Vol. 207, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 6, 1995 Pages 375-381

AN ATYPICAL ANION TRANSPORTER FUNCTIONING AT ACID pH IN NEUROBLASTOMA CELLS

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Received December 19, 1994

At pH 7.4, $^{36}Cl^-$ uptake by neuroblastoma cells was Na^+-independent, saturable and blocked by submicromolar concentrations of DIDS. This suggests that at this pH, Cl^- transport is mediated by an exchanger analogous to erythroid band 3. At pH 6.2, $^{36}Cl^-$ uptake was markedly activated by external carboxylate anions such as acetate. Acetate-stimulated $^{36}Cl^-$ uptake was blocked by DIDS (IC_{50} = 0.15 µM). Saturation by external $^{36}Cl^-$ was observed with $K_{0.5}$ = 8 mM. $K_{0.5}$ was not modified by acetate. As ^{36}Cl efflux is also activated by acetate, we suggest the presence, in neuroblastoma cells, of an anion exchanger activated by carboxylic anions. This exchanger is active when the extracellular pH is 6.0 - 6.5.

Electroneutral, Na^+-independent anion exchangers (AE) are an important and probably ubiquitous family of membrane proteins. The first and most extensively studied was the "band 3" protein of erythrocyte membranes (for review, see Ref. 1). This AE1 exchanger catalyzes Cl^-/HCO_3^- or Cl^-/Cl^- exchange and is blocked by submicromolar concentrations of the stilbenedisulfonate DIDS (2 - 4). In addition to AE1, two other forms of the exchanger, AE2 and AE3 have been cloned (5, 6). It seems that mRNAs of the AE3 form are essentially found in neurons and heart (5). Both AE1 and AE3 are very sensitive to DIDS, while AE2 was reported to be less sensitive (7, 8). Both AE2 and AE3 exhibit a steep dependence on internal pH between

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Abbreviations used: AE, anion exchanger; DIDS, 4,4'-diisothiocyanostilbene-2-2'-disulfonic acid; NMDG, N-methyl-D-glucamine; pH_i, intracellular pH.

0006-291X/95 $5.00

7 and 8 : maximum activity is observed at $pH_i \geq 7.4$ while the exchange is much slower at $pH_i \leq 7.0$ (7). AE2 and AE3 are believed to play a role in the regulation of cytosolic pH by exchanging internal HCO_3^- for external Cl^- in case of alkaline load (7, 9, 10). If this is the main role of the exchanger, its activity should be near zero at pH_i lower than physiological; otherwise, HCO_3^- extrusion would further acidify the cell. In this report, we show that a Na^+-independent chloride transporter, with atypical properties, is active at acid pH.

MATERIALS AND METHODS

Chemicals

Bumetanide, 4,4'-diisothiocyanostilbene 2,2'-disulfonic acid (DIDS) and N-methyl-D-glucamine (NMG) were from Sigma. [$^{36}Cl^-$]NaCl was from Amersham.

Determination of $^{36}Cl^-$uptake

Mouse neuro 2A cells (11) were cultured as described (12). Cells were transferred to multidish 6 wells (Gibco) and grown for 3 days in a humidified atmosphere of 5 % CO_2/95 % air, until the protein content was 0.25-0.5 mg per well. Prior to the experiment, the cells were washed with 3 x 2 ml sucrose medium containing 0.29 M sucrose, 3 mM K-gluconate, 0.5 m M $MgCl_2$, 10 mM glucose, 10 mM Hepes-Tris (pH 7.4) or 10 mM Mes-Tris (pH 6.2). 1 ml of sucrose medium at 37° was added, with 10 µl of various anions or DIDS, followed by 20 µl of $^{36}Cl^-$ (final concentration = 0.2 µCi/ml). After various time intervals (90s for initial influx measurements), the uptake was stopped by rapid removal of the incubation medium and washing with 3 x 2 ml ice-cold saline (145 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM Hepes-Tris pH 7.3. The cells were then dissolved in 1 ml 0.1 M NaOH. 800 µl of the extract were neutralized with 80 µl of HCl 1N and counted by liquid scintillation. The remaining 200 µl were used for protein determination by the method of Peterson (13).

