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Reconstitution of Carrier-Mediated Choline Transport in Proteoliposomes Prepared from Presynaptic Membranes of *Torpedo* Electric Organ, and its Internal and External Ionic Requirements

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Summary. Proteoliposomes made by a butanol-sonication technique from electric organ presynaptic membranes showed choline transport activity. In contrast to intact nerve terminals, the uptake of choline was dissociated from its conversion to acetylcholine in this preparation. The kinetics of choline uptake by proteoliposomes was best described by two Michaelis-Menten components. At a low concentration of choline, uptake was inhibited by hemicholinium-3 and required external Na^+ and, thus, closely resembled high-affinity choline uptake by intact cholinergic nerve terminals. Choline transport could be driven by the Na^+ gradient and by the transmembrane potential (inside negative) but did not directly require ATP. External Cl^-, but not a Cl^- gradient, was needed for choline transport activity. It is suggested that internal K^+ plays a role in the retention of choline inside the proteoliposome. Proteoliposomes should prove a useful tool for both biochemical and functional studies of the high-affinity choline carrier.

Key Words choline transport · liposomes · acetylcholine synthesis · ionic gradients · transmembrane potential · carrier

Introduction

A specific carrier mechanism with a high affinity for choline is characteristic of cholinergic nerve terminals, and its properties *in situ* have been extensively defined (*see* reviews, Kuhar & Murrin, 1978; Jope, 1979). Nevertheless, the metabolism of choline is complex and it has been suggested that the activity of the choline carrier is affected by intracellular levels of choline and of acetylcholine (ACh) (Jope et al., 1978; Marchbanks et al., 1981), the principal product of choline metabolism in cholinergic nerve terminals. In order to study the uptake of choline in the absence of subsequent metabolic transformation and to do so under conditions where the composition of the internal medium can be controlled, several groups have used shocked and resealed synaptosomal ghosts (Adam-Vizi & Marchbanks, 1983; Breer & Lueken, 1983); however, these protocols do not appear to cause a complete dissociation between choline uptake and ACh synthesis (Adam-Vizi & Marchbanks, 1983; and *personal observation*).

A different approach has been to reconstitute the choline carrier into artificial lipid vesicles, and reports on the formation of proteoliposomes by dialysis of cholate-solubilized cortical nerve terminal membranes (King & Marchbanks, 1982*a*; Meyer & Cooper, 1983) show that the choline carrier can be studied in such a system. In the experiments reported in this paper a similar approach was taken, but with two important differences. Firstly, the starting material used here to prepare presynaptic membranes was the electric organ of *Torpedo marmorata* which is rich and homogeneous in its cholinergic innervation (*cf.* Morel et al., 1979). Secondly, proteoliposomes were prepared using a butanol-sonication technique which has been developed to reconstitute another important presynaptic function, ACh release (Israël et al., 1983, 1984). This technique permits the formation of larger liposomes that facilitate studies on a transport process (e.g. King & Marchbanks, 1982*b*).

The results of the present experiments demonstrate that the reconstituted choline carrier closely resembles the carrier in its native state in that it is saturable, with high and low affinity constants, and that at low choline concentrations uptake is Na^+ dependent and sensitive to low concentrations of hemicholinium-3 (HC-3). Proteoliposomes were then used to test the effects of changes in the internal and external media on choline accumulation in order to clarify the ionic and energy requirements of the choline carrier.

Abbreviations: ACh (acetylcholine); HC-3 (hemicholinium-3); ChAT (choline acetyltransferase).

