ± 0.01	−5
+ bumetanide	0.87 ± 0.02	

As shown in Table I, this difference in ATP requirement was also reflected in the bumetanide-sensitive volume changes that can be induced by manipulating the external K^+ concentration [11]. For the experiment of Table I, cells were either made to swell by activation with cyclic AMP at an elevated K^+ concentration, or to shrink by hypertonic activation at a lowered K^+ concentration. After depletion of cellular ATP by preincubation with dinitrophenol, the cyclic-AMP-dependent response was completely abolished, whereas the hypertonically-induced response at least partly persisted (Table I). A similar result was obtained when the transport system was activated with epinephrine instead of cyclic AMP (not shown).

These results are in agreement with the data presented in Ref. 10. They indicate that the activation pathways by which hypertonic shock and epinephrine induce transport activity differ in a rate-limiting step that requires ATP. Moreover, since the ATP dependence of transport was equal after activation by epinephrine and by cyclic AMP (Fig. 1), this step must have been distal to the synthesis of cyclic AMP.

In its classical mode of action, cyclic AMP activates a protein kinase that, either directly or through a kinase cascade, phosphorylates the target protein. Such a mechanism has been proposed for cyclic-AMP-induced bumetanide-sensitive transport in bird red cells as well [2,8]. In an attempt to gain more insight in the underlying activation mechanism, we decided to establish the relationship between bumetanide-sensitive transport and cyclic AMP concentration in two different setups: (i) after activating the bumetanide-sensitive system with either cyclic AMP or epinephrine, and (ii)

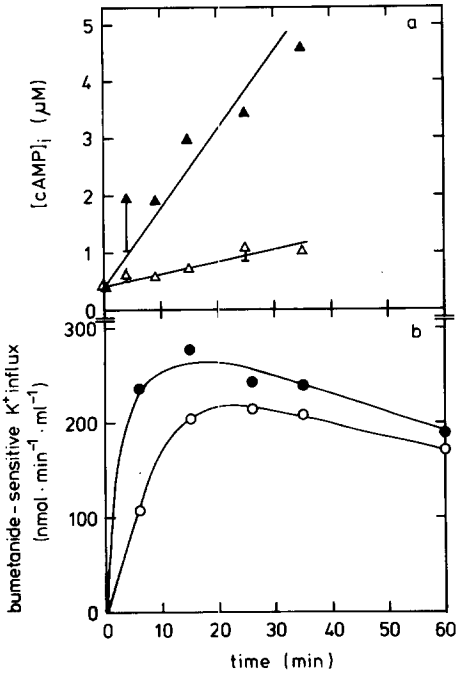

Fig. 2. Time-course of (a) intracellular cyclic AMP-concentration and (b) bumetanide-sensitive K^+ influx after addition of two different concentrations of cyclic AMP: (a) △, 0.12 mM; ▲, 1.3 mM; (b) ○, 0.20 mM; ●, 2.0 mM. 0.5 mM MIX was added to all incubations. Cyclic AMP concentrations and K^+ influx were determined as described in Materials and Methods; K^+ influx was measured over a 10-min interval centered around the indicated time points.

