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Kinetics of Irreversible Inhibition of Choline Transport in Synaptosomes by Ethylcholine Mustard Aziridinium

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Summary. Ethylcholine mustard aziridinium (ECMA) inhibits choline transport in synaptosomes at a half-maximal concentration of about 20 μM. The rate of inhibition falls off rapidly after 10 min and the concentration dependency reaches a plateau at about 100 μM. The inhibition is not removed by washing the synaptosomes, and choline and hemicholinium-3 protect the carrier against attack by the mustard. Choline efflux, particularly that stimulated by choline in the medium (transactivation) is also inhibited by the aziridinium compound. Similarly choline influx activated by preloaded internal choline is inhibited by ECMA. The mustard can enter the synaptosomes in an active form but most of the carrier is alkylated when facing the outside. Prior depolarization of the synaptosomes causes an increase in the rate of inhibition by ECMA which is proportionally about the same as the increase in choline influx also caused by depolarization. At low ECMA concentrations the rate of inhibition is that of a first-order reaction with the carrier but at high ECMA concentrations the translocation of the carrier to the outward-facing conformation controls the rate of inhibition. Using a model of choline transport with some simplifying assumptions it is possible to estimate the amount of carrier; cholinergic synaptosomes carry about six times the concentration of carrier found in noncholinergic ones. In noncholinergic synaptosomes the carrier faces predominately out, the reverse in cholinergic ones. The rate constant of carrier translocation is increased by combination with choline some six- to sevenfold to about 3.5 min^{-1}. The rate constant of ECMA attack on the carrier is about 440 M^{-1} sec^{-1}.

Key Words ethylcholine mustard · choline carrier · affinity label · translocation rates · synaptosomes

Introduction

Transmembrane transport of choline is a ubiquitous feature of tissues. It serves to provide all cells with choline for the synthesis of phospholipids and additionally the synthesis of acetylcholine in cholinergic neurons. Choline uptake has been shown to have the classical features of carrier-mediated transport in squid axon (Hodgkin & Martin, 1965), erythrocytes (Martin, 1968; Devés & Krupka, 1979) and in isolated nerve terminals (Marchbanks, 1968*b*; Marchbanks, Wonnacott & Rubio, 1981). Considerable progress has been made in elucidating the general mechanistic features of carrier-mediated choline transport (*see*, for example, Martin, 1969; Krupka & Devés, 1980) and the carrier has been solubilized and reincorporated into liposomes (King & Marchbanks, 1980, 1982; Meyer & Cooper, 1983). However, there are certain kinetic aspects of carrier action which require specific irreversible inhibitors for their elucidation and in addition such an inhibitor of choline transport could be used as an affinity label to aid in its isolation and purification.

The description by Rylett and Colhoun (1980) of ethylcholine mustard aziridinium (ECMA) suggested that it inhibited choline transport in isolated nerve terminals (synaptosomes). The inhibition appeared to be insensitive to washing but aspects of specificity and kinetic mechanism were not investigated. In this paper we present details of the specificity and kinetics of ECMA inhibition and offer a tentative mechanistic interpretation of its effect on the choline carrier in synaptosomes. We have chosen to use synaptosomes from cerebral cortex because all the traditional features of choline transport can be readily demonstrated in this preparation. These include saturability, Na^+-K^+ dependence, HC-3 inhibition, transactivation and marked exchange fluxes (Marchbanks, 1968*b*; Marchbanks et al., 1981). An additional reason is that the cerebral cortex synaptosome preparation would contain representatives (if they exist) of both the putative 'low'- and 'high'-affinity choline carrier which might therefore be usefully distinguished by the mustard. We have used ECMA rather than the exact analogue (choline mustard aziridinium) because of the commercial availability of the former. In fact, substitution of the ethyl group produces no significant change in the inhibitory potency of the mustards (Tables 1 & 2, Rylett & Colhoun, 1980).