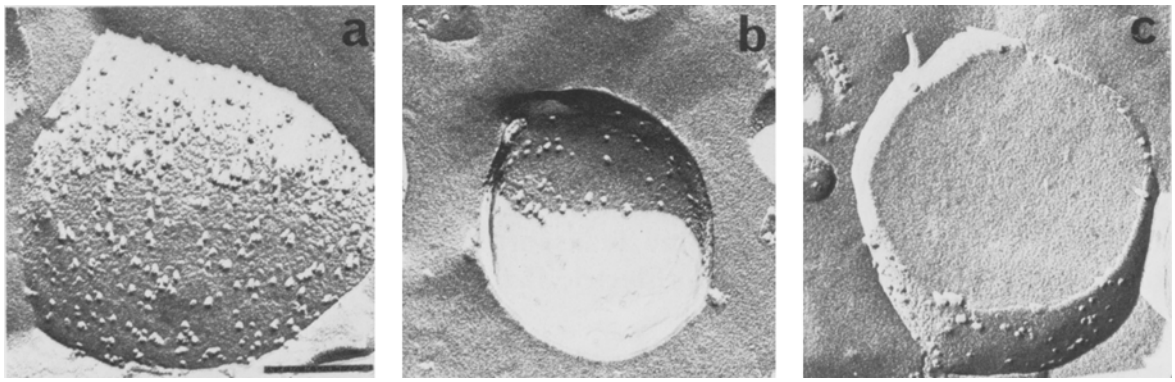

Fig. 1. Electron micrographs of freeze-fractured presynaptic membrane proteoliposomes. Both the convex (*a*) and concave (*b*) faces of the single leaflet membrane show intramembrane particles, while the interior (*c*) appears empty. Bar equals 0.1 μm. Magnification 145,000×

Materials and Methods

Preparation of Synaptosomes and Proteoliposomes

Electric organ synaptosomes were prepared by the procedure described by Morel et al. (1977) using a discontinuous sucrose gradient. The band of material enriched in synaptosomes was used to prepare presynaptic membranes and, therefrom, proteoliposomes by the method of Israël et al. (1983, 1984).

In brief, synaptosomes derived from approximately 15 g of electric organ tissue were hypo-osmotically shocked at alkaline pH (8.1), centrifuged, and the pellet was lyophilyzed. The lyophilyzed membrane was mixed with 2 to 4 mg of L-dipalmytoyl phosphatidylcholine (Sigma) in 1 ml butanol, and the solvent evaporated under a stream of N_2. The proteolipid mixture was suspended in 500 μl of the internal medium consisting of 25 mM K_2 succinate, 250 mM KCl, 2 mM $MgCl_2$, 100μM dithiothreitol, 100 μM EGTA and 10 mM Tris buffer, pH 7.1. The mixture was then sonicated using a probe sonicator (type 20-2105, Bioblock, France) for 15 sec. The proteoliposomes formed were passed through Sephadex G50 (coarse) columns equilibrated with the external medium (300 mM NaCl, 25 mM Na_2SO_4 and 10 mM Na phosphate buffer, pH 7.1) and were recovered in 600 μl. The internal and external media were modified as indicated in some experiments.

Measurement of Choline Uptake

Aliquots (50 μl) of proteoliposomes containing about 10 μg of protein were allowed to equilibrate with the ambient temperature (18 to 21°C). Choline uptake was initiated by the addition of 0.2 μM (^{3}H) choline chloride (78 Ci/mmol, Amersham, U.K.). Samples were either filtered immediately (i.e. 15 sec) to obtain blank values or allowed to incubate for 15 min (unless stated otherwise) at room temperature and then filtered. The filtration procedure was as has been described by Morel et al. (1977) except that Millipore filters of 0.3 μm pore diameter were used. Test compounds were added just prior to the addition of choline in the indicated experiments. In those experiments where proteolytic enzymes were used, pronase (1.5 mg/ml, Boehringer, FRG) was added either to the proteolipid mixture 20 min before sonication or to the proteoliposomes following sonication and 20 min before choline uptake was measured. Neuraminidase (E.C.3.2.1.18; 0.5 U/ml, Nakari Chemicals, Japan) was added to the proteoliposomes 10 min before choline uptake was measured.

Measurement of Internal Volume

To measure the volume trapped inside the proteoliposomes, 250 nCi of (U-^{14}C) sucrose (584 mCi/mmol, Amersham, U.K.) was added to the proteolipid mixture before sonication. The amount of radioactivity retained by the filters and its concentration in the internal medium were measured and the internal volume was calculated as dpm per sample/dpm per μl which gave μl/sample.

Kinetics Analysis of Choline Uptake

Proteoliposomes were incubated with ^{3}H-choline (1 μCi) and varying concentrations of nonradioactive choline at final concentrations ranging from 0.4 to 100 μM and the rate of choline uptake was measured as described above. The resulting data were analyzed using a Digital PDP-8 computer programmed to approximate nonlinear functions by the least-squares method. Two equations were tested:

$$v = \frac{V_{\max} \cdot S}{K_m + S} + K_D \cdot S \tag{1}$$

and

$$v = \frac{V_H \cdot S}{K_{mH} + S} + \frac{V_L \cdot S}{K_{mL} + S} + K_D \cdot S \tag{2}$$

where v and S represent velocity and substrate concentration, K_m and V_{max} are the classical Michaelis-Menten parameters, H and L subscripts represent high and low affinity components. The term K_DS was included due to the nonsaturable aspect of the substrate-velocity plot; K_D was estimated by taking the slope of the final portion of the substrate-velocity curve.

