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AMILORIDE AND 5-N,N-DIMETHYLAMILORIDE INHIBIT THE CARRIER MEDIATED UPTAKE OF CHOLINE IN EHRLICH ASCITES TUMOR CELLS

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Abstract—Amiloride and 5-*N*,*N*-dimethylamiloride (DMA) inhibit the choline uptake of Ehrlich ascites tumor cells. The inhibition by DMA is competitive with a K_I value of 20 μM. The apparent K_M value for choline was determined as 15 μM. Amiloride is approximately three times less potent. Amiloride uptake is not antagonized by choline or impaired in cells characterized by a deficient choline carrier. This indicates that amiloride is not transported into the cell by the choline carrier. The inhibition of the choline uptake by DMA cannot be attributed to a depression of choline kinase (EC 2.7.1.32) and is therefore considered to be due to a direct interaction between DMA and the choline carrier. DMA does not compete with sodium ions for its effect on the choline carrier. It is suggested that the choline carrier of Ehrlich ascites tumor cells exhibits a binding site for DMA similar to the one on the Na^+/H^+ antiporter.

The carrier mediated uptake of choline has been studied in most detail in cholinergic nerve endings [1] but has also been described in proliferating cells [2–5] and erythrocytes [6]. In addition to its role in the uptake of choline, the carrier is involved in the uptake of *N*-mustard [3]. Here we show that amiloride and its congener DMA† competitively inhibit the choline carrier of Ehrlich ascites tumor cells.

Amiloride (3,5-diamino-6-chloro N-(diaminomethylene)-pyrazinecarboxamide) has been shown to effect a variety of plasma membrane and intracellular systems [7–16]. The diuretic property of this drug has first been attributed to the inhibition of Na^+ channels of tight epithelia as shown by [17]. Another important system which is impaired by amiloride is the Na^+/H^+-antiport [18]. Besides its function in the renal proximal tubulus, where it promotes extrusion of protons into the tubular fluid [19], the Na^+/H^+-antiport has been demonstrated to be important in regulation of intracellular pH as reviewed by [20, 21]. DMA is a congener of amiloride with increased affinity for the Na^+/H^+-antiport system [22–25]. The observed high affinity of amiloride and DMA to the choline carrier demonstrated here raises the possibility that the carrier contains a cation binding site similar to the one exhibited by the Na^+/H^+-antiporter [26–28].

MATERIALS AND METHODS

Materials. Amiloride and 5,5-*N*-dimethylamiloride were a gift of the Austrian branch of Merck, Sharp & Dohme. Methyl-[^{14}C] choline chloride (50 mCi/mmol), [^{3}H]inulin and [^{3}H]H_2O were obtained from the Radiochemical Centre (Amersham, U.K.).

Cell culture. Ehrlich ascites tumour cells were cultured [25] and cells resistant to *N*-mustard were obtained as described [29].

Choline uptake measurement. The amount of [^{14}C]choline/l cell water was determined by the silicon oil layer technique in a double labelling experiment with [^{3}H]H_2O and [^{14}C]choline chloride. One- to two-hundred microlitres of radioactive labelled cells were layered on the top of 400 μl tubes containing a lower phase of 20 μl detergent solution (2 M NaCl, 40 mM EDTA, 0.2% sodium *N*-lauryl sarcosine, pH 10) and an upper phase of 100 μl silicon oil (10 parts AR 200/3 parts AR 20) and centrifuged in a Beckman Microfuge B for 15 sec at 10,000 *g*. The amount of ^{3}H and ^{14}C in the cell pellet and the supernatant was measured by liquid scintillation counting. The water volume in the pellet was calculated from the distribution of ^{3}H between pellet and supernatant and the known volume of the supernatant. The amount of extracellular water was determined as 15% of total water volume in separate experiments with [^{3}H]inulin and subtracted from the water volume and the obtained value taken as cell volume. The amount of radioactive labelled choline in the extracellular water space of the pellet was subtracted from the total amount of [^{14}C]choline found in the pellet and the nmoles choline/l cell water in the pellet were calculated with these data. All experiments were performed at 37°.