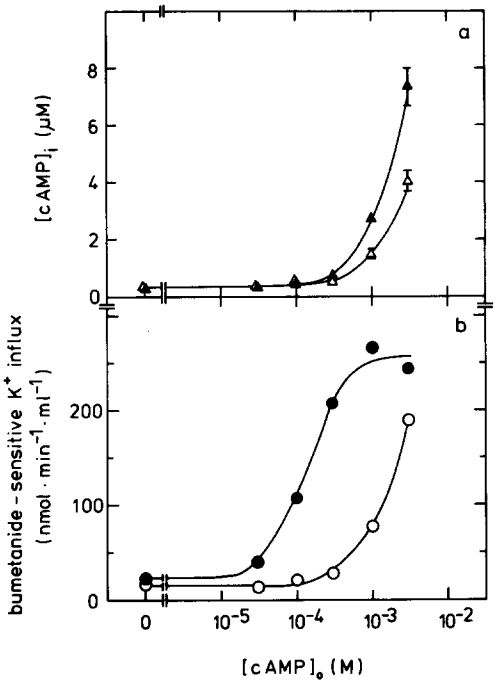

Fig. 3. (a) Intracellular cyclic AMP-concentration and (b) bumetanide-sensitive K^+ influx as a function of extracellular cyclic AMP concentration in the absence (open symbols) or presence (closed symbols) of 0.5 mM MIX. Cells were prewarmed for 2 min to 41°C and the incubation period started with the addition of cyclic AMP. After 15 min samples were withdrawn for determination of the cyclic AMP concentration; $^{86}Rb^+$ influx was allowed to proceed for 10 min, centered around this time point. For further details, see Materials and Methods.

after 'switching off' the system by washing away cyclic AMP or epinephrine from pre-activated cells.

Fig. 2 shows the time-course of the effect of two concentrations of externally-added cyclic AMP on both the intracellular cyclic AMP concentration (a) and bumetanide-sensitive transport activity (b). The intracellular cyclic AMP concentration increased linearly over 35 min, with a rate that was approximately proportional to its concentration (Fig. 2a and data not shown). A similar observation has been made by Garay [5] from human erythrocytes. By contrast, transport activity levelled off 10–20 min after addition of cyclic AMP (Fig. 2b).

Characteristically, the optimal influx was higher as the extracellular cyclic AMP concentration was raised. In our initial experiments, the transport rate declined again (up to 40% within 20 min) after reaching its optimum; more recently, transport activity has been stable over at least 60 min. We do not understand the reason for this discrepancy; we suppose it could be related to seasonal variations in hormonal balance of our donor turkeys. In the following, we have concentrated on the transport optimum, leaving the subsequent decline out of consideration.

In Fig. 3 we have plotted the intracellular cyclic AMP concentration and the transport activity, both measured 15 min after addition of cyclic AMP, as a function of extracellular cyclic AMP concentration. In the presence of MIX (closed symbols), a phosphodiesterase inhibitor, both curves were shifted to the left compared to controls without MIX (open symbols). Similar sets of curves were obtained when transport activity was stimulated by increasing concentrations of epinephrine (up to 10^{-4} M; results not shown, see also Ref. 14).

In Fig. 4, we have replotted bumetanide-sensitive transport activity after activation with cyclic AMP or epinephrine in the absence or presence of MIX as a function of intracellular cyclic AMP concentration. Although we cannot exclude a slight effect of MIX on the exact position of the curve (the closed symbols appear to be systematically positioned to the left compared to the open symbols), the relationship between transport activity and intracellular cyclic AMP concentration was largely independent of the mode of activation (cyclic AMP or epinephrine; circles and squares,

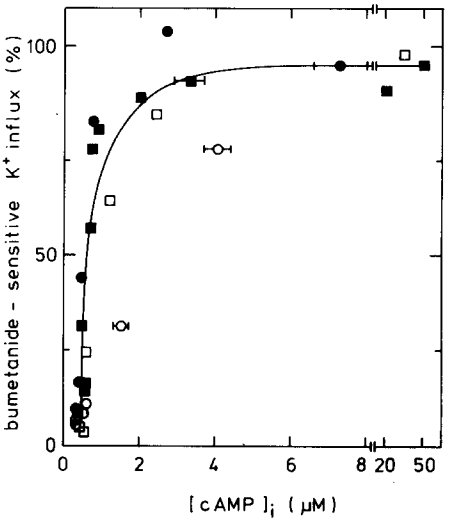

Fig. 4. Bumetanide-sensitive K^+ influx as a function of intracellular cyclic AMP concentration after activation with cyclic AMP or epinephrine. K^+ influx and intracellular cyclic AMP concentration were determined 15 min after addition of increasing concentrations of cyclic AMP (circles; data replotted from Fig. 3) or epinephrine (squares; separate experiment, see text) in the presence (closed symbols) or absence (open symbols) of 0.5 mM MIX. For further details, see Materials and Methods. 100% values correspond to 269 and 243 nmol K^+ per min and per ml cells for epinephrine- and cyclic-AMP-induced activation, respectively. Intracellular cyclic AMP concentrations measured for the three points to the right were 22, 38 and 47 μM, respectively.

respectively), or the absence or presence of the phosphodiesterase inhibitor. This observation confirms that in this system both epinephrine and MIX act through their effects on the intracellular cyclic AMP concentration. Note that bumetanide-sensitive transport was halfmaximally activated at an intracellular cyclic AMP concentration of about 0.5 μM. A similar value can be estimated from data reported by Kregenow [15].