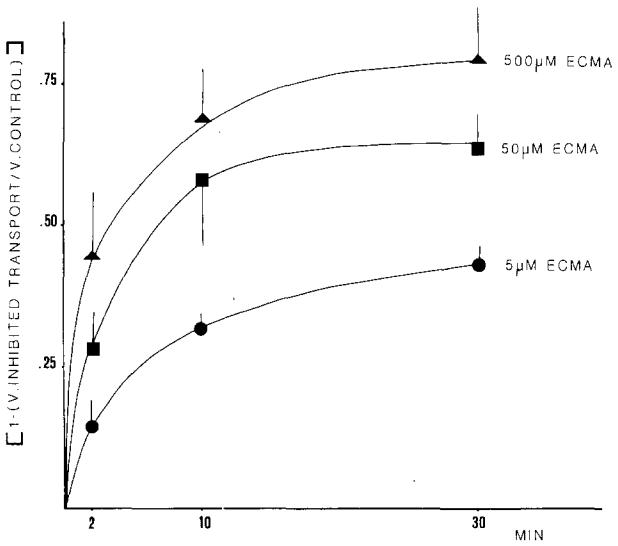

Fig. 1. Time dependency of inhibition of choline transport by ECMA. Synaptosomes (approx. 1 to 2 mg protein in 0.5 ml) were washed and incubated at 37°C for the time shown on the abscissa in solutions of normal medium containing various concentrations of ECMA. The incubation was terminated by dilution of the sample and immediate sedimentation (30 sec) in a Janetski TH-11 centrifuge followed by three washes. The synaptosomes were then resuspended in normal medium and ^{3}H-choline uptake measured. Control synaptosomes were submitted to the same incubation and washing but without ECMA. The choline transport in controls incubated for 2 min was 10.4 ± 0.69 (n = 6) pmol min^{-1} mg^{-1} and was not significantly different in the case of 10- or 30-min pre-incubation. In the presence of 100 μM hemicholinium-3 (HC-3) the choline transport in controls was reduced to 1.73 ± 0.203 (n = 5) pmol min^{-1} mg^{-1}, indicating that about 85% of the transport was HC-3 sensitive, i.e. carrier-mediated. Results are expressed as fractional inhibition, i.e. (1-(inhibited uptake/control uptake)) and the bars represent the range or standard error of two or three separate experiments

Materials and Methods

Preparation of Synaptosomes

Synaptosomes were prepared from scraped guinea pig cerebral cortex by the method of Gray and Whittaker (1962) with the minor modification described by Wonnacott and Marchbanks (1976). The P_2B fraction was collected from the gradient, diluted once with normal medium (3 K^+ medium) containing (in mM): 5 sodium phosphate buffer, pH 7.2, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 180 NaCl. The diluted fraction was then centrifuged at 10,000 rpm in a Sorvall RC5 centrifuge, rotor SS34, for 20 min. The final pellet from 1 g original tissue was resuspended in 1.0 ml normal medium. Aliquots of about 500 to 700 μg of protein were removed, centrifuged briefly in an MSE Micro Centaur and resuspended in the appropriate medium. The synaptosomal suspension contained about 4 to 6 mg protein per ml as measured by the method of Lowry et al. (1951).

Preparation of ECMA

The precursor N-2-acetoxyethyl-N-2′-chloroethyl-ethylamine was dissolved in distilled water and allowed to stand at room temperature for at least 50 min to generate the aziridinium species. The pH of the solution was then adjusted to 11.5 by addition of NaOH. The solution was maintained at this pH for 10 min prior to readjustment to pH 7.5 with HCl.

Aziridinium concentration in the solution was determined by the iodine-thiosulfate titration (Golumbic, Fruton & Bergmann, 1946; Gill & Rang, 1976) using 5 mM thiosulfate, 2.5 mM iodine and 0.1% freshly made starch solution. An aliquot of ECMA was treated with an amount of thiosulfate approximately twice the expected amount (in moles) of aziridinium. After acidification with HCl and standing at room temperature for 15 min the excess unreacted thiosulfate was determined by titration with the iodine solution. The yield of aziridinium was 55 ± 7 (n = 6) of theoretical and all results are expressed in terms of cyclized derivative measured in each experiment.

Preincubation with ECMA

Synaptosomes were always washed before preincubation at 37°C for 2, 10 or 30 min with different concentrations of ECMA ranging from 0.5 μM to 2 mM. At the end of preincubation, synaptosomes were centrifuged for 10 sec in a bench microcentrifuge, washed twice and resuspended in the normal medium to test for choline uptake.

Choline Transport

^{14}C choline was added to the synaptosomes to give a final concentration of 2 μM and a radioactive concentration of 0.1 μCi/ml. The samples were incubated at 37°C for 4 min. At the end of the preincubation period, synaptosomes were centrifuged, washed twice and resuspended in a fixed volume (0.2 ml) of normal medium. The radioactivity of the resuspended pellet was estimated in a liquid scintillation counter (LKB Rack BETA) and the scintillant was toluene containing 5 g of 2,5-diphenyloxazole (PPO) and 0.2 g of 1,4-*bis*-(phenyloxazole-2-yl) benzene (POPOP) per liter/synperonic NXP/2 M NH_4OH (100/50/1 by vol). The counting efficiency for ^{3}H was 20% and ^{14}C 50%.

Materials

The radiolabeled compounds were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Ethylcholine mustard aziridinium precursor was purchased initially from Salford Fine Chemicals (Industrial Centre Building, Salford M5 4WT, U.K.) and subsequent batches of this compound were purchased from Research Biochemicals Inc., P.O. Box 181, Wayland, Mass. 01778. The concentration dependency of choline transport inhibition by the compound from both sources was superimposable.

Results

Time and Concentration Dependency of ECMA Inhibition

In Fig. 1 is shown the dependency of time and ECMA concentration of inhibition of choline transport. The initial rate of inhibition falls off rapidly after 2 min and inhibition is 80 to 90% complete after about 10 min. Thereafter the rate of inhibition only slowly increases.

