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ACTIVE ACCUMULATION AND EXCHANGE TRANSPORT OF CHLORIDE IN ASTROGLIAL CELLS IN CULTURE

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Chloride transport in primary cultures of astroglial cells from rat brain shows saturation kinetics, is inhibited by SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), and exchanges with HCO_3^-. These properties are similar to those of the anion exchange system in erythrocytes. Estimates of intracellular Cl^- concentration($[Cl^-]_i$) in the primary cultures give values in the range of 31 to 43 mM, which are 3- to 5-times greater than predicted from equilibration with an average measured membrane potential of −70 mV, suggesting that these cells also actively accumulate Cl^-.

Introduction

Astroglial cells occupy approx. 20% of the total cell volume of the grey matter in the mammalian central nervous system [1], and their processes are to be found in many areas of the central nervous system such as around synapses and blood vessels [2]. One of the proposed roles for astroglia is an involvement in chloride transport, which may perhaps be related to acid-base control and cerebrospinal fluid production in the central nervous system not involving the choroid plexus [3–6].

We have already presented some evidence that Cl^- transport in primary cell cultures from neonatal rat brain [7] occurs by a mediated process which shows many similarities to the electrically neutral Cl^- exchange process in red blood cells [8]. Such primary cultures from neonatal rat brain have been shown by many workers to consist predominantly of astroglia [9], and in our hands these cultures show 70 to 90% positive staining for the specific astroglial marker, glial fibrillary acidic protein [10]. In this report I present more detailed data on the kinetics of Cl^- uptake and SITS inhibition in these cultures and show that $^{36}Cl^-$ efflux from the cells is stimulated by the presence of Cl^- or HCO_3^- in the medium. Also, intracellular Cl^- concentrations ($[Cl^-]_i$) in these cultures, calculated from steady-state $^{36}Cl^-$ levels and estimates of intracellular volume, are several-fold higher than would be expected if $[Cl^-]_i$ were in equilibrium with membrane potentials of −65 to −75 mV that we have measured in these cultures [11]; values within the range found for astroglial cells in vivo [12].

Methods

Cell culture. Cells were started from the cerebral hemispheres of 1-day-old rats and grown in 60-mm diameter plastic petri dishes in supplemented Eagle's basal medium plus 20% fetal calf serum, as previously described [7,10,11].

Measurement of Cl^- uptake. These experiments were performed as previously described [7]. Growth medium was poured off and 2.5 ml of the following medium was added (mM): NaCl 122; KCl 3; $CaCl_2$ 1.3; $MgSO_4$ 1.2; KH_2PO_4 1.2; Hepes (titrated to give a final pH of 7.4 with NaOH at 37°C) 20; glucose 10.

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

To reduce medium Cl^-, sodium chloride was replaced with sodium isethionate. After preincubation in this medium for 1 h at 37°C in an air atmosphere, 3 μCi of $^{36}Cl^-$ as NaCl at a specific activity of 4.4 mCi/g was added. After appropriate times at 37°C the dishes were rapidly washed 7 times with 3 ml of ice-cold 0.32 M sucrose using a repipet (Labindustries). The total wash time took about 20 s. After the final wash the cells were removed from the dishes by scraping into 2.5 ml distilled water and were then sonicated for 15 s in a test tube in a bath sonicator. 2-ml aliquots were taken for counting $^{36}Cl^-$, and 50 μl for determining protein.

Measurement of Cl^- efflux. Cultures were allowed to equilibrate at 37°C in medium identical to that described for the uptake experiments containing 3 μCi $^{36}Cl^-$ per dish. After 1 h at 37°C the cells were washed as described above and then warm media of the same composition but with Cl^- replaced by isethionate, or media containing normal Cl^- or isethionate plus 10 mM $NaHCO_3^-$, was added. At various times dishes were again washed as before and the amount of cpm/mg protein remaining in the cells was determined.

Materials. These were obtained and used as previously described [7].