In another set of experiments, we investigated the 'switch-off' behaviour of bumetanide-sensitive transport in relation to the intracellular cyclic AMP concentration. To this end, we loaded the cells with cyclic AMP by pretreatment with epinephrine, and then washed away the hormone. Fig. 5 shows that immediately after removal of epinephrine, both the intracellular cyclic AMP concentration and the bumetanide-sensitive transport activity dropped sharply, and that the decline of both parameters was retarded by MIX. The residual

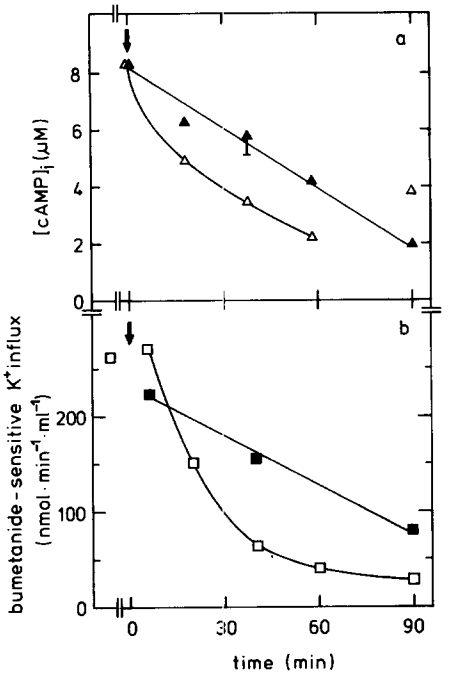

Fig. 5. Decline of (a) intracellular cyclic AMP concentration and (b) bumetanide-sensitive K^+ influx in the absence or presence of MIX after removal of epinephrine. Cells were preincubated for 10 min with 10 μM epinephrine, washed three times with 50 volumes of ice-cold standard medium and resuspended at 0°C in saline without (open symbols) or with (closed symbols) 0.5 mM MIX. At $t = 0$ cells were rewarmed to 41°C. Intracellular cyclic AMP concentrations (triangles) and K^+ influx (squares) were determined as described in Materials and Methods. $^{86}Rb^+$ was allowed to proceed for 10 min centered around the indicated time points.

decrease in intracellular cyclic AMP concentration in the presence of MIX was largely due to efflux of this nucleotide: from the extracellular cyclic AMP concentrations we calculated that after 1 h 40% of the original cyclic AMP content had been broken down in the control cells, compared to less than 10% in the MIX-treated cells.

In a similar experiment we preincubated the cells for 40 min with 1 mM cyclic AMP. Although the loading with cyclic AMP was only half as effective in this experiment (final concentration, 4 μM vs. 8 μM in that of Fig. 5), the subsequent decline of both transport activity and cyclic AMP concentration looked similar to Fig. 5 (not shown).

In Fig. 6, we have for both experiments replotted bumetanide-sensitive transport activity as a function of the remaining intracellular cyclic AMP concentration. In contrast to Fig. 4 different curves emerged for the two experiments. Also in a series if additional incubations, in which cells had been pretreated with increasing concentrations of epinephrine for different times, we failed to obtain a single relationship between residual transport activity and cyclic AMP concentration (not shown). By contrast, the presence or absence of MIX (closed and open symbols, Fig. 6) did not affect the individual relationships. These observations indicate that also in the 'switch-off' setup MIX affected bumetanide-sensitive transport mainly or exclusively through its effect on the cyclic AMP concentration; however, under these circumstances the latter parameter was clearly not the sole factor determining transport activity. Significantly, transport activity had declined by 50% or more at a cyclic AMP concentration (5 μM) that by far exceeded the concentration required to half-saturate transport activity in the 'switch-on' setup (cf. Figs. 6 and 4). Since the cells did not change their volume to any appreciable extent during the