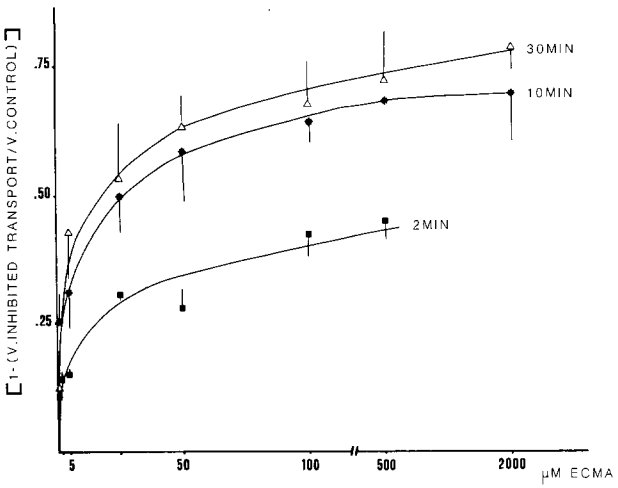

Fig. 2. Concentration dependency of inhibition of choline transport by ECMA. Experimental details as in Fig. 1

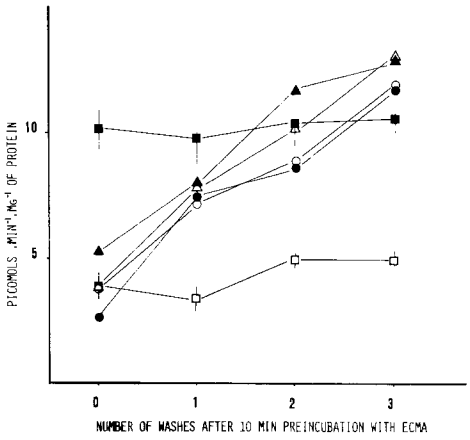

Fig. 3. Protection from ECMA inhibition by substrate analogues. Synaptosomes (1 to 2 mg protein/ml, 0.5 ml) were incubated for 10 min at 37°C with and without 50 μM ECMA and 2 μM HC-3 or, 2 mM choline also with and without 50 μM ECMA. The synaptosomes were sedimented and then washed with 1.0 ml normal medium for the number of times shown on the abscissa. After each wash an aliquot (1 mg protein) was taken for the estimation of choline transport activity with results shown on the ordinate. Points are mean and SEM (n = 4) in the case of control and 50 μM ECMA conditions. Others are the average of two separate determinations [■ Control, ● + 2 mM choline, ▲ + □ + 50 μM ECMA, ● choline + ECMA, ▲ HC + 3 + ECMA]

The initial rate of inhibition increases with ECMA concentrations. However, as Fig. 2 shows even at high ECMA concentration (2.0 mM) the inhibition is only 75% complete. The transmembrane flux of choline is always composed of at least two elements, that mediated by a carrier and that resulting from Fick's law diffusion. Only the former is inhibited by the substrate analogue hemicholinium-3 (HC-3). In these experiments the flux in the presence of 100 μM hemicholinium was 16% of the control; therefore the carrier-mediated component is about 84% of the total shown in Figs. 1 and 2, and so it is likely that 100% inhibition of the carrier does eventually occur at high ECMA concentrations and periods in excess of 30 min. An explanation of this slow approach to complete inhibition is offered later. The cyclized derivative was stable for at least 6 hr at 4°C as measured by thiosulfate titration. The expected degradation product is ethyldiethanolamine but chromatography (*not shown*) revealed the presence initially and a subsequent increase in the concentration of various quaternary derivatives. These quaternary compounds predominate in preparations left for more than 12 hr at 4°C. However, they were shown not to be responsible for inhibition of choline transport as follows: a preparation of freshly prepared ECMA inhibited choline transport to 55% ± 4 (n = 4) of control but the same preparation tested one day later only inhibited to 86% ± 5 (n = 4) of control.

Protection from ECMA Inhibition

The aziridinium ion is an active alkylating agent and can be expected to interfere with a wide variety of metabolic processes. The specificity of its effect on choline transport has been investigated by protecting the carrier by its substrate (choline) or a structural analogue (HC-3) during the incubation with ECMA. These compounds were then removed by serial washing of the synaptosomes which was followed by estimations of ^{3}H-choline uptake. Figure 3 shows the results of this experiment. If ^{3}H-choline uptake is measured without any washes then ECMA, choline, HC-3 and combinations thereof all inhibit uptake greater than 50%. Washing removes the inhibition associated with HC-3, excess choline and 50 μM ECMA in the presence of these compounds. The inhibition caused by 50 μM ECMA alone is not affected by washing and the control values remain stable. It is evident that the carrier can be protected from inhibition by its substrate and an inhibitory substrate analogue. This suggests that the action of ECMA is specifically directed at the carrier and that it does not affect other aspects of nerve terminal metabolism necessary for the transport activity of the carrier. As others have observed (Rylett & Colhoun, 1980) the action of ECMA is not reversed by its removal on washing.

Effect of ECMA on Carrier-Mediated Efflux of Choline

Carrier-mediated efflux can be demonstrated in synaptosomes (Marchbanks et al., 1981) as in erythro-

Table 1. Inhibition of ^{14}C-choline efflux by ECMA[a]

Measurement	Pre-incubation condition	Efflux (dpm/mg protein)		Inhibition ($1 - V_i/V_c$)
		Control	+50 μM ECMA	
Efflux of ^{14}C-choline during 2-min pre-incubation	—	1104 ± 95	1500 ± 191	−0.34 ± 0.068 (5)
↓ 2 washes				
Efflux of ^{14}C-choline during 4-min incubation	—	921 ± 70	789 ± 34	0.134 ± 0.035 (5)
	+ Choline 2 mM	834 ± 78	771 ± 115	0.062 ± 0.17 (3)
Influx of ^{3}H-choline during 4-min incubation	—	7734 ± 804	5154 ± 461	0.310 ± 0.078 (5)
Efflux of ^{14}C-choline during 4-min incubation in presence of 2 mM choline	—	1375 ± 164	950 ± 131	0.271 ± 0.101 (5)
	+ Choline 2 mM	896 ± 157	889 ± 56	0.037 ± 0.14 (3)