Determination of Other Biochemical Parameters

ACh content was measured by the chemiluminescent method described by Israël and Lesbats (1981). Total LDH activity was measured by the method of Johnson (1960). ChAT was estimated by the acetylation of choline from (^{14}C)-acetylCoA by the method of Fonnum (1975). Conversion of (^{3}H)-choline to (^{3}H)-ACh was measured following extraction and thin-layer chromatography such as has been described by O'Regan (1982). Protein was determined by the method of Bradford (1976).

Freeze-Fracture Electron Microscopy

The "sandwich technique" of Gulik-Krzywicki and Costello (1978) was used to obtain freeze-fracture sections of proteoliposomes which had been concentrated beforehand (Israël et al., 1984). The replicas were examined at 40,000× direct magnification with a Siemens Elmiskop 104 electron microscope.

Results

Morphological and Biochemical Characteristics of Proteoliposomes

Appearance Following Freeze-Fracture Electron Microscopy

Proteoliposomes formed during sonication of a suspension of a synthetic phospholipid (phosphatidylcholine dipalmytoyl) and presynaptic membrane components were predominantly unilamellar and ranged in size from 0.1 to 1.0 μm in diameter. The membrane phase of these proteoliposomes shows both large and small particles which appear to be distributed on the concave and convex faces of the membranes following freeze fracture (Fig. 1); these particles very probably represent membrane proteins (*cf.* Israël et al., 1984). That the interior of the proteoliposomes is empty can be seen when the plane of fracture goes through the proteoliposomes.

Comparison of Biochemical Markers in Electric Organ Synaptosomes and Proteoliposomes

In contrast to synaptosomes which were rich in choline acetyltransferase (ChAT, E.C. 2.3.1.6.), the proteoliposomes contained only small amounts of this enzyme and undetectable amounts of other cytoplasmic markers (Table 1). In view of these results, it is not surprising that proteoliposomes were unable to convert choline to ACh. Proteoliposomes thus appeared to be a useful tool for the study of choline transport independent of the acetylation reaction driven by ChAT.

Table 1. Biochemical markers in synaptosomes and proteoliposomes[a]

	Synaptosomes	Proteoliposomes
LDH (E/min/g ml^{-1})	3.3 ± 0.6	Not detectable
ChAT (nmol/hr/g tissue)	170.2 ± 20.5	22 ± 4
ACh content (nmol/g tissue)	47.3 ± 6.4	Not detectable
ACh synthesis (% conversion of ^{3}H-choline to ^{3}H-ACh)	23.0 ± 1.7	Not detectable

[a] Results are expressed as mean ± SEM of at least three experiments.

Main Characteristics of Choline Uptake by Proteoliposomes

Dependence of Presynaptic Membrane Proteins

In a representative experiment, proteoliposomes incubated with 0.2 μM ^{3}H-choline for 15 min at room temperature accumulated at least 10 times more choline than at 15 sec, and this time-dependent accumulation was blocked in the presence of HC-3 (Fig. 2), a competitive inhibitor of choline transport in intact preparations. In subsequent experiments, choline uptake under control conditions (0.2 μM ^{3}H-choline, 15 min incubation) was calculated following subtraction of the 15-sec values and found to be 0.28 ± 0.04 pmol/min/mg protein (n = 25). This accumulation of choline by proteoliposomes was inhibited by 81 ± 3% (n = 12) by 10 μM HC-3. Furthermore, when the filters on which proteoliposomes had been deposed were rinsed with cold distilled water, the accumulated choline was found to have been localized in an osmotically labile compartment (*data not shown*). In contrast, liposomes prepared exclusively with the synthetic lipid did not show a time-dependent uptake of choline and were not affected by HC-3 (n = 5).