Assay for choline kinase activity. An enriched fraction of choline kinase was prepared. Cells from culture were washed in phosphate buffered saline and centrifuged 5 min at 600 *g*. The following steps were carried out at 0°. Ten volumes of buffer A (25 mM Tris/HCl, pH 8.0, 2 mM $MgSO_4$, 0.5 mM dithio-

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† Abbreviations used: DMA, 5-*N*,*N*-dimethylamiloride.

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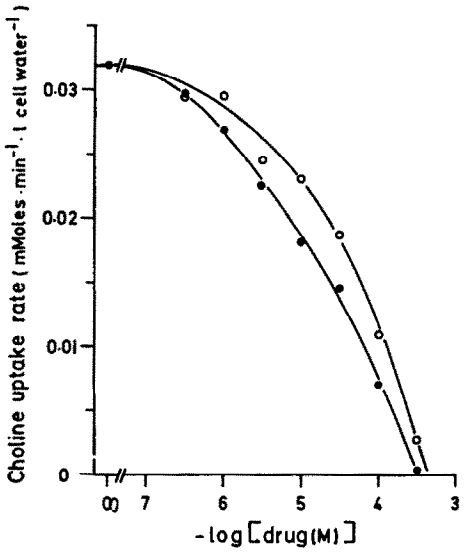

Fig. 1. Concentration–response curves of inhibition of choline uptake in sensitive Ehrlich ascites cells. Exponentially growing cells from culture were concentrated to a density of 1.6×10^6 cells/ml by centrifugation (5 min at 600 g) and resuspension in Eagle's minimal essential medium with Earle's salts buffered with 20 mM Mops at pH 7.35. 10 μCi/ml tritiated water was added to the suspension. The concentration of choline in this medium was taken as 7.1 μM. After a preincubation period of 10–40 min at 37° 135 μl aliquots of the cellular suspension were transferred to test tubes with 150 μl of the same medium and mixed rapidly. Ten minutes later the cells were separated from the medium by the silicon oil layer method, and the amount of choline/l cell water was calculated as described under Materials and Methods. Each point represents the mean of two determinations. Data were plotted by the method of Lineweaver and Burk [38]. The uptake of [^{14}C]choline was measured after at 12.6 μM choline. The inhibitors were added together with the [^{14}C]choline at time 0: ●—●, DMA; ○—○, amiloride. Each point represents the mean of two determinations.

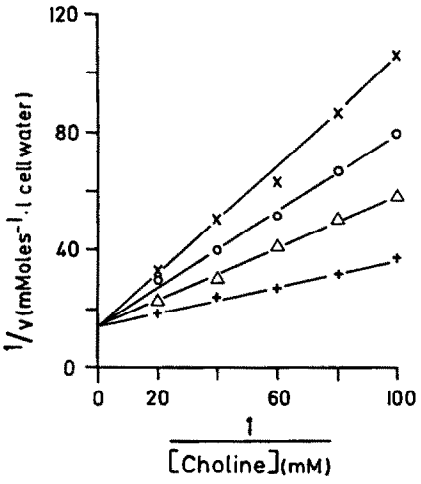

Fig. 2. Competitive inhibition of choline uptake by DMA in sensitive Ehrlich ascites cells. The experiment was performed as described in the legend to Fig. 1 in the presence of 10, 12.4, 16.6, 25 and 50 μM choline in the medium. The inhibitor was added together with the [^{14}C]choline. Controls: +—+; 20 μM DMA: △—△; 40 μM DMA: ○—○; 60 μM DMA: ×—×. Each point represents the mean of two determinations. Data were plotted by the method of Lineweaver and Burk [38]. The K_I value for DMA from the three plots with inhibitors was calculated by linear regression analysis as 20 ± 1.5 μM (mean ± SEM).

threitol and 250 mM KCl) were added to 1 vol. of packed cells and cells lysed by three cycles of freezing and thawing. After centrifugation at 10,000 *g* for 1 min 10 vol. of the supernatant were applied to 1 vol. of a DEAE-cellulose column (Whatman DE 52) equilibrated in buffer A. More than 90% of choline kinase activity applied to the column do not bind on the column under these conditions and were used as enzyme source in the assays. The assay was performed at 37° in 20 μl of a mixture containing 5 mM $MgSO_4$, 25 mM Hepes/KOH pH 7.4, 100 mM KCl and the concentrations of ATP or [^{14}C]choline described in the individual experiments. After an incubation period of 15 to 30 min, the reaction was stopped by decreasing the temperature to 0°. Aliquots (5 μl) of the mixture were applied to cellulose coated thin layer plastic sheets (Merck, thickness of layer 0.1 mm). After chromatography in a mixture (v/v) of 70% ethanol and 30% 1 M ammonium acetate, pH 6.0, the spots containing phosphorylcholine and choline were cut out and radioactivity was determined by liquid scintillation counting. Formation of phosphorylcholine was dependent linearly on concentration and time up to 30 min.