[a] Synaptosomes (approx. 1 to 2 mg protein in 0.5 ml) were preloaded with ^{14}C-choline (0.2 μCi/ml, 4 μM) for 30 min at 37°C and then washed twice by sedimentation. This was followed by a 2-min pre-incubation at 37°C with or without 50 μM ECMA and choline as shown in 0.5 ml medium which was terminated by dilution and two further washes. After resuspension in 0.5 ml normal medium containing 0.5 μCi/ml ^{3}H-choline (2 μM or 2 mM as shown), efflux of ^{14}C into the supernatant and influx of ^{3}H-choline into the pellet was measured during the subsequent 4 min. Mean and SEM with number of experiments in parentheses are shown. The figures for inhibition are the means and SEM of matched pairs of control and inhibited samples.

cytes (Martin, 1968). The inhibition of this process by ECMA was investigated as follows. Synaptosomes were first preloaded with 0.2 μCi/ml of ^{14}C-choline for 30 min. They were then washed and submitted to 50 μM ECMA for 2 min at 37°C. During this period the efflux of ^{14}C-choline was monitored. As can be seen (Table 1, row 1) ECMA at this stage actually increased efflux. After washing out the ECMA further efflux of ^{14}C choline was measured for a 4-min period and at the same time the influx of ^{3}H-choline was measured. There was a small, but significant ($P < 0.02$) inhibition of efflux of ^{14}C-choline and a larger also significant inhibition of ^{3}H-choline influx. If efflux was carried out in the presence of 2 mM choline, transactivation (Marchbanks et al., 1981, *cf.* Table 8) occurred and efflux of ^{14}C-choline was increased by about 50%. Inhibition by ECMA was also increased. No anti-cholinesterase was present during these experiments nor was the radioactivity separated into choline and acetylcholine so a component of the radioactive efflux will include spontaneous release of ^{14}C-ACh synthesized within the synaptosome. However, such release is not stimulated by external choline (*unpublished results*).

This suggests that choline efflux, particularly the component that can be transactivated is inhibited *pari passu* with choline influx. As Table 1 also shows the inhibition can be prevented by the presence of choline during the incubation with ECMA. The increase of efflux during the incubation with ECMA can be explained by the presence of quaternary degradation products of ECMA (or perhaps even ECMA itself) that are effectively transported; and therefore like choline cause transactivation of efflux while they are present in the medium.

We have also investigated the effect of ECMA on transactivation of influx (Table 2). Synaptosomes were preloaded with either 4 or 400 μM choline, washed and then incubated with 50 μM ECMA for 2 min. As we have previously reported increasing the internal choline concentration by preloading does increase subsequent choline influx by transactivation. There is also an increase in inhibition by 50 μM ECMA though not very great. However, if the transactivated component of influx is obtained by difference the inhibition of this is seen to be substantially higher. When influx is measured in 2 mM choline at which concentration the carrier will be saturated and its activity swamped by diffusion no inhibition by ECMA can be observed. We concluded that transactivation effects which we attribute to an increased rate of translocation of the carrier caused by choline transport are also inhibited by ECMA.

Uptake of ECMA into Synaptosomes

The occurrence of transactivation by ECMA itself or its decomposition products prompted us to investigate its uptake across the synaptosomal membrane. Synaptosomes were incubated with ECMA, washed and then osmotically lysed. The lysate was investigated for its inhibitory effect on choline

Table 2. Effect of ECMA on transactivated influx[a]

Influx choline conc.	Preloading	dpm/mg protein · 4 min		Inhibition $(1 - V_i/V_c)$
		Control	+50 μM ECMA	
2 μM	—	7737 ± 804	5310 ± 325	0.288 ± 0.073
	+	9231 ± 1436	6007 ± 1626	0.380 ± 0.124
	Difference (+ −)	1494	697	0.533
2 mM	—	1406 ± 158	986 ± 314	0.283 ± 0.22
	+	2079 ± 142	2169 ± 123	−0.058 ± 0.08
	Difference (+ −)	673	1153	−0.71

[a] Synaptosomes (approx. 1 to 2 mg protein in 0.5 ml) were preloaded with choline at concentrations of either 4 μM (−) or 400 μM (+) for 30 min at 37°C and then washed twice by sedimentation. After incubation with 50 μM ECMA as described in the legend to Table 1 they were washed and the influx of ^{3}H-choline (0.5 μCi/ml) measured at the choline concentrations indicated. Mean and SEM of five experiments are shown. The figures for inhibition are derived from matched pairs of control and inhibited samples.

transport into synaptosomes by pre-incubation and washing in the normal way. The results are shown in Table 3 and it can be seen that the lysate from synaptosomes incubated with ECMA did contain inhibitory activity that was not present in lysates from synaptosomes never previously exposed to ECMA. The inhibitory effect of the lysates increased with the period of incubation with ECMA and its concentration. This qualitative test suggests that ECMA can enter synaptosomes in significant amounts though we have not established that it is taken up by the agency of the choline carrier or that it remains in its original form.

