Biochimica et Biophysica Acta, 1110 (1992) 199–201

BBAMEM 75764

Activation of the $Na^+/K^+/Cl^-$ cotransport system by reorganization of the actin filaments in Ehrlich ascites tumor cells

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(Received 23 April 1992)

Key words: Sodium/potassium/chloride cotransport; Volume regulation; Actin; Microfilament; Cytochalasin B; (Ehrlich ascites tumor cell)

Reorganization (disassembly) of the actin filaments in Ehrlich ascites tumor cells, either by hypotonic treatment in the presence of Ca^{2+} or by addition of cytochalasin B, results in activation of the $Na^+/K^+/Cl^-$ cotransport system. However, other regulatory processes, some of which may be dependent on an intact filament system, are responsible for the activation of the $Na^+/K^+/Cl^-$ cotransport system after cell shrinkage.

Introduction

In common with many other mammalian cells, Ehrlich ascites tumor cells show a volume recovery (regulatory volume increase) when hypotonic pretreatment, resulting in a net loss of KCl, is followed by restoration of tonicity (see Ref. 1). After the initial osmotic shrinkage, the cells recover their volume with an associated KCl uptake. The primary process is an activation of an otherwise quiescent bumetanide-inhibitable anion/cation cotransport system (with subsequent replacement of Na^+ by K^+ via the Na^+/K^+-pump, stimulated by the Na^+ influx) [2], demonstrated to be a $Na^+/K^+/Cl^-$ cotransport system [3,4]. It was recently demonstrated, that the $Na^+/K^+/Cl^-$ cotransporter is actually activated all ready during the hypotonic pretreatment, and that maximal activity was obtained within 10 min after transfer to hypotonic medium (unpublished results).

In the period 1–15 min after transfer of Ehrlich ascites tumor cells to hypotonic medium, a dramatic change is observed in the organization of the microfilament network [5] as also described in shark rectal gland by Ziyadeh et al. [6]. Microfilaments, as seen by the Immunogold/Silver staining method using anti-actin antibodies, appear in Ehrlich cells as a dense network distributed in the whole cytoplasm. After 15 min in hypotonic medium, the cytoplasm appears poorly stained with patches of actin, present essentially only close to the membrane. Thus, actin reorganization accompany volume regulation in hypotonic medium [5].

When the hypotonic medium is Ca^{2+} free and contains EGTA, no osmotically induced reorganization of the microfilament is seen [5]. We, therefore, examined whether activation of the $Na^+/K^+/Cl^-$ cotransport system is affected by the presence of external Ca^{2+} in the hypotonic medium.

Materials and Methods

The activation of the $Na^+/K^+/Cl^-$ cotransport system in all experiments is measured as the bumetanide-inhibitable K^+ influx using 30 μM bumetanide. At this concentration, bumetanide inhibits the $Na^+/K^+/Cl^-$ cotransporter more than 80% [7] whereas the K^+/Cl^- cotransporter is only expected to be inhibited by about 5% [8]. Furthermore, the bumetanide-inhibitable K^+ influx has been demonstrated to be strictly Na^+-dependent during hypertonic conditions as well as during hypotonic conditions (unpublished results).

Results and Discussion

Fig. 1 shows that after 10 min in hypotonic medium the $Na^+/K^+/Cl^-$ cotransporter is activated when ex-

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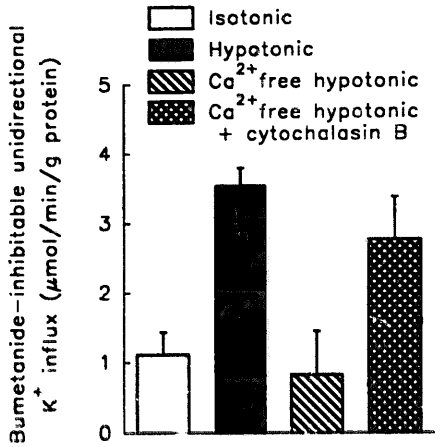