Efflux of $^{36}Cl^-$

The cells were preincubated 2 h in 1 ml gluconate medium containing 145 mM Na-gluconate, 3 mM K-gluconate, 0.5 mM Mg-acetate, 0.5 mM Ca-acetate, 10 mM glucose, 10 mM Mes-tris pH 6.2 and 0.5 µCi $^{36}Cl^-$. After washing with cold sucrose medium, $^{36}Cl^-$ efflux was initiated by addition of 2 ml of efflux medium at 37°. After various time intervals, the radioactivity remaining in the cells was measured as described above.

RESULTS AND DISCUSSION

As shown in Fig. 1A, neuro 2 A cells take up $^{36}Cl^-$ at a relatively high rate in sucrose medium at pH 7.4. External Na^+ was not required and saturation by Cl^- was observed with $K_{0.5}$ = 5 mM. As expected, $^{36}Cl^-$ influx was slower at pH 6.2 (Fig. 1A). However, when the experiments were

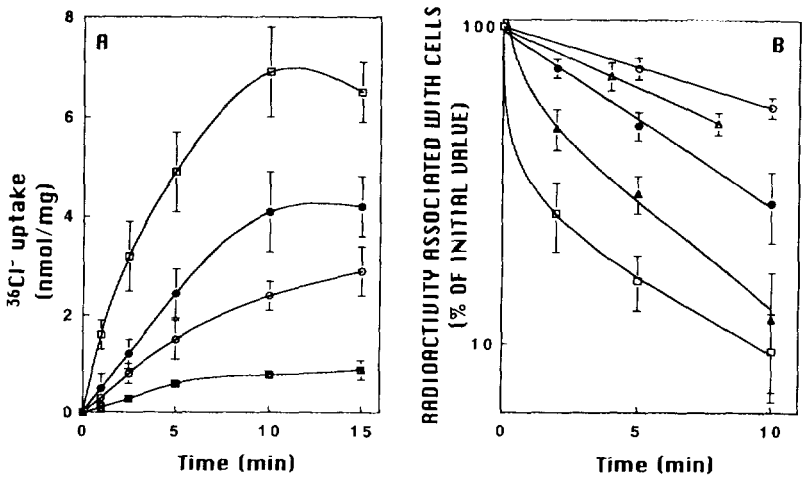

Fig. 1. **(A)** $^{36}Cl^-$ uptake as a function of time in neuroblastoma cells at pH 7.4 (filled symbols) and 6.2 (open symbols) in the presence (squares) or absence (circles) of sodium acetate, 10 mM. For experimental protocol, see Materials and Methods. Each point represents the mean ± SD for 3 experiments. **(B)** $^{36}Cl^-$ efflux from neuroblastoma cells at pH 7.4 (filled symbols) or 6.2 (open symbols). Circles, control sucrose medium; squares, NaCl 20 mM; triangles, Na-acetate, 10 mM. The cells were loaded with $^{36}Cl^-$ as described in Materials and Methods. The composition of efflux media was K-gluconate, 3 mM; Mg-gluconate, 1 mM; Glucose, 10 mM; Hepes-Tris (pH 7.4) or Mes-Tris (pH 6.2), 10 mM; sucrose, 0.29 M in the absence of added salts; in the presence of salts, the sucrose concentration was decreased to maintain constant osmolarity. Each point represents the radioactivity remaining in the cells at the time indicated. The error bars represent the mean ± SD for 4 experiments.