Sidedness of Inhibition by ECMA

For the purposes of mechanistic studies it is important to know on what side of the membrane the carrier has to be in order to be susceptible to attack by ECMA under the conditions that we have used. This question acquires more relevance because of our finding that inhibitory concentrations of ECMA enter synaptosomes within a 2-min period of incubation. We have attempted to resolve the problem by examining the rate of inhibition in the presence of high concentrations of choline added just immediately prior to ECMA. In such circumstances any carrier facing outward will be protected from inhibition by choline, whereas carrier inside will not be protected except by choline already present inside the synaptosome.

As Fig. 4 shows "inside inhibition" (i.e. in presence of 2 mM choline outside) does occur within the 2- and 10-min incubation period but it is only about 10 to 20% of the total inhibition that can be achieved at the same time and ECMA concentration when the outside-facing carrier is unprotected.

Table 3. Uptake of ECMA into synaptosomes[a]

Time of incubation (min)	Inhibition by lysate $(1 - V_i/V_c)$	
	50 μM ECMA	500 μM ECMA
2	0.0784 ± 0.006 (5)	0.153 ± 0.018 (3)
60	0.120 ± 0.07	0.192 ± 0.082 (3)

[a] Synaptosomes (approx. 1 to 2 mg protein in 0.5 ml) were washed and then incubated at 37°C with ECMA at the concentrations shown for the time indicated. After washing twice and resuspension in 100 μl of water for 5 min they were spun down and the supernatant was taken, diluted one-half with normal medium of double strength and used to resuspend a synaptosome pellet. After 2-min incubation the pellet was diluted and washed and the uptake of ^{14}C-choline determined in the normal way. Controls were treated with a lysate from synaptosomes treated in exactly the same way except that the initial incubation medium did not contain ECMA. The control values were 14.4 ± 2.9 pmol min^{-1} mg protein^{-1} (n = 6). Mean inhibition and SEM with number of experiments in parentheses are given.

We also attempted to protect "inside inhibition" with choline introduced into the synaptosome by preloading. The experiments (Fig. 4, inset) suggest (but are not statistically significant) that preloading decreases "inside inhibition" while increasing total inhibition. The interpretation of this experiment is complicated by the probability that ECMA is prevented from entering the synaptosome by choline. If ECMA alkylated carrier from the inside only but access was prevented by choline it would be expected that the initial rate of inhibition would be sustained. This is not the case so we conclude that the initial inhibitory effect of ECMA under the conditions we have employed is predominately but not totally on the outward-facing carrier.

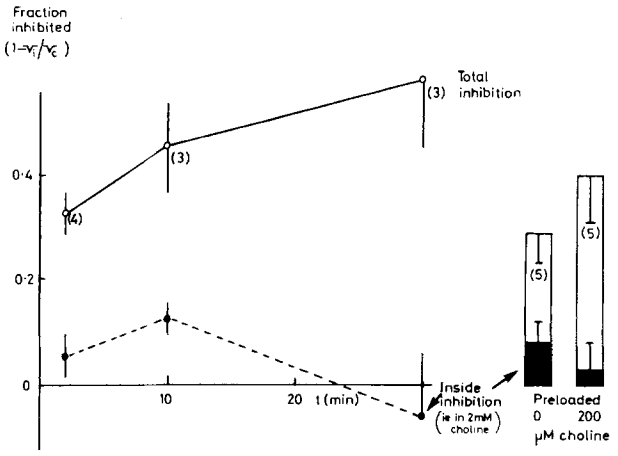

Fig. 4. Sidedness of inhibition by ECMA. Synaptosomes (1 to 2 mg protein/ml, 0.5 ml) were incubated with and without 50 μM ECMA with and without the immediate prior addition of 2 mM choline at 37°C for the periods of time shown on the abscissa. They were then diluted, washed twice and the uptake of ^{14}C-choline (0.1 μCi/ml, 2 μM) measured as in Fig. 1. The figures for total inhibition (—○—) are derived from experiments without a protecting concentration (2 mM) of choline and those for inside inhibition (—●—) when 2 mM choline was added immediately prior to the ECMA to prevent its inhibition of any outward-facing sites. The histogram columns show the result of another set of experiments in which the inside concentration of choline was increased by preloading for 37°C with the concentration of choline shown. Mean and SEM (number of experiments in parentheses) of fraction inhibited calculated from matched pairs of inhibited and control samples are shown

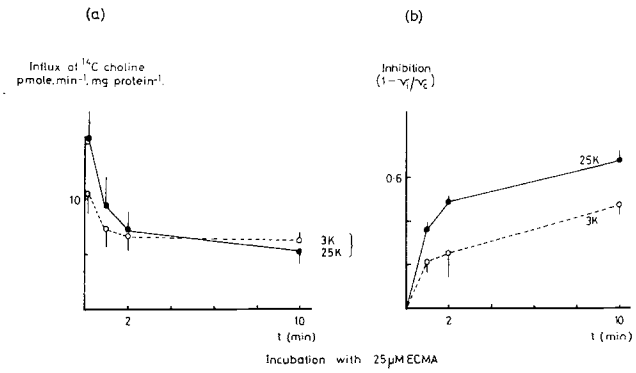

Fig. 5. Effect of prior depolarization on inhibition by ECMA. Synaptosomes in the usual amounts were first incubated in a normal medium (3 K --○--) or a medium containing a depolarizing concentration of K^+ (25 mM, —●—) for 30 min at 37°C. They were then washed and resuspended in normal medium with or without 25 μM ECMA and incubated for the time shown at 37°C. The control without ECMA was incubated for 1 min. After dilution and two washes ^{14}C-choline uptake was measured in the usual way. (*a*) shows the data expressed as an influx of choline, with the control placed at $t = 0$ for clarity; (*b*) the data is expressed as the fractional inhibition. Mean and SEM of three separate experiments are given