Fig. 1. Effect of hypotonic treatment in the presence and absence of calcium on the bumetanide-inhibitable potassium influx in Ehrlich ascites tumor cells. The bumetanide-inhibitable K^+ influx was measured as the difference between the influx in the absence and the presence of bumetanide (30 μM). $^{86}Rb^+$ was used as tracer for K^+. The cells were preincubated for 15 min. At time zero, the flux was initiated by addition of 1/3 volume flux-medium, containing isotope and bumetanide when required. The suspensions were incubated at 37°C and samples were removed at intervals for separation of cells from medium accomplished by cation exchange chromatography [16]. The samples of cell suspensions with tracer were applied to chilled ion-exchange columns and washed through with a ice-cold isotonic sucrose-bovine serum albumin solution. Radioactivity of cell lysates was determined by liquid scintillation counting. The fluxes were determined from samples taken at 1.0, 1.5, 2.0 and 2.5 min by linear regression. Isotonic: both preincubation medium and flux medium were isotonic standard medium (300 mOsm) with the following composition (mM): Na^+, 150; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 150; sulfate, 1; inorganic phosphate, 1; 3-(*N*-morpholino)propane sulfonic acid (Mops), 3.3; *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (Tes), 3.3; *N*-2-Hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes), 5 (pH 7.4). Hypotonic: after preincubation in isotonic standard medium, the cells were incubated for 10 min in hypotonic standard medium (150 mOsm) with the following composition (mM): Na^+, 75; K^+, 2.5; Mg^{2+}, 0.5; Ca^{2+}, 0.5; Cl^-, 75; sulfate, 0.5; inorganic phosphate, 0.5; Mops, 3.3; Tes, 3.3; Hepes, 5 (pH 7.4), before the flux was initiated in this medium. Ca^{2+}-free hypotonic: after preincubation in Ca^{2+}-free isotonic standard medium containing EGTA (1 mM) the cells were incubated for 10 min in Ca^{2+}-free hypotonic standard medium containing EGTA (1 mM), before the flux was initiated in this medium. Ca^{2+}-free hypotonic + cytochalasin B: the procedures and media were the same as under Ca^{2+}-free hypotonic conditions, but the cells were incubated with cytochalasin B (42 μM) for 1 min before the flux was initiated. The vertical bars represent S.E. of 7, 3, 3 and 3 experiments in the isotonic, hypotonic, Ca^{2+}-free hypotonic and Ca^{2+}-free hypotonic + cytochalasin B groups, respectively.

ternal Ca^{2+} is present, but not in its absence. Since reorganization of the microfilaments is seen when external Ca^{2+} is present, but not in its absence, this supports the hypothesis that the activation of the $Na^+/K^+/Cl^-$ cotransporter is related to reorganization of actin filaments. Cytochalasin B is known to cause reorganization of microfilaments (see Ref. 9). Addition of cytochalasin B (42 μM) to Ehrlich cells in the hypotonic, Ca^{2+} free medium results within 1 min in activation of the cotransporter (Fig. 1). This is in clear contrast to the effect of cytochalasin B on the K^+/Cl^- cotransporter. Garay et al. [10] found in human red cells that cytochalasin B strongly depressed the hypoosmotically induced K^+/Cl^- cotransporter. Moreover, it is found [5] that cytochalasin B strongly depresses the hypoosmotically-induced activation of K^+ and/or Cl^- channels in Ehrlich cells. This is also in contrast to the effect of cytochalasin B on the $Na^+/K^+/Cl^-$ cotransport system demonstrated here.

From the results of Cornet et al. [5] and the results in Fig. 1, it is strongly suggested that the actin reorganization after swelling in hypotonic medium was caused by an increase in free intracellular Ca^{2+} resulting from Ca^{2+} influx. It has recently been shown that nonspecific, cation-selective channels which are permeable to Ca^{2+} are activated after swelling of Ehrlich cells in hypotonic medium [11]. Whether such channels allow the entry of sufficient Ca^{2+} to cause the reorganization of actin filaments is not known. A significant cell depolarization during regulatory volume decrease has been observed in Ehrlich cells [12]. Hypothetically, such membrane depolarization might also result in Ca^{2+} entry via activation of voltage gated Ca^{2+} channels. It is well-known from other systems that Ca^{2+} can promote reorganization of actin filaments (see, for example, Ref. 9).

It should be noted that intracellular Cl^- is decreased after 10 min in hypotonic medium and it has previously been suggested that this decrease may be an important factor in activating the $Na^+/K^+/Cl^-$ cotransporter [13]. However, in Ehrlich cells the loss of Cl^- and volume in hypotonic medium is found to be independent of the presence of external Ca^{2+} [14] ruling out Cl^- as an important factor.