The addition of pronase to the proteoliposomes reduced choline uptake by 45%, and a similar treatment with neuraminidase caused a 25% reduction in choline uptake. These results are consistent with observations in intact tissues (Massarelli et al., 1982). Moreover, when pronase was added to membrane components before incorporation, the resulting proteoliposomes lost their ability to accumulate

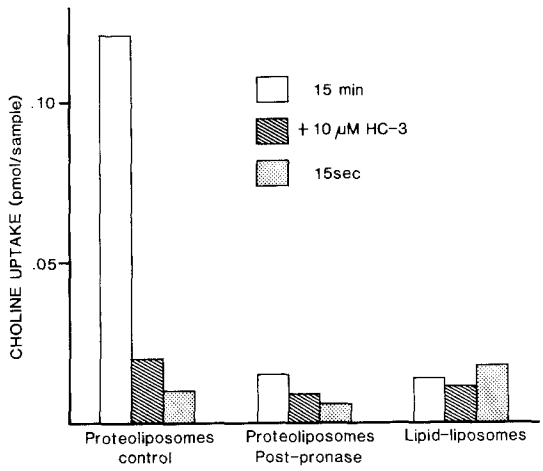

Fig. 2. Time-dependent, HC-3-sensitive choline uptake by proteoliposomes as a result of incorporation of presynaptic proteins. Aliquots of liposomes made using either presynaptic membrane components and synthetic lipid, or protease-treated membrane components and lipid, or lipids only, were incubated with ^{3}H-choline (0.2 μM) for the indicated times in the presence or absence of HC-3 (10 μM). Only proteoliposomes showed time-dependent, HC-3-sensitive choline uptake. Results are expressed as pmol/sample as not all samples contained protein

choline in a time-dependent, HC-3 sensitive manner (Fig. 2).

Although no attempt was made in these experiments to separate protein from other components of the presynaptic membrane, these results indicate that protein and perhaps glycoprotein moieties are involved in the transport of choline.

Time and Temperature Effects

When a more detailed study of the time dependence of choline uptake by proteoliposomes was made, it was observed that choline was taken up at a constant rate for up to 45 min, after which time there occurred a slow loss of accumulated choline (Fig. 3). The internal volume of the proteoliposomes was measured and found to be 5.8 ± 1.0 μl/mg protein (n = 6); hence, the internal concentration of choline at the peak of its accumulation can be estimated to be 0.4 μM. This value is higher than the external choline concentration (0.2 μM).

Incubation of proteoliposomes with choline at room temperature gave near optimal transport, as it was observed that both raising and lowering the incubation temperature resulted in a loss of activity (Fig. 3).

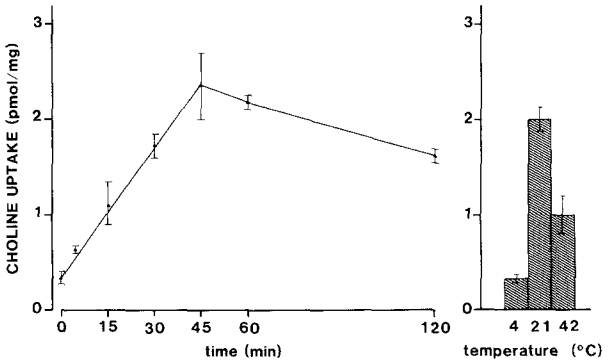

Fig. 3. Time and temperature dependence. Aliquots of proteoliposomes were incubated with choline for varying lengths of time at 21°C or for 30 min at different temperatures and choline uptake was measured. Results are expressed as means ± SEM of 4 to 6 values

Kinetics

As discussed by Yamamura and Snyder (1973) for choline transport by rat brain synaptosomes, the dependence of the rate of choline uptake by proteoliposomes on external choline concentrations is better described by an equation containing two Michaelis-Menten components (Fig. 4*B*) rather than one (Fig. 4*A*). The most striking difference between the two curves is that the bottom curve better predicts the data points for choline uptake at the lowest choline concentrations tested (*see* insets). An analysis of variance showed that the residual variance was decreased when two Michaelis-Menten terms were considered (F = 4.49, $P < 0.05$). An Eadie-Hofstee analysis of the same data (Fig. 4*C*) also shows a clear discontinuity, supporting the case for at least two uptake components.