reproduced at a higher ionic strength (see also Table I) initial $^{36}Cl^-$ influx values were increased sometimes to such an extent that $^{36}Cl^-$ uptake was higher at pH 6.2 than pH 7.4. This activation was not due to the presence of a particular cation in the medium but the nature of the external anion appears to be important. At pH 6.2 , as at pH 7.4, $^{36}Cl^-$ influx was essentially Na^+-independent. The uptake was generally slightly larger in the presence of Na^+ than with substituent cations (K^+ or NMG^+) but the difference was not statistically significant (not shown). This predominance of Na^+-independent transport is somewhat surprising as it was recently shown (14) that, in differentiated mammalian neurons, Na^+-dependent Cl^-/HCO_3^- exchange was the main mechanism for recovery from acid load. Results obtained at pH 6.2 with chloride or bicarbonate were difficult to interpret as those anions compete with $^{36}Cl^-$ for transport. We therefore used carboxylate anions, which are known to be poor substrates (if transported at all) for AE1 (1). As shown in Table I , acetate ($K_{0.5}$ = 5 mM), as well as a number of other carboxylate ions, was a very effective activator of $^{36}Cl^-$ uptake at pH 6.2, whereas it was moderately inhibitory at pH 7.4.

Table I. Effect of various anions and DIDS on initial $^{36}Cl^-$ uptake in neuro 2A cells at pH 6.2 and 7.4

	pH = 6.2	pH = 7.4
Control	3.0 ± 1.4	10 ± 2
+ DIDS (1 μM)	0.5 ± 0.2	1.9 ± 0.3
Na-formate	9.9 ± 0.7	-
Na-acetate (10 mM)	11 ± 2	7.3 ± 0.9
+ DIDS (1 μM)	2.1 ± 0.5	1.9 ± 0.3
Na-acetate (20 mM)	12 ± 3	-
Na-pyruvate (20 mM)	7 ± 2	-
Na-lactate (20 mM)	9 ± 2	-
Na-gluconate (100 mM)	9 ± 3	-
NMG-gluconate (20 mM)	5 ± 2	-
NMG- gluconate (100 mM)	12 ± 4	-
Na-isethionate (145 mM)	3.4 ± 0.3	-

The incubation medium (37°C) contained 3 mM K-gluconate, 1 mM Mg-gluconate, 10 mM glucose, 10 mM Hepes-Tris (pH 7.4) or Mes-Tris (pH 6.2) and 0.29 M sucrose in controls; in the presence of salts, the sucrose concentration was decreased to maintain constant osmolarity. DIDS was added to the well immediately before the tracer, so that irreversible covalent binding was minimized. Initial uptake is expressed in nmol $^{36}Cl^-$ taken up per mg protein per min, as mean ± SD for 3 to 6 experiments.

Interestingly, the transporter at acid pH has a certain specificty for carboxylic anions. Isethionate (but not gluconate) for instance is without significant effect, thus, being an appropriate "spectator" anion in our conditions. $^{36}Cl^-$ uptake was strongly inhibited by low (1 μM) concentrations of DIDS (see Table I). Under all conditions (pH = 6.2, pH = 7.4, presence and absence of acetate), the IC_{50} for DIDS was around 0.15 μM. No preincubation with the inhibitor was required, suggesting that DIDS forms a high-affinity reversible complex with the transporter. External chloride protected against DIDS inhibition (not shown).

We could find no evidence for Na^+-K^+-$2Cl^-$ cotransport in neuro 2A cells. The specific inhibitor bumetanide (15) had no effect on $^{36}Cl^-$ uptake even in the presence of external Na^+ and K^+; $^{22}Na^+$ uptake was very slow and was not activated by external KCl (not shown). Some ouabain-insensitive $^{86}Rb^+$ uptake was observed but it was insensitive to bumetanide (not shown).

We investigated $^{36}Cl^-$ efflux, with the results shown in Fig. 1B. First of all, at pH 7.4, $^{36}Cl^-$ efflux was stimulated about twice by 20 mM external Cl^-. This is in contrast with mammalian erythrocytes, where the AE1-mediated chloride efflux is strictly dependent of the presence of Cl^- or other anions that can be exchanged (1). However, a $^{36}Cl^-$ efflux independent of external anions is observed in many other cell lines (9). The mechanism underlying this flux remains unclear and might involve Cl^- channels. Our results