Results and Discussion

Saturation kinetics for Cl^- uptake

The dependence of the initial rate of Cl^- uptake, measured with $^{36}Cl^-$ for a 1-min period, on varying external Cl^- concentrations is shown in Figs. 1A and 1B. Fig. 1A also shows the inhibition of this Cl^- uptake by 1 mM SITS. In Fig. 1A the continuous lines show the best fit to the Michaelis-Menten equation for v versus s, using a computer program following the method of Bliss and James [13], and double reciprocal plots of these data are shown in Fig. 1B. The mean value of V and apparent $K_{1/2}$ values are given in Table I.

Saturation kinetics for Cl^- transport and SITS sensitivity has been found for a number of cells including erythrocytes [8,14–16], Ehrlich Ascites tumor cells [17–19] and frog cornea [20]. $K_{1/2}$ value of 36.2 mM for total Cl^- uptake is close to the apparent $K_{1/2}$ value of 23.4 mM for external Cl^- reported for Cl^- transport in Ehrlich ascites cells

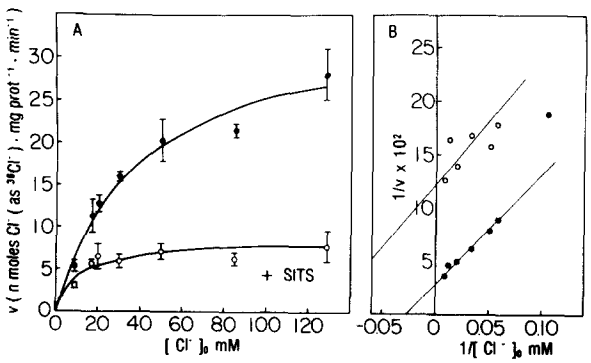

Fig. 1. A. Rate of uptake of Cl^- (v) as a function of external Cl^- concentration ($[Cl^-]_o$). Uptake of $^{36}Cl^-$ was measured as described in Methods in Hepes medium and is expressed as nmol $Cl^- \cdot (mg\ protein)^{-1} \cdot min^{-1}$ based on the specific activity of $^{36}Cl^-$ in the external medium. SITS at a final concentration of 1 mM was added 15 min prior to addition of $^{36}Cl^-$ for the (o) data. To correct for retention of $^{36}Cl^-$ after washing, dishes plus cells were treated in an identical manner but washed immediately after adding $^{36}Cl^-$. The remaining counts were found to be 112 ± 54 cpm per dish (±S.D., $n = 24$) and this was used as a zero-time uptake. Uptake after 1 min without SITS was 1 359 ± 189 cpm for 17 mM Cl^- and 503 ± 37 cpm for 127 mM Cl^-, representing the maximum and minimum uptakes in terms of cpm, respectively. All values shown are the means ± S.D. for $n = 3$ or 4 and have the zero-time uptake subtracted and are therefore taken to represent intracellular $^{36}Cl^-$ content [7]. The lines shown are the best fit of the data to the Michaelis-Menten equation as described in the text. The age of the cultures was 18 days. The average protein content of all the dishes used in the experiment was 0.91 ± 0.13 mg (±S.D., $n = 93$). B. The mean data and fitted lines shown in A plotted as double reciprocal plots. •———•, No SITS; o———o, 1 mM SITS present.

[19]. The analysis of the kinetic constants of Cl^- exchange in erythrocytes is quite complex; the inside and outside Cl^- transport sites show different affini-

TABLE I

KINETIC CONSTANTS FOR Cl^- UPTAKE

Data obtained from graphs shown in Fig. 1. Figures presented as mean ± S.E.