RESULTS

The dose effect curves for the impairment of choline uptake by amiloride and DMA at 12.6 μM external choline are depicted in Fig. 1. Compared to amiloride, DMA is approximately three times more effective in the inhibition of the choline uptake system. Inhibition is almost complete at a drug concentration of 300 μM. A kinetic evaluation of the type of inhibition of choline uptake by DMA was performed. As illustrated in Fig. 2, we observe a competitive inhibition of choline uptake with a K_I of 20 μM. The apparent K_M value for choline was calculated from this experiment as 15 μM.

A capacity of cells to accumulate amiloride has been demonstrated [10, 14]. The time course of amiloride accumulation in Ehrlich cells at an external concentration of 0.2 mM is shown in Fig. 3. There is no significant difference in amiloride uptake rates between a choline carrier deficient cell line, which was selected by its ability to grow in the presence of *N*-mustard ([29] and unpublished results) and the parental line. Increasing choline in the external medium from 7.1 μM to a concentration of 1 mM or 10 mM has no effect on amiloride accumulation. The results shown in Fig. 3 provide evidence that amiloride is not transported by the choline carrier and indicate that the reduction of choline uptake by amiloride is due to an inhibition of the choline carrier but not the result of competition between choline and amiloride for the amiloride transporting system.

The uptake of choline is achieved by two subsequent processes. Carrier mediated transport of choline into the cell and trapping of choline inside the cell by phosphorylation by the choline kinase

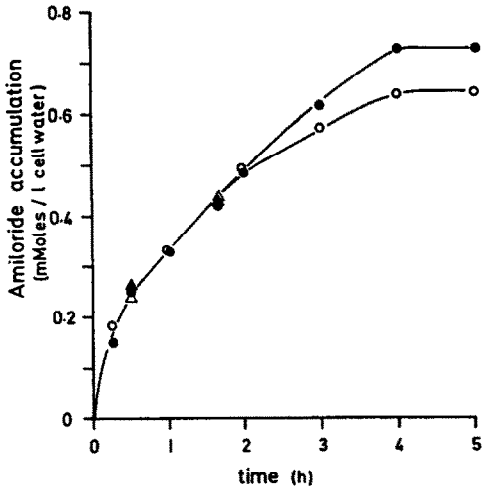

Fig. 3. Time course of amiloride-accumulation. The amount of amiloride binding and uptake was determined by taking advantage of the fluorescent properties of amiloride [14]. To a suspension of 2.8×10^6 cell/ml in complete culture medium plus sera a 0.1 M solution of amiloride in DMSO was added to give a final concentration of 0.2 mM. At the time points after addition of amiloride indicated in the diagram, 130 µl aliquots of the suspension were separated from the medium by centrifugation through a silicon oil layer into 20 µl of detergent as described in Methods under "choline uptake measurement". The cell pellet together with the detergent solution was diluted by addition of 3 ml 10 mM Tris/HCl, pH 7.35. After centrifugation at 1000 *g* for 5 min the fluorescence of amiloride in the supernatant was measured in a Perkin-Elmer 650–10S fluorescence-spectrophotometer at an excitation of 363 nm (slit width 5) by detecting the emission at 420 nm (slit width 15). Results are expressed as mMoles amiloride/l cell water. The amount of cell water/cell pellet was determined as 0.5 µl in separate samples with tritiated water. The fluorescence intensity was a linear function of amiloride concentration in the range of 0.03 to 1.0 mmoles amiloride/l cell water: ●—●, Ehrlich cells, sensitive to *N*-mustard; ○—○, Ehrlich cells, resistant to *N*-mustard; △, sensitive cells, 1 mM choline in the medium; ▲, sensitive cells, 10 mM choline. Each point represents the mean of three determinations.