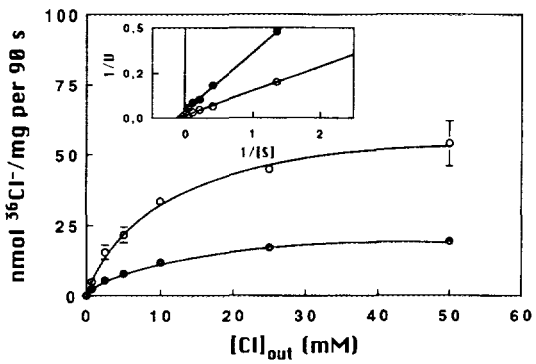

Fig. 2. Initial $^{36}Cl^-$ uptake at pH 6.2 as a function of extracellular chloride concentration. Uptake was measured in sucrose medium (composition as in legend to Table I) containing the indicated concentration of NaCl. Sucrose concentration was decreased to maintain isosmolarity. Filled circles, control; open circles, 10 mM Na-acetate present in the incubation medium. Error bars represent the mean SD for 4 experiments.

nonetheless indicate that at least 50 % of the chloride efflux at pH 7.4 is mediated through Na^+-independent Cl^-/Cl^- exchange. As already reported by Olsnes *et al.* (9) using other cell lines, the efflux was slower at pH 6.2 than pH 7.4. Addition of external NaCl (20 mM) increased the rate of efflux but it remained relatively slow. However, addition of external Na-acetate strongly activated $^{36}Cl^-$ efflux. This result suggest that carboxylate anions stimulate an anion exchanger which has particular properties. The known homologs of the erythrocyte anion exchanger (AE2 and AE3) are activated at alkaline intracellular pH (5,7,16), and as Na-acetate should decrease the internal pH (9), it is not likely that an AE1 or AE3 type of exchanger is activated under those conditions. Furthermore, when neuro 2a cells were preincubated in 125 mM K^+ and nigericin (1 µg/ml) at various pH values, $^{36}Cl^-$ uptake was still highest at acid and lowest at alkaline pH (not shown). Under these conditions, the membrane potential is close to 0 mV (12) and nigericin (which carries out electroneutral K^+/H^+ exchange) abolishes any existing pH gradient: i.e; the intracellular pH is clamped at the extracellular pH value (17). Thus intracellular pH seems to be less important than the extracellular pH, and no activation is observed at alkaline pH_i, in contrast to what is observed with AE2 and AE3.

Our data suggest that chloride transport at pH 7.4 is mediated through a Na^+-independent Cl^-/Cl^- exchanger of the band 3 family, possibly AE3 as the latter may be expressed in neuronal cells (5) and is blocked by low concentrations of DIDS (7). Our experimental data would be consistent with the idea that, even at pH 6.2, $^{36}Cl^-$ is mediated by an exchanger of the AE1-AE3 family : as shown in Fig. 2, chloride influx is saturable with $K_{0.5}$ around 8 mM. The same value was found in the absence or in the presence of 10

mM acetate. Another similarity is the reversible inhibition by low concentrations of DIDS : as shown in Table I, 1 μM DIDS was effective in the presence of acetate, without preincubation. DIDS may react covalently with unprotonated amino groups but this is unlikely to occur at acid pH in less than 2 min; thus, DIDS presumably forms a non-covalent high-affinity complex with the transporter activated by acetate. It can be concluded that acetate stimulates $^{36}Cl^-$ uptake by an allosteric mechanism without interacting directly with Cl^- or DIDS binding.

It has been known for a long time that acetate, at high concentrations, is a non-competitive blocker of anion exchange in erythrocytes (18). Activation of chloride exchange (by anions which are not transported) has never been observed for exchangers of the AE1 type. Our results cannot be attributed to an experimental artifact as in Vero cells, which have been extensively characterized with respect to chloride transport (9,10, 16), we did not find any activation of $^{36}Cl^-$ uptake by acetate at acid pH.

The possibility that Cl^- is exchanged for carboxylate anions at acid pH needs further investigation. Such a mechanism was recently described in epithelial cells of the distal colon (19). In neuronal cells, such transporters might be involved in the regulation of the internal concentrations of various anions and thus indirectly modify the amount of current carried when anion channels open.

ACKNOWLEDGMENTS

We thank the Belgian National Funds for Scientific Research (FNRS) for a grant to L. Bettendorff. P. Wins is research associate at the FNRS.

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