Conditions	Apparent $K_{1/2}$ (mM Cl_o^-)	V (nmol $Cl^- \cdot$ (mg protein)$^{-1} \cdot$ min^{-1})
Control	36.2 ± 5.3	34.2 ± 2.1
+1 mM SITS	9.7 ± 2.9	8.1 ± 0.6

ties for Cl^- and modifier sites for Cl^- are also present [8,14]. In erythrocytes the actual $K_{1/2}$ outside is only around 3 mM [8,14]. Thus, if the Cl^- transport system in the astroglial cultures is similar to the erythrocyte exchange system, the outside site will be saturated at $[Cl^-]_o$ of 36 mM. In the absence of significant levels of other anions transported on the same carrier, the transport of intracellular Cl^- outwards will thus be rate-limiting for uptake of external Cl^-, since the anion transport system in erythrocytes shows obligatory 1 : 1 exchange. Based on our previous data I would estimate that at $[Cl^-]_o = 36$ mM, internal Cl^- would be around 0.08 μmol Cl^-/mg protein [7]. This represents 17 to 23 mM $[Cl^-]_i$ depending on whether values of 4.8 or 3.5 μl/mg protein for the internal cellular volume are used (these values are discussed later). These values are close to the values of 26 to 44 mM for $K_{1/2}$-in quoted for red blood cells at an outside pH of 7.2 [15,16]. In recent experiments we have found that complete omission of external Cl^- had no effect on the measured membrane potential of cultured astrocytes (Hirata, H. and Kimelberg, H.K., unpublished observations) suggesting that the effects of varying $[Cl^-]_o$ on the rate of Cl^- uptake are not due to changes in the membrane potential.

Cl^-/Cl^- and Cl^-/HCO_3^- exchange

The exchange system in erythrocytes is known to also transport HCO_3^- and OH^- [8,14–16]. The experimental data in Fig. 2 shows that addition of Cl^- or HCO_3^- to cultures that had been preloaded with $^{36}Cl^-$ and suspended in a Cl^--free medium increased the rate of efflux of $^{36}Cl^-$. The rate constants obtained in the presence of Cl^- or HCO_3^- of 0.16 to 0.19 min^{-1}, respectively, are much lower than those found for red blood cells but comparable to the values of 0.35 min^{-1} found in Friend erythroleukemic cells [21]. These results suggest either that the transport system in astroglial cell cultures is different from or is present at a much lower density than in red blood cells. The above data also imply that in the astroglial cultures the exchange component only comprises one half of the total Cl^- flux. This is in marked contrast to the case of red blood cells where exchange is four orders of magnitude faster than net flow [8].

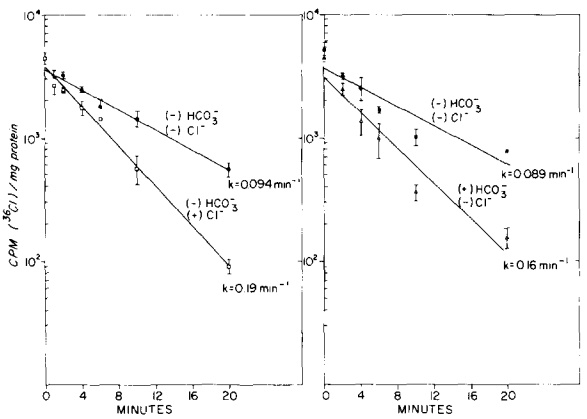

Fig. 2. Efflux of $^{36}Cl^-$ from preloaded cells into Cl^- and HCO_3^--free media and effects of added Cl^- or HCO_3^-. Experiments were performed as described in Methods. The different conditions are shown on the graphs. Each point represents values for three dishes and is shown as the mean ± S.D. The lines are the best fit from linear least squares regression analysis and the values for *k* represent the first-order rate constants calculated from the slopes. 18-day-old cultures were used.