Effect of Prior Depolarization on Inhibition by ECMA

The finding that ECMA mainly attacked the carrier when it was in the outside-facing position prompted us to investigate whether there was a relationship between the rate of inhibition and the rate at which the carrier returns to the outside conformation and thereby increases the time spent or its effective concentration facing outward. In order to return the carrier at a faster rate we have utilized the maneuver of prior depolarization. This releases intrasynaptosomal ACh from cholinergic synaptosomes which would otherwise immobilize the carrier on the inside of the synaptosomal membrane (Marchbanks et al., 1981). We and others, have previously shown that prior depolarization increases the rate of choline influx. As Fig. 5 shows, it also increases the rate of inhibition by ECMA. The increase in the rate of inhibition (1.69×) is comparable to the increase in the rate of hemicholinium-sensitive choline influx (1.58). In the Discussion we draw some conclusions about the distribution of choline carrier between cholinergic and noncholinergic synaptosomes from this data.

Kinetic Analysis of the Action of ECMA

The finding that the rate of inhibition is related to the rate of choline uptake and that both are related to the rate of return to the outward-facing conformation of the carrier immediately suggests a reason for the biphasic nature of the concentration dependence of the rate of inhibition. At low ECMA concentration first-order kinetics apply in that the initial rate of inhibition is the product of the inhibitory first-order rate constant, the ECMA concentration and the concentration of outward-facing carrier. However, at high ECMA concentrations the rate of fractional inhibition will be limited by the rate of translocation of the carrier to the outward-facing conformation. The asymptote at high ECMA concentration also indicates the amount of carrier that can be alkylated by the first-order reaction and is thus facing outside.

In Fig. 6 is given a plot of the rate of inhibition as a function of ECMA concentration over the range 0.5 to 500 μM with dotted lines to indicate the above extrapolation. The rate constant of inhibition and the composite rate constants of translocation may then be derived by adopting a much simplified model of choline transport as described in the legend to Fig. 6. The proportion of outward-facing carrier is $2 \times 0.27 = 0.54$ and the first-order rate constant of ECMA inhibition can be obtained directly from the slope at low ECMA concentrations. Its value is 440 M^{-1} sec^{-1}, rather similar to that for the

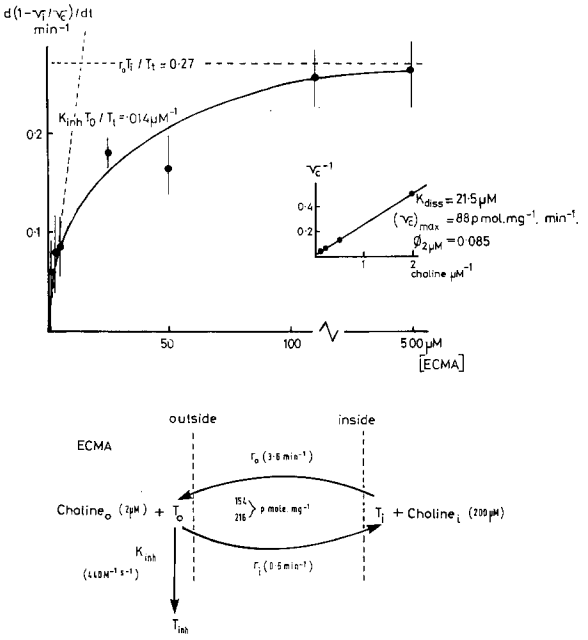

Fig. 6. Kinetic analysis of ECMA inhibition. Synaptosomes were pre-incubated for 2 min at 37°C with the concentrations of ECMA shown and then washed and choline uptake measured in the normal way. The fractional rate of inhibition (as defined in Fig. 1) per minute is shown ± SEM of four observations. The control value of choline influx was 10.4 ± 0.69 (n = 6) pmol min^{-1} mg^{-1} synaptosomal protein and in the presence of 20 μM HC-3 was 1.76 ± 0.19. The 'hemicholinium blank' has been subtracted from all values.

In the simple model shown below the main Figure the translocating rate constants (r_o, r_i) have been corrected for the inhibition of flux in cholinergic synaptosomes. They are shown as composite rates (i.e., the weighted average of free carrier and that bound to choline) resulting from the inside and outside concentrations of choline as shown. The carrier is denoted with amounts facing outside T_o and inside T_i. The upper center figure represents the total amount of carrier in cholinergic synaptosomes and the lower in the remaining 90%.