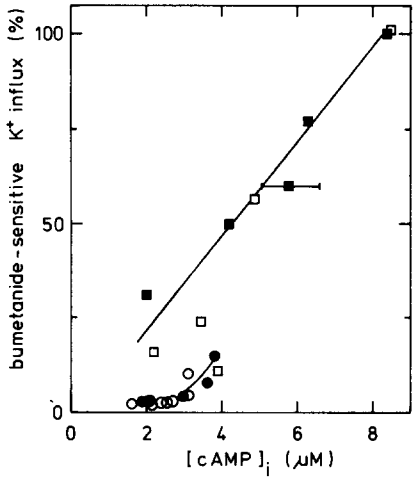

Fig. 6. Bumetanide-sensitive K^+ influx as a function of intracellular cyclic AMP concentration after removal of epinephrine or extracellular cyclic AMP. K^+ influx and intracellular cyclic AMP concentration were determined at different time points after washing away epinephrine (squares; data replotted from Fig. 5) or cyclic AMP (circles; separate experiment, see text), in the presence (closed symbols) or absence (open symbols) or 0.5 mM MIX. For further details, see Materials and Methods. 100% values (measured immediately before removal of epinephrine or cyclic AMP) correspond to 262 and 342 nmol $K^+ \cdot min^{-1} \cdot (ml\ cells)^{-1}$, respectively.

'switch-off' incubations, cell swelling cannot have been a cause [11,2] of this premature inactivation. This is confirmed by the observation that in these inactivated cells transport activity was restored to its original level by the readdition of epinephrine or cyclic AMP (not shown).

Discussion

The main findings presented here are the following: (i) bumetanide-sensitive transport in turkey red cells was ATP-dependent. The ATP level required for hypertonically-induced transport was significantly lower than that required for either epinephrine- or cyclic AMP-induced transport (Fig. 1b, Table I).

(ii) There was no unequivocal relationship between bumetanide-sensitive transport activity and cyclic AMP concentration. Specifically, after the removal of epinephrine or extracellular cyclic AMP from preactivated cells, transport activity had declined by 50% or more at a cyclic AMP concentration that was approx. 10-fold that required to activate the transport system (Figs. 4,6).

The first finding is in agreement with the data reported in [10]. Clearly, our experimental setup (activation of transport after ATP depletion) makes it impossible to distinguish between any effects of ATP on the activation process or on transport activity per se. It therefore may hardly come as a surprise that epinephrine- or cyclic AMP-induced bumetanide-sensitive transport was ATP-dependent: this is what would be expected for an activation cascade involving at least one, per definition ATP-requiring, protein kinase. However, the ATP dependence of the hypertonically-induced transport (which apparently does not act through a rise in the intracellular cyclic AMP concentration [2]) indicates that this nucleotide is required in a more fundamental capacity as well. As has been proposed for other cell types [4], ATP may bind to some regulatory site on the transport protein.

Although our first finding is in agreement with a classical mode of action of cyclic AMP, the other observation suggests that the effect of cyclic AMP is modulated by secondary cyclic-AMP-involving processes. Specifically, once fully activated, bumetanide-sensitive transport appeared to decline irrespective of the actual intracellular cyclic AMP concentration, whenever this concentration decreased in time: be it by phosphodiesterase activity, leakage out of the cell, or a combination of both (Figs. 5, 6 and text). How exactly the disappearance of cyclic AMP independent of its absolute concentration may affect bumetanide-sensitive transport is unclear at present. Because of the huge buffer capacity of hemoglobin a pH effect can be excluded. Our data do tie in to some extent with those obtained by Goldberg et al. [16]. These authors have established that cyclic GMP turnover rather than the cyclic GMP concentration itself is the key parameter in determining the hyperpolarization of the photoreceptor.

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