In both models a "diffusion" component was included for which the constant was estimated by determining the slope of the substrate-velocity plot between 50 and 100 μM choline. However, the value obtained, 0.026 pmol · min^{-1} · mg^{-1} · μM^{-1}, is likely to be an overestimation of the contribution of simple diffusion to choline uptake by proteoliposomes at room temperature since no time-dependent choline uptake was observed either at 0°C (Fig. 3) or when pure lipid liposomes were used (Fig. 2). The difficulty in distinguishing between low affinity and diffusion components of uptake has been discussed by others (*cf.* Wheeler, 1979).

From three separate experiments at low choline concentrations (0.4 to 5 × 10^{-6} M) the high-affinity Michaelis-Menten constant (K_{mH}) was estimated to be 1.28 ± 0.38 μM, which is in good agreement with

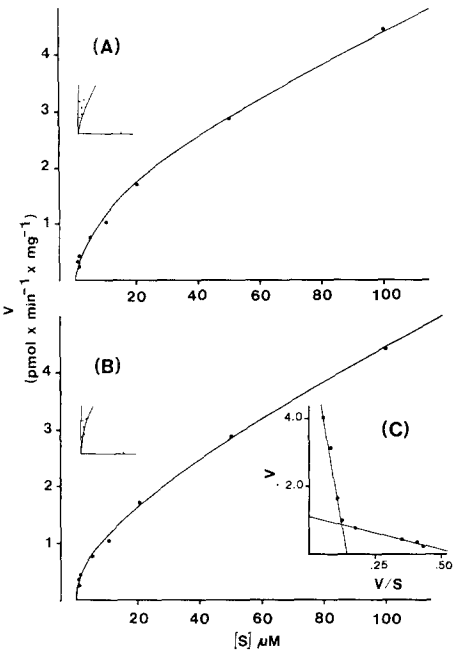

Fig. 4. Kinetic characteristics. Choline uptake into proteoliposomes was measured following 30 min of incubation at room temperature at varying concentrations of choline. The data are plotted as the velocity of choline accumulation as a function of the choline concentration in *A* and *B*. The two curves represent equations (*see* Materials and Methods) containing: *A*) one Michaelis-Menten term (K_m = 12.7 ± 3.0 μM) and a diffusion component, or *B*) two Michaelis-Menten terms (K_{mH} = 1.42 ± 0.36 and K_{mL} = 45.6 ± 9.0) and a diffusion component. The Michaelis-Menten constants were determined using a program which approximates nonlinear functions by the least-squares method. An Eadie-Hofstee plot of the results (*C*) also indicates the existence of two components

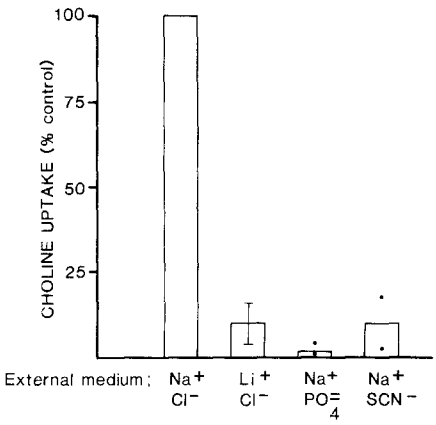

Fig 5. Dependence on external NaCl. Choline uptake was measured following 15 min of incubation at room temperature in the indicated media. Equimolar substitution of LiCl, Na phosphate or NaSCN for NaCl (300 mM) in the external medium blocked choline uptake by proteoliposomes

results obtained on electric organ synaptosomes (Morel et al., 1977).

Ionic Requirements for Choline Uptake by Proteoliposomes

Effects of Changes in Ionic Composition of the External Medium

Since the predominant ions in the external medium under control conditions were Na^+ and Cl^-, the next experiments were done to test whether these ions were required for choline transport activity. Either replacement of Na^+ by Li^+ or replacement of Cl^- by phosphate or thiocyanate caused a sharp loss in choline uptake (Fig. 5), indicating that both Na^+ and Cl^- must be present in the external medium for choline to be transported by these proteoliposomes.

The Na^+ dependence of choline uptake is consistent with the identification of this property of proteoliposomes with the choline carrier in intact cholinergic nerve terminals (i.e. Yamamura & Snyder, 1973). Likewise, high-affinity choline transport in rat brain synaptosomes (Kuhar & Zarbin, 1978) and electric organ T-sacs (Rothlein & Parsons, 1979) has been described as Cl dependent, although this is not true for all preparations tested (O'Regan & Collier, 1981).