Cytochalasin B is known to inhibit actin assembly by inhibiting the addition of actin monomers to the filaments (see Ref. 9) and is found to cause a dramatic change in the microfilament organization in Ehrlich cells [5]. Fig. 2 demonstrates that addition of cytochalasin B (42 μM) to the cells in isotonic medium, within 1 min results in a 4-fold activation of the $Na^+/K^+/Cl^-$ cotransporter, indicating that reorganization of the filament system also in isotonic medium is able to activate the cotransporter. It should be noted that pharmacological stabilization of F-actin microfilaments prevents cAMP-stimulated Cl^- secretion by T84 cells [15]; the authors suggest as one possibility that the effect might be on the basolateral $Na^+/K^+/Cl^-$ cotransporter.

A more dramatic activation of the cotransporter is seen after cell shrinkage in hypertonic solution. Cell shrinkage does not seem to result in any reorganization of the microfilaments [5]. Fig. 3 demonstrates that the activation of the cotransporter by hypertonic treatment is not affected by the presence or absence of external

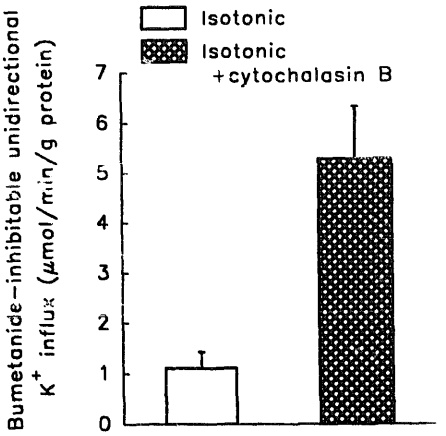

Fig. 2. Effect of cytochalasin B on the bumetanide-inhibitable unidirectional potassium influx. The cells were preincubated in an isotonic standard medium for 15 min. The K^+ influxes were measured, as described in Fig. 1, under isotonic conditions (open bar) and after incubation with cytochalasin B (42 μM) for 1 min (shaded bar). The vertical bars represent S.E. of 7 and 5 experiments in the isotonic and isotonic + cytochalasin B groups, respectively. The bumetanide-insensitive flux was slightly (14 ± 3% S.E., $n = 5$) inhibited by cytochalasin B.

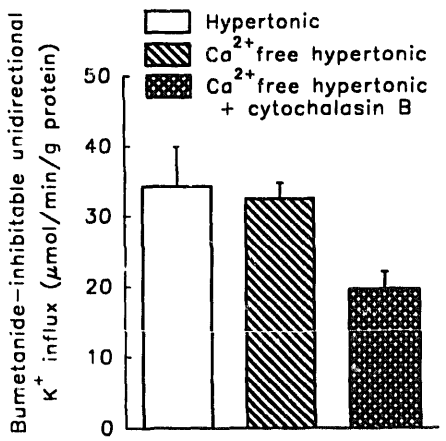

Fig. 3. Effect of hypertonic treatment in the presence and absence of calcium on the bumetanide-inhibitable potassium influx. The experiments were performed as described in Fig. 1. Hypertonic: after preincubation in isotonic standard medium, the flux was measured during the first two minutes in hypertonic medium (400 mOsm) with the following composition (mM): Na^+, 200; K^+, 6.7 Mg^{2+}, 1.3; Ca^{2+}, 1.3; Cl^-, 200; sulfate, 1.3; inorganic phosphate, 1.3; Mops, 3.3; Tes, 3.3; Hepes, 5 (pH 7.4). Ca^{2+}-free hypertonic: after preincubation in Ca^{2+}-free isotonic standard medium containing EGTA (1 mM), the flux was initiated in Ca^{2+}-free hypertonic medium containing EGTA (1 mM). Ca^{2+}-free hypertonic + cytochalasin B: The media were the same as under Ca^{2+} hypertonic conditions, but the cells were incubated with cytochalasin B (42 μM) for 1 min before the flux was initiated. The vertical bars represent S.E. of three experiments in all groups, respectively.

Ca^{2+}. Under these circumstances, disruption of the filament system with cytochalasin B prior to the hypertonic treatment results in a less dramatic activation. It is, thus, obvious that other regulatory processes, some of which may even be dependent on an intact filament system, are responsible for the activation of the $Na^+/K^+/Cl^-$ cotransport system after cell shrinkage.

In conclusion, disruption of the microfilament system in Ehrlich cells results in a partial activation of the $Na^+/K^+/Cl^-$ cotransporter. However, maximal activation of the cotransport system requires other regulatory pathways that are currently under investigation.

Acknowledgements

We are grateful to Ms. Marianne Schiødt for expert technical assistance. This work was supported by grants from the Danish Natural Science Research Council (11-6835 to E.K.H.) and by a Carlsberg Foundation research fellowship (F.J.).

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