Active accumulation of Cl^- and effects of ouabain in relation to measured membrane potentials and cell volume

The results in Fig. 3A compare the uptake of $^{36}Cl^-$ measured in buffered salt solution to uptake in the complete medium in which the cells were grown. The differences seen are quite small. The results shown in Fig. 3B were for cells pretreated with dibutyryl cyclic AMP, with and without ouabain. Again, the uptake expressed as content of Cl^- per mg protein was similar to that seen in Fig. 3A. The important values, however, are the ion activities, or failing that exchangeable Cl^- expressed as intracellular concentrations. We have reported that the volume of distribution of K^+ for dibutyryl cyclic AMP-treated cells in a buffered salt solution based on an internal K^+ concentration of 127 mM obtained from electrophysiological studies and a K^+ content of 0.61 μmol/mg protein was 4.8 μl/mg protein [11]. Using equilibration with ^{14}C-labeled 3-*O*-methyl-D-glucose as a measure of internal volume [22], we have recently found an average value of 3.5 μl/mg protein for untreated cultures in HCO_3^- buffered medium. The same value was obtained after treatment with norepinephrine, which has similar effects on cell morphology as dibutyryl cyclic AMP [23]. The internal chloride

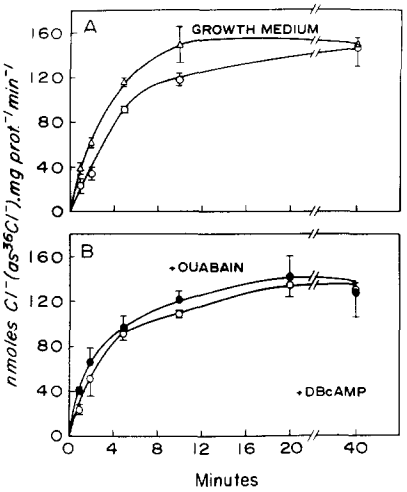

Fig. 3. Uptake of Cl^- in the absence and presence of ouabain and dibutyryl, cyclic AMP (DBcAMP). A. Uptake in growth or reaction medium in absence of ouabain or DBcAMP. Uptake of Cl^- was measured with $^{36}Cl^-$ as described in Methods at constant $(Cl^-)_o$ = 127 mM, and the values shown were corrected for zero-time uptake. For the data shown as (△) the complete growth medium was first poured off and 2.5 ml of fresh complete growth medium, or the medium defined in Methods plus 10 mM $NaHCO_3$ for the data shown as (○), was added to each petri dish, which was then incubated at 37°C for 1 h in a 5% CO_2/95% air incubator. 3 μCi of $Na^{36}Cl$ in 30 μl volume was then added to each dish and the time course of uptake measured at 37°C. Each value was the mean ± S.D. of three separate determinations of 27-day-old cultures. Average protein content of dishes in mg was 1.13 ± 0.10 (△) and 1.13 ± 0.12 (○) (±S.D., n = 18). B. Growth media was poured off from each dish and 2.5 ml of the medium defined in Methods plus 10 mM $NaHCO_3$ and containing 1 mM dibutyryl cyclic AMP (DBcAMP) was added to each dish. For the data marked (●) 1 mM ouabain was also present. The dishes were then left for 2 h in a 5% CO_2/95% air incubator. Cl^- uptake was then measured as described above. Each value was the mean ± S.D. of three separate determinations of 23-day-old cultures. Protein content of the dishes in mg was 0.81 ± 0.12 for both (●) and (○) (±S.D., n = 21 for both).

concentration, calculated using an average steady state level of 0.15 μmol Cl^-/mg protein would be 31 mM for 4.8 μl/mg protein and 43 mM for 3.5 μl/mg protein. Using microelectrodes [11] we have previously obtained an average value of −70 mV when we measured the membrane potentials of these cells under the same conditions (pretreated with dibutyryl cyclic AMP and $[K^+]_o$ = 4.5 mM). More recently we have found values that ranged from −70 to −80 mV, also at $[K^+]_o$ of 4.5 mM (Hirata, H. and Kimelberg, H.K., unpublished observations). To be in equilibrium with a membrane potential of −70 mV at $[Cl^-]_o$ = 127, $[Cl^-]_i$ would have to be 8.7 mM, or 3.6 to 4.9 times smaller than the values for $[Cl^-]_i$ calculated above for values of 4.8 and 3.5 μl/mg protein respectively. With an external chloride concentration of 127 mM, these values give Cl^- equilibrium potentials of −37 mV and −29 mV respectively. This assumes the activity coefficient of Cl^- inside the cell is the same as outside [24]. Therefore intracellular Cl^- is not in electrochemical equilibrium and shows active accumulation.