Choline accumulation was not improved by the addition of 1 to 5 mM Ca^{2+} to the external medium (n = 3, *data not shown*), which is in agreement with findings on isolated nerve terminals from *Torpedo* (Rothlein & Parsons, 1979).

Effects of Changes in Ionic Composition of the Internal Medium

One of the advantages of working with proteoliposomes is that the internal, as well as the external, ionic environment can be controlled. The principal ionic species in the normal internal milieu were K^+ and Cl^-. When Cs^+ replaced K^+, choline transport activity fell to one-third of the control value, and both Li^+ and Na^+ were even less effective substitutes for K^+ (Fig. 6). Choline uptake was not affected by the substitution of phosphate for Cl^- in the internal medium. The lack of a stimulatory effect upon lowering internal Cl^- argues against a role for an inwardly directed Cl^- gradient in regulating choline transport and suggests instead that the pres-

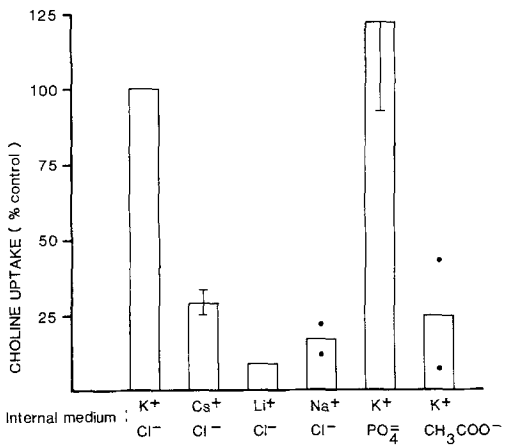

Fig. 6. Dependence on internal KCl. Equimolar substitution of CsCl, LiCl or NaCl for KCl (250 mM) in the internal medium all caused decreases in choline uptake, while replacing KCl with K phosphate was without effect. When acetate replaced Cl^-, choline uptake was inhibited.

ence of external Cl^- suffices for the expression of choline transport activity. Nonetheless, a decrease in choline uptake was observed when acetate replaced Cl^-, but this may have been due to an inhibitory action of acetate on the uptake mechanism.

The importance of minor constituents of the internal medium was also examined. It was found that the presence of an impermeant anion such as succinate was necessary for optimal accumulation of choline, but that sulfate was as effective as succinate in producing this effect (Table 2). Neither Mg^{2+} nor EGTA were found to be essential; however, the marked inhibitory effect of internal Ca^{2+} suggested to us that their inclusion was a useful precaution.

Energy Requirements for Choline Uptake by Proteoliposomes

To see whether choline transport was directly dependent on an internal source of ATP, this compound was added to the internal medium. Unexpectedly, 5 mM ATP caused an inhibition of choline uptake (to 26% of control values) and this effect was reversed to some extent by the addition of 0.5 mM ouabain to the external medium (to 41% of control values). This observation clearly indicates that choline transport does not require ATP; however, the basis for the inhibitory action of ATP remains an unresolved problem.

Under physiological conditions, the Na^+ and K^+ gradients are maintained at the expense of ATP, but in liposomes these gradients are imposed upon the system by the choice of internal and external media. The results presented in Figs. 5 and 6, showing that external Na^+ was required for choline uptake while Na^+ on both sides of the membrane blocked uptake, are consistent with the Na^+ gradient being a driving force for choline transport. Indeed, it was found that the addition of either gramicidin or monensin, which abolish the Na^+ gradient (Pressman, 1976), led to a reduction in choline uptake (Table 3).

Table 2. Effects of changes in ionic composition of the internal medium on choline accumulation by proteoliposomes[a]

Condition	Choline uptake (test/control)
No succinate	0.22 ± 0.06
Succinate replaced by sulfate	0.88
No $MgCl_2$	0.89
No EGTA	0.72 ± 0.30
Added $CaCl_2$ (5 mM)	0.09

[a] Results are expressed as either mean of duplicate determinations or mean ± SEM of three experiments.