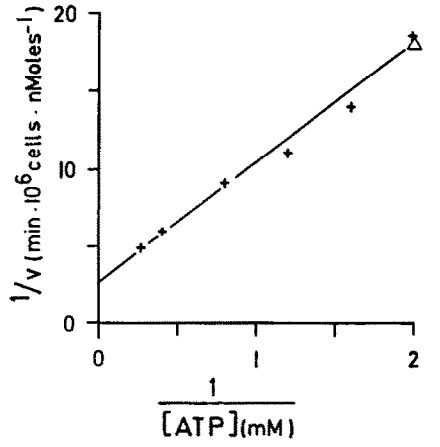

Fig. 4. Choline kinase activity in sensitive Ehrlich ascites cells as a function of ATP concentration. Choline kinase activity was measured as described in Materials and Methods. Each point represents the mean of at least two experiments: +—+, no inhibitor; △, 0.4 mM DMA. Choline concentration was 200 µM. Data were plotted by the method of Lineweaver and Burk [38]. K_M value for ATP as determined by linear regression analysis is 2.6 mM.

[5, 30, 31]. Our data on inhibition of choline uptake by DMA presented so far cannot distinguish whether DMA acts directly on the choline carrier by reducing the uptake rate or by impairment of the choline kinase. Therefore, the effect of DMA on the choline kinase was analysed in a cell free preparation. It has been reported that amiloride acts as an inhibitor of protein kinases by competition with ATP [14]. Figure 4 demonstrates that 0.4 mM DMA, which cause a complete block of choline uptake (Fig. 1), does not effect choline kinase activity at saturating choline concentrations and an ATP concentration of 500 µM. At low choline concentrations, DMA exerts an slight competitive inhibition (Fig. 5) with a K_I value of 1.4 mM. The K_I value of DMA for the competitive inhibition of choline kinase is 70-fold higher than the value for inhibition of choline uptake (1.4 mM vs 20 µM). The K_M/K_I ratio, which represents a measure of the affinity of the drug relative to choline, is 0.76 in the case of choline uptake and only 0.047 in the case of the choline kinase.

These striking differences in sensitivity lead to the conclusion that the inhibition of choline uptake by DMA is caused by a depression of the choline carrier activities and not due to an impairment of choline kinase.

As shown in Fig. 6, a decrease of the sodium concentration in the medium from 130 mM to 1 mM does not shift the concentrations required for the

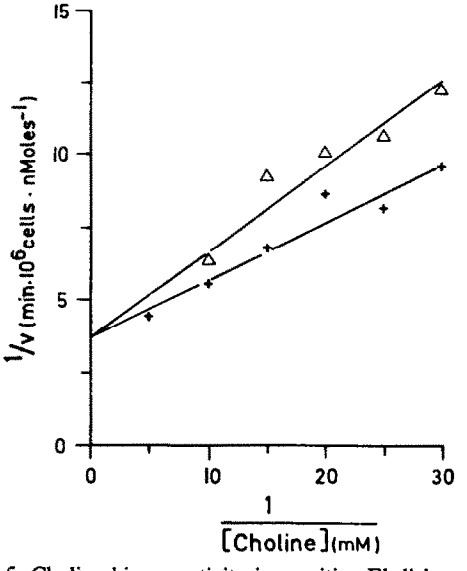

Fig. 5. Choline kinase activity in sensitive Ehrlich ascites cells as a function of choline concentration. The concentration of ATP was 4 mM. Choline kinase activity was measured as described in Materials and Methods. Each point represents the mean of at least two experiments. Data are plotted as described in Fig. 4: +—+, no inhibitor; △, 0.6 mM DMA.