Another test of whether Cl^- is passively distributed would be to alter the membrane potential and see if $[Cl^-]_i$ is affected. We have shown that addition of ouabain to the astroglial cultures leads to exchange of intracellular K^+ for Na^+ [11] and therefore should result in depolarization. Recently we have indeed found that the membrane potential depolarizes from −71 to −39 mV after treating the cells with 1 mM ouabain for 1 hour (Hirata, H. and Kimelberg, H.K., unpublished observations). Such depolarization would be expected to increase intracellular Cl^- levels if Cl^- is passively distributed. However, the rate of uptake of $^{36}Cl^-$ and the final steady state content of Cl^- were unaffected by pretreating the cells with 1 mM ouabain for 2 h (Fig. 3B). Thus, either the initial Cl^- content and/or that found in the presence of ouabain were not in equilibrium, or there were marked changes in cell volume. Using the ^{14}C-labeled 3-*O*-methyl-D-glucose method we have obtained a reduced volume of 2.8 μl/mg protein after treatment with ouabain for 2 h (unpublished observations). Using a Cl^- content of 0.15 μmol/mg protein this gives a value for $[Cl^-]_i$ of 54 mM giving a Cl^- equilibrium potential of −23 mV. This value is still lower than the value for the membrane potential of −39 mV found after ouabain treatment although the difference is less than in the absence of ouabain (see above).

The above data substantiate that these characterized primary cultures from neonatal rat brains, which consist predominantly of astroglial cells, have a Cl^- exchange system similar to that described for erythrocytes and other cells and tissues [8,14–19, 21,24,25]. In red blood cells the conductance to Cl^-, although low, is still much greater than that for

cations so that Cl^- determines and is therefore in equilibrium with the membrane potential [8]. In contrast, in astroglial cells Cl^- shows exchange transport but is also out of equilibrium with the membrane potential which is mainly determined by K^+ [11,26,27] whose conductance is presumably much greater than that of Cl^-. Active transport of Cl^- is becoming an increasingly common finding in many cells, especially epithelia (e.g., Refs. 20 and 28). An important point to be determined for astroglia as well as other cells is whether the anion exchange system is solely responsibe for active accumulation of Cl^- as seems to be the case in heart muscle [24], presumably by net exchange of anions other than Cl^- out for Cl^- in, or if a Cl^- pump or neutral cation plus Cl^- co-transport are also present. We have found that 5 mM furosemide, but nor SITS, markedly lowers the Cl^- levels and inhibits K^+ transport in astroglial cultures (unpublished observations), similar to its effects in Ehrlich ascites cells [29]. This suggests that active accumulation may be due to a coupled KCl co-transport system rather than the exchange system, although by analogy with the properties of Ehrlich ascites cells [29] the mechanism may not use metabolic energy directly and thus may be a secondary rather than a primary active transport system.

Addition of ouabain to cells usually results in swelling, due to influx of Na^+ and Cl^- because of Donnan forces now unopposed by the inhibited $(Na^+ + K^+)$-pump, but swelling is not seen in all cells and tissues [30]. The lack of swelling in our cells after ouabain treatment is similar to that found for astroglial cells in vivo [31], and could also be explained by a very low free Cl^- permeability, as well as a low Na^+ permeability, operating in parallel with much faster exchange or co-transport systems. In addition, if the Cl^- conductive pathway of the astroglial cells though normally low is also variable, then a number of the conflicting reports on the electrophysiological properties of astroglia in vivo in different preparations and under different conditions [27] could be due, in part, to alterations in the relative contribution of this pathway to the overall electrical properties of the membrane.

Acknowledgements

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