# Mechanisms Controlling Choline Transport and Acetylcholine Synthesis in Motor Nerve Terminals during Electrical Stimulation

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ABSTRACT Electrical stimulation of the chick ciliary nerve leads to a frequency-dependent increase in the $Na^+$-dependent high affinity uptake of [$^3$H]choline (SDHACU) and its conversion to acetylcholine (ACh) in the nerve terminals innervating the iris muscle. The forces that drive this choline (Ch) uptake across the presynaptic membrane were evaluated. Depolarization with increased $[K^+]_{out}$ or veratridine decreases Ch accumulation. In addition to the electrical driving force, energy is provided by the $Na^+$ gradient. Inhibition of the Na,K-ATPase decreased the Ch taken up. Thus, changes in the rate of Ch transport are dependent on the electrochemical gradients for both Ch and $Na^+$. Ch uptake and ACh synthesis were increased after a conditioning preincubation with high $[K^+]_{out}$ or veratridine. As is the case for electrical stimulation, this acceleration of Ch uptake and ACh synthesis was strongly dependent on the presence of $Ca^{++}$ in the incubation medium. $Na^+$ influx through a TTX-sensitive channel also contributed to this acceleration. Inasmuch as membrane depolarization reduces the initial velocity of Ch uptake and ACh synthesis, their increases during electrical stimulation therefore cannot be the direct effect of the depolarization phase of the action potential. Instead they are the result of the ionic fluxes accompanying the presynaptic spike. It is concluded that stimulation of Ch uptake and ACh synthesis by nerve activity depends first, on the ACh release elicited by $Ca^{++}$ influx after depolarization and second, on the activation of the Na,K-ATPase due to $Na^+$ entry. Furthermore, it is suggested that the release of ACh after stimulation drives translocation of cytoplasmic ACh into a protected compartment (probably vesicular). This recompartmentation of intraterminal ACh stimulates ACh synthesis by mass action, allowing further accumulation of Ch.

## INTRODUCTION

The functioning of cholinergic nerve terminals depends on their ability to replace the acetylcholine (ACh) released. At rest, radio-labelled choline (Ch) is not converted to ACh very rapidly; however, electrical stimulation increases the rate of Ch acetylation several fold (Birks and MacIntosh, 1961; Collier and MacIntosh, 1969; Potter, 1970). Furthermore, during nerve stimulation, the store of ACh in the terminals is maintained at a level near that of the resting preparation, even when the total amount of ACh released exceeds that of the

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initial store (Brown and Feldberg, 1936; Birks and McIntosh, 1961). Therefore, it is clear that cholinergic nerve terminals have the ability to accelerate ACh synthesis in order to compensate for its loss by stimulus-coupled secretion.

The search for the nature of the mechanism regulating ACh synthesis has focused on the synthesizing enzyme, acetyl-CoA:choline O-acetyl-tranferase E.C.2.3.1.6, (CAT). However, there is no convincing evidence for modulation of this enzyme as a regulatory influence, for it is usually present in excess in nerve endings (see review: Haubrich and Chippendale, 1977). Previous work (Birks, 1963) has suggested that $Na^+$ may be involved in choline transport. It has since been confirmed the cholinergic nerve endings possess a sodium-dependent, high-affinity transport system for Ch (SDHACU) (Haga and Noda, 1973; Yamamura and Snyder, 1973). This uptake system appears to be closely coupled to ACh synthesis, leading several authors to suggest that Ch transport may be rate-limiting for ACh synthesis (Barker and Mittag, 1975; Simon et al., 1976; but see also Collier and Ilson, 1977). In any case, the availability of precursor is a likely mechanism to regulate neurotransmitter synthesis. There is evidence to suggest that the rate of Ch transport is increased by electrical nerve stimulation (Simon et al., 1976; Collier and Ilson, 1977). Although the processes which mediate this effect are not well understood, it is clear that $Ca^{++}$ is involved (Murrin and Kuhar, 1976; Collier and Ilson, 1977).

Previous work from this laboratory has demonstrated the presence of SDHACU in the terminals of the ciliary nerve innervating the chick iris muscle (Suszkiw and Pilar, 1976). The kinetics of this uptake has been characterized as have the effects of various ionic and metabolic perturbations (Suszkiw and Pilar, 1976; Beach et al.[1]). In the present study the ciliary nerve-iris preparation is used to investigate the energetics of SDHACU and its relation to ACh synthesis during electrical nerve stimulation, to clarify the regulatory steps necessary to maintain a steady supply of ACh during synaptic activity. It is concluded that the stimulation of Ch uptake and ACh synthesis are due to the aftereffects of action potential depolarization, and that these effects are mediated through both $Ca^{++}$ and $Na^+$ fluxes.