Theoretical Treatment: The fractional rate of inhibition is equal to the rate of formation of alkylated carrier divided by the total available carrier. At low ECMA concentration first-order rate kinetics apply but at high ECMA the return of the carrier to the outside conformation becomes rate controlling. The dotted lines show extrapolations to obtain numerical values for the first-order inhibition and the rate of carrier return. The fraction of carrier facing outwards can be determined by extrapolating the first-order rate to estimate the point at which all the externally facing carrier is alkylated and multiplying by the time of exposure to ECMA (2 min).

The rate of translocation of carrier can be regarded as the product of the amount of available carrier inwardly (T_i) or outwardly facing (T_o) with a composite rate constant (r_o) outward and (r_i) inward. The composite rate constant makes no distinction between the rates of translocation of free carrier and that bound to choline. This is a gross oversimplification because there is every reason to suppose that 'r' is strongly affected by the binding of carrier to choline or other compounds (*see below* and Marchbanks et al., 1981). However, choline concentrations (2 μM outside, about 200 μM average inside) have been kept the same in this experiment so that although the value of 'r' may not be immediately interpretable in terms of the rates of free and bound carrier their relativities will have been kept constant. With the assumption that translocation rates are controlling (i.e., much lower than the rates of association and dissociation to substrate) and the usual steady state ($r_iT_o = r_oT_i$) and conservation assumptions ($T_t = T_o + T_i$) we get $T_o/T_t = r_o/(r_o + r_i)$.

T_o/T_t is the fraction of carrier facing outside and this can be calculated from Fig. 6 as 2 × 0.27 = 0.54. Therefore, T_i/T_t = 0.46 and values for the composite rate constants r_o (0.59 min^{-1}) and r_i (0.50 min^{-1}) may be obtained from the limiting rate of inhibition. These composite rate constants can be approximately separated into those associated with free carrier and that bound to choline as follows. Figure 5 shows that prior depolarization increases the rate of choline uptake and inhibition by factors of 1.58 and 1.69, respectively (avg. 1.63). This implies an increase in the average fraction of outward-facing carrier (T_o/T_t) from 0.54 to 0.54 × 1.63 = 0.88. Only in cholinergic synaptosomes will T_o/T_t have changed and the removal of their internal ACh allows the carrier to adopt the conformation found in the majority of noncholinergic synaptosomes. Although the change in orientation is actually caused by the dissociation of a nontranslocating carrier-ACh complex it can be regarded as an increase in the true rate constant of translocation from inside to out (r_o). Since $T_o/T_t = r_o/(r_o + r_i)$ we can calculate (assuming no change in r_i at 0.5 min^{-1}) a true value for r_o of 3.66 min^{-1}. These rate constants apply to the noncholinergic synaptosomes and cholinergic synaptosomes when they have lost their acetylcholine. Since the concentration of choline inside is about 200 μM (>90% saturating) as opposed to 2 μM (<10% saturating), comparison of the inward and outward rate constants suggests that combination of the carrier with choline increases its translocation rate by about seven times.

The steady-state influx of choline under control conditions is;

$$v_c = T_o r_i \theta_{2\,\mu M}$$

where $\theta_{2\,\mu M}$ is the fraction of outward-facing carrier binding choline. $\theta_{2\,\mu M}$ can be determined from the concentration dependence of influx, shown in the inset to Fig. 6 as a reciprocal plot. With a K_{diss} of 21.5 μM, $\theta_{2\,\mu M}$ has a value of 0.085. As given above the uninhibited influx due to carrier (i.e., HC-3-sensitive) at choline concentrations of 2 μM was 8.64 ± 0.71 pmol min^{-1} mg^{-1} synaptosomal protein. From this a value of 376 pmol carrier per mg synaptosomal protein is obtained applicable to the total of cholinergic and noncholinergic synaptosomes. This is likely to be an overestimate since combination of carrier with choline increases 'r' (*see* Discussion) and thus decreases the estimate of T_o. The distribution of carrier between cholinergic (154 pmol mg^{-1}) and noncholinergic (216 pmol mg^{-1}) synaptosomes can be approximated as described in the Discussion

alkylation of choline dehydrogenase (395 M^{-1} sec^{-1}, Barlow & Marchbanks, 1984).

Discussion

The aziridinium compound that results by cyclization of β-halo-alkylamines can alkylate —NH_2,

—COOH and SH groups, polymerize with itself or undergo various decompositions to tertiary and quaternary amines (Fruton et al., 1946). Thin-layer chromatography (*data not shown*) of the cyclized derivative suggest that at least five compounds are formed even in the absence of tissue. We have established that the agent that irreversibly inhibits choline transport does lose its activity on a time scale consistent with the decomposition of the aziridinium ion. However, any preparation of ECMA will contain several other compounds that affect choline transport. This is suggested by the pronounced transactivation of choline efflux by the ECMA preparation during the pre-incubation (Table 1, row 1) that disappears after washing. It follows from this that any investigation of the irreversible inhibitory effect of the aziridinium compound requires the removal of its various decomposition products either by washing or some other means.

The concentration for half-maximal inhibition taken at the 10-min time points is about 20 μM. This is higher than the apparent K_i (2.7 μM) observed by Rylett and Colhoun (1980) probably because in their studies ECMA was not washed out prior to the uptake studies. Increased concentrations of nucleophilic protein used in these experiments might dilute the ECMA and account for the apparent difference but without kinetic studies it is not possible to say whether it is real. Inhibitory effects in this concentration range are what might be expected if the toxicity of ECMA is related to its inhibition of choline transport but it is puzzling that neither Fisher et al. (1982) nor Mantione et al. (1983) were able to observe any chronic decrease in tissue choline as a result of ECMA dosages in a comparable range.