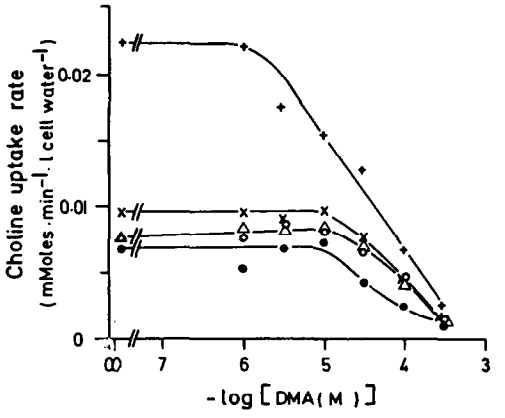

Fig. 6. Effect of sodium ions on the inhibition of choline uptake by DMA. Cells were washed three times with Hepes buffered salt solution, containing 130 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$ and 20 mM Hepes/Tris pH 7.4 and suspended in this medium. For the measurement of choline uptake at lower sodium concentrations the appropriate amount of NaCl was replaced by KCl. Cells were incubated for 30 min at 37° and the choline uptake was determined at 21.5 μM choline of a 10 min period as described in Materials and Methods. DMA was added together with [^{14}C]choline. Sodium concentrations in the uptake medium: ●—●, 1 mM; ○—○, 4 mM; △—△, 15 mM; ×—×; 40 mM; +—+, 130 mM.

inhibition of choline uptake by DMA to lower DMA concentrations. This indicates that in contrast to the inhibition of the Na^+/H^+-antiport by amiloride and DMA [19, 22, 32] sodium ions do not antagonize the effect of DMA in a competitive manner. The observed impairment of choline uptake rate in Ehrlich ascites cells after replacing sodium ions by potassium ions depicted in Fig. 6 is a property also described for the high affinity choline uptake system of cholinergic neurons [1].

DISCUSSION

A part of the effects of amiloride described in the literature can be attributed to interactions with plasma membrane systems involved in the transport of sodium ions. The inhibitions of sodium channels and the Na^+/H^+-antiport by amiloride are observed at μM concentrations [7, 24]. The affinity of amiloride for the choline carrier is lower than described for the Na^+/H^+ antiport but is higher when compared with the affinity for the (Na^+/K^+)ATPase (EC 3.6.1.37, ref. 15) and the Na^+/Ca^{2+} exchange [16].

Amiloride and DMA may either bind on the same site of the choline carrier as choline or may recognize a separate binding site similar to the one on the Na^+/H^+ antiporter. Support for the first assumption comes from the competitive nature of inhibition of choline uptake by DMA. However, in the case of two separate but topographically closely related sites the binding of amiloride to a different site than the choline binding site would also yield the same pattern of inhibition. The existence of two binding sites would offer an explanation for the high affinity of hemicholinium and other bisquaternary ammonium compounds for the choline carrier [33, 34]. One charged group could bind the choline binding site, while the other recognizes the amiloride binding site. An effect similar to the one of amiloride on choline uptake in Ehrlich ascites tumor cells has been reported for harmala alkaloids, which competitively inhibit the sodium-dependent high affinity choline uptake in rat striatal synaptosomes [35]. Like amiloride, harmala alkaloids are known to act as inhibitors of the Na^+/H^+-antiport [36]. These findings suggest but do not prove that the choline carrier exhibits a cation binding site similar to the one recognized by amiloride on the Na^+/H^+-antiport system. It should be noted that we also find differences between the amiloride binding site of the choline carrier and the Na^+/H^+ antiporter. As shown in Fig. 6, DMA does not compete with sodium ions for inhibition of choline uptake, which is in contrast to the inhibition of the Na^+/H^+ antiport [22]. However, Vigne *et al.* have reported [28] that in solubilized membrane preparations of rabbit renal microvilli tritium labeled ethylpropylamiloride does not compete with sodium for binding but does compete with protons, indicating that the observed interference between amiloride and sodium Na^+/H^+ antiport in intact cells is not due to a direct competition between amiloride and sodium for the same site. Another difference is a 40-fold higher affinity of DMA compared to amiloride for the Na^+/H^+ antiport system [25], whereas there is only a 3-fold difference in their effect on the choline carrier (Fig. 1).

Amiloride has also been shown to be responsible for inhibition of intracellular enzymes and processes such as protein kinases [11–14], phosphatases [37] or protein synthesis [10]. In cell-free systems the concentrations needed for an impairment of these intracellular processes are in the range between 100 μM and 1 mM. As depicted in Fig. 5, an additional side effect of DMA is the inhibition of the choline kinase activity at mM concentrations. Amiloride can be accumulated in the cell as shown for Ehrlich ascites cells (Fig. 3), hepatocytes [10] and A431 epidermoid carcinoma cells [14]. Therefore, when using intact cells, side effects are more likely to be observed at lower concentrations of amiloride in the medium than in cell free systems. Even when taking this into consideration, the effect of DMA on choline uptake cannot be attributed to an impairment of choline kinase activity.

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