Preliminary reports of some of these results have been presented (Vaca and Beach, 1977; Vaca and Pilar, 1977).

## METHODS

Tissues were isolated from white leghorn chickens, 9–12 d old, which were killed by decapitation. Two slightly different preparations of the ciliary nerve-iris muscle were used. In most of the experiments, the iris was dissected out from the ciliary body and sclera, with the consequent section of the ciliary nerve branches at the outer rim of the iris, as previously described (Beach et al.[1]). If the isolated iris was stimulated, the preparation folded over and crumpled; to prevent this in the experiments in which the ciliary nerves were to be stimulated electrically, the muscle was left attached to the scleral ring. In this case, eyes with surrounding connective tissues were excised and transferred to oxygenated Tyrode's solution maintained at room temperature. The ciliary ganglion

[1] Beach, R., K. Vaca, and G. Pilar. Metabolic requirements for high affinity choline uptake and acetylcholine synthesis in nerve terminals at the neuromuscular junction. Manuscript submitted for publication.

was then freed from the surrounding tissue until the ciliary nerves were exposed; the oculomotor nerve was sectioned proximal to the ganglion while the ciliary nerves were left attached to the iris and ciliary body. The posterior portion of the sclera, the choroidal coat, the retina, and the vitreous humor were removed, and the lens was teased away from the iris. The preparation was thus reduced to the iris, attached to the ciliary body, with both remaining inserted into a scleral ring.

### *Ch Uptake and ACh Synthesis in Control Preparations*

A systematic discrepancy between the intact ciliary nerve-iris preparation and the free iris preparation was introduced by the dissection procedures. In the free iris preparation, there was a partial activation of the $Na^+$-dependent uptake relative to the $Na^+$-independent uptake, when compared with the experiments done with the ciliary nerve intact. In the latter preparation, typical control values (Fig. 1, 0 Hz) were 0.84 pmol/8 min per iris for Ch uptake and 0.08 pmol/8 min per iris for ACh synthesis. In one experiment, the ciliary nerve and iris were dissected as usual for electrical stimulation. The postganglionic nerve was then sectioned with the remainder of the preparation left intact. In this unstimulated control, 1.87 pmol Ch were taken up in 8 min with 0.63 pmol being converted to ACh. This was fairly typical of the values for uptake usually observed in free irises (compare with Figs. 2 A, 8 A, or 9 A), although somewhat higher than the levels of ACh synthesis usually seen. There may also have been a small nonspecific reduction in uptake in the intact preparation due to greater diffusional barriers.

### *Incubation Conditions*

Preparations were first equilibrated at 37°C for 2–3 min in normal Tyrode's solution. Where appropriate, the solution bathing the irises was then replaced with a preincubation solution as described in the Results section. In experiments where irises were preincubated in solutions containing elevated $K^+$ concentrations, a 2-min wash in normal Tyrode's followed the preincubation. Finally, preparations were transferred to solutions containing ([$^3$H]methyl)-choline (specific activity 10.1 Ci/mol, 98% radiochemical purity, Amersham/Searle Corp., Arlington Heights, Ill.) and incubated for 8 min with exceptions indicated in the text. For electrical stimulation experiments, the ciliary ganglion including a short length of postganglionic nerve was pulled into a suction electrode at this point, and supramaximal stimuli were applied using trains of bipolar pulses, 0.2 ms in duration, at the desired frequencies delivered from an electrical stimulator. After incubation in the experiments where electrical stimulation was used, the preparation was transferred to ice-cold (4°C) Tyrode's solution, and the iris was dissected in 30–40 s and homogenized. In experiments with free irises, uptake was terminated with a 10-ml wash of ice-cold Tyrode's solution on a filter paper under suction. This "stop-wash" cold Tyrode's solution procedure was shown previously to eliminate most of the extracellular [$^3$H]Ch (Beach et al.[1]).

### *Assay Conditions*

Each iris was then transferred to a tight-fitting glass homogenizer kept at near-ice temperature, containing 120 $\mu$l of 0.2% acetic acid in 95% ethanol and 10 $\mu$l each of 0.1 M choline chloride, 0.1 M acetylcholine chloride and