The lack of strong effects on other components of the cholinergic systems is also puzzling yet fortunate for its potential as an affinity agent. Choline acetyltransferase is only weakly inhibited by ECMA at mM concentrations (Rylett & Colhoun, 1979; Barlow & Marchbanks, 1984). Choline kinase is also only slightly affected in the mM range (Barlow & Marchbanks, 1984) and acetylcholinesterase likewise (Clement & Colhoun, 1975). Indeed the only other enzyme of choline metabolism affected by ECMA at μM concentration appears to be choline dehydrogenase (Barlow & Marchbanks, 1984).

At low concentrations of ECMA the rate of inhibition falls off well below the theoretical maximum (Fig. 1) presumably due to competing reactions. However, at higher ECMA concentration the fraction of choline uptake inhibited approaches 0.8; when the diffusion (HC-3 insensitive) component of uptake is taken into account this represents almost the theoretical maximum inhibition. If, as is sometimes supposed, there are two types of choline carrier the mustard does not appear to distinguish between them by any differences in its inhibitory potency.

The protection experiments (Fig. 3) suggest that the mustard acts at the binding site of choline on the carrier which would be necessary if it is to be used as an affinity reagent. However, such experiments do not guarantee that the carrier is the only compound affected. In this connection it is to be noted that significant amounts of aziridinium enter the synaptosome in an active form (Table 3) which would expose the intrasynaptosomal proteins to alkylation. Even though the mustard gets inside synaptosomes the most part of its inhibitory action is by attack on the outside (Fig. 4).

Choline transport is mediated by a translocating carrier in synaptosomes as well as in erythrocytes. This is demonstrated by the transactivation of choline influx and efflux. Consistent with this is the inhibition by ECMA of the transactivated components of both efflux and influx (Tables 1 & 2). It follows from the translocating nature of the carrier and that ECMA alkylates the carrier predominately when it is in the outward-facing conformation that procedures that release the carrier to face outward should increase the rate of inhibition. That this indeed happens (Fig. 5) is consistent not only with our assumption as to the mechanism of ECMA inhibition but also the explanation we have offered the activation of choline influx by depolarization of cholinergic synaptosomes (Marchbanks et al., 1981). Acetylcholine binds the choline carrier on the inside of the membrane but is not itself transported. When the acetylcholine is released by depolarization-evoked Ca^{2+} entry into the terminal the carrier being no longer bound is free to translocate to the outward-facing confirmation. There it is available to mediate choline influx or is accessible to alkylation by ECMA.

From the data in Fig. 5 we may attempt to make a tentative estimate of the distribution of choline carrier between cholinergic and noncholinergic terminals. Concentrations of ACh in cholinergic synapses are in the mM range (Marchbanks, 1968*a*; Dunant et al., 1974; Katz & Miledi, 1977). This is sufficient to block choline fluxes almost completely (Marchbanks et al., 1981). The orientation of the carrier in the nondepolarized cholinergic synaptosome will therefore be exclusively on the inside unable to mediate choline influx. Since cholinergic terminals constitute rather less than 10% of the total (Richardson, 1981) and the activation of their carrier produces a 63% increase in rate of choline flux (Fig. 5) they must have about 6.3 times the concentration of carrier present on noncholinergic synap-

tosomes. Applying this to the figure for average content of choline carrier given in Fig. 6 we can estimate that cholinergic synaptosomes contain about 1540 pmol/mg synaptosomal protein and the rest about 240. For a molecule of mol wt 10^4 this would represent 0.2 to 1.5% of synaptosomal protein content.

In the development of the arguments given in the legend to Fig. 6 we have used a number of simplifying assumptions so that the treatment is illustrative and the figures are tentative. It is clear that the assumption of equality of translocation rates of carrier free and bound to choline is incorrect; there is a sixfold difference in the rate of inward orientation of essentially free carrier compared with the outward orientation of carrier bound to choline. A systematic study of the rate of ECMA inhibition using different concentrations of preloaded choline would reveal details of the differences in the rate of translocation of carrier free or bound to choline. As a consequence of this inapposite assumption the total amount of carrier is likely to be overestimated. However, the other major assumption, that the binding reaction will be much faster than the translocating rate, is supported by the rate of ECMA attack. The rate of ECMA attack will be analogous to the rate of choline binding to the carrier and it has a rate constant at least an order of magnitude higher than that for translocation when the volume of the membrane is taken into account.

Despite these uncertainties there is a general reason for using, as here, an intuitive approach to carrier kinetics rather than the formal treatment of an exact model. The choline carrier binds to a variety of different cations (Martin, 1972) as well as substances as yet unknown. Since these will affect the translocation rate of the carrier the kinetic treatment will be incorrect unless they can be eliminated from the experimental system or included in the model. The former is not possible with the synaptosomes, the latter leads to equations of such opacity as to be self defeating. In this study with an irreversible inhibitor we have arrived at some tentative figures for amounts, distributions and translocation rates of the choline carrier in synaptosomes using a simple model. We believe that further experiments on the same line but with the elimination of inconsistencies from the model will offer progress to more definitive figures.

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