To further explore the possibility that the K^+ gradient, as well, might constitute a driving force for choline transport, the effect of adding a highly specific K^+ ionophore, valinomycin (Pressman, 1976), was tested. At 2 μM, valinomycin more than doubled choline accumulation and the increased accumulation was as sensitive to HC-3 as under control conditions (Table 2). This result suggests that the K^+ gradient influences choline transport as a function of the transmembrane potential created by the gradient in the presence of a permeability to K^+. Furthermore, the effect of valinomycin was not only to increase the rate at which choline was accumulated, but also to raise the plateau concentration of choline inside the proteoliposomes (Fig. 7). At 60 min the internal concentration of choline can be estimated to be 2.1 ± 0.3 μM (n = 3); hence, 10-fold higher than in the external medium.

To study this phenomenon in more detail, choline accumulation by proteoliposomes was measured at different external K^+ concentrations in the presence and absence of valinomycin (0.2 μM). In the absence of valinomycin, the addition of KCl to the external medium was without effect until its concentration exceeded 20 mM (Fig. 8). On the other hand, in the presence of a low concentration of valinomycin, choline uptake was most affected by small changes in the concentration of KCl in the low range. This latter finding is consistent with the hypothesis that choline uptake is stimulated by valinomycin because valinomycin creates a membrane potential under control conditions which is attenuated upon reducing the K^+ concentration gradient.

Table 3. Effect of ionophores on choline uptake by proteoliposomes[a]

Condition	Choline uptake (test/control)
Gramicidin (5 μM)	0.16 ± 0.02
Monensin (5 μM)	0.26
Valinomycin (2 μM)	2.49 ± 0.30
+ HC-3 (10 μM)	0.30 ± 0.02

[a] Results are expressed as either mean of duplicate determinations or mean ± SEM of three to five experiments.

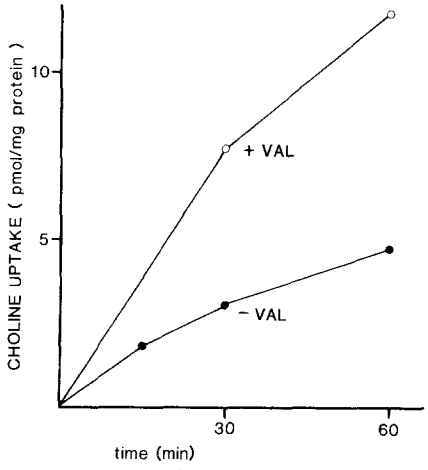

Fig. 7. Effect of adding valinomycin (0.2 μM) on choline uptake. In the presence of valinomycin, both the initial rate of choline uptake and the total accumulation of choline by proteoliposomes were increased

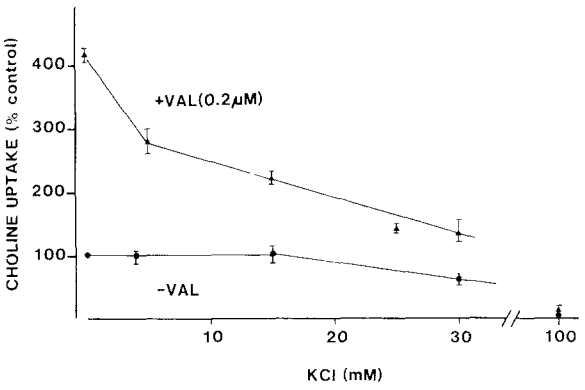

Fig. 8. Effect of increasing external K^+ on choline uptake in the presence and absence of valinomycin (0.2 μM). External K^+ was much more inhibitory for choline uptake in the presence than in the absence of valinomycin, although choline uptake was blocked at 100 mM external K^+ in both cases. Results are expressed as means ± SEM of three experiments

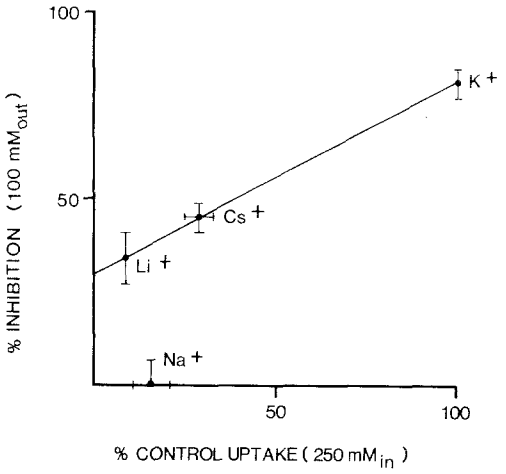

Fig. 9. Correlation between the inhibitory effect of K^-, Cs^+ and Li^+ on choline uptake when they were present in the external medium and their suitability as principal constituents of the internal medium. The addition of 100 mM NaCl to the external medium was without effect

Nonetheless, it was surprising to us that at 100 mM KCl, even in the absence of valinomycin, choline uptake was almost completely blocked. This result could suggest that, as for choline transport in erythrocytes (Martin, 1972), K^+ may have a direct inhibitory effect on choline uptake. In the next set of experiments a series of monovalent cations were tested for their inhibitory effect on choline uptake. The order of potency for the tested cations was found to be $K^+ \gg Cs^+ > Li^+$, while the addition of 100 mM NaCl to the external medium had no effect whatsoever on choline uptake. The specificity of this effect recalled that established in Fig. 6 when substitutions were made for K^+ in the internal medium. Indeed, when the results of these two sets of experiments are compared (Fig. 9), there appears to be a strong correlation between the suitability of a cation as the main constituent of the internal medium to demonstrate choline uptake and the inhibitory effect of the same cation when added to the external medium. These results could be explained by a different role for internal K^+ other than determination of the membrane potential, namely that internal K^+ prevents the efflux of choline. Such a role would be less well filled by Cs^+ and Li^+, while the effect of substituting Na^+ for K^+ in the internal medium is complicated due to the concomitant destruction of the Na^+ gradient.

Discussion

In this paper, we show that proteoliposomes made from presynaptic membranes of the electric organ

and a synthetic lipid are capable of concentrating choline. The characteristics of choline uptake by this preparation closely resemble those described for choline uptake in cholinergic tissues. Thus, the kinetics of choline uptake by proteoliposomes is best described by two Michaelis-Menten components and at low concentrations, choline uptake is both HC-3 sensitive and Na^+ dependent. That these properties are maintained in proteoliposomes is all the more remarkable since choline uptake is no longer coupled to ACh synthesis in this preparation. As neither pure lipid liposomes nor pronase-pretreated proteoliposomes showed time-dependent, HC-3-sensitive choline accumulation, it seems likely that a presynaptic membrane protein is responsible for choline transport.

In proteoliposomes, choline transport activity was optimal when: 1) Na^+ was present outside, but not inside, 2) K^+ was present inside, but not outside, and 3) Cl^- was present outside, whether or not Cl^- was present inside. What, then, are the possible roles for these three ions in regulating choline transport activity?

A dependence for solute transport on an inwardly directed Na^+ gradient has been established for many transport systems (Schultz & Curran, 1970), including choline transport (Breer & Lueken, 1983; Meyer & Cooper, 1983), which are sometimes called secondary active transports due to their indirect dependence on intracellular ATP (Eytan, 1982). The above-mentioned dependence on external Na^+ and the inhibition observed with gramicidin and monensin for choline uptake by proteoliposomes are all consistent with the hypothesis that the concentration gradient for Na^+ can be used to drive choline transport.

While the K^+ concentration gradient may also be directly involved with the activity of the choline carrier, the results presented here show that choline transport was most sensitive to the K^+ gradient when proteoliposomal membrane permeability to K^+ was increased by the addition of valinomycin. In other words, it seems possible that choline transport can also be driven by a transmembrane potential (inside negative); such a proposal was made previously on the basis of *in situ* studies on choline accumulations that show it is decreased during depolarization (Carroll & Goldberg, 1975; Murrin & Kuhar, 1976; Beach, Vaca & Pilar, 1980; O'Regan & Collier, 1981). The possibility that internal K^+ competes with choline and prevents its efflux could explain the selectivity of choline uptake with respect to other internal cations, and such a role for K^+ is not incompatible with its role in determining the membrane potential. Nonetheless, measurements of the efflux of choline from proteoliposomes as a function of the membrane potential would also be necessary to clarify the role of internal K^+ and the membrane potential in determining choline transport rates. The observation that a Cl^- gradient was not important for choline transport activity, although its presence in the external medium was required, suggests that Cl^- might affect only the binding of choline to its carrier and not its translocation across the membrane.

In conclusion, these proteoliposomes show promise of being a useful tool for both the biochemical characterization and a functional understanding of the choline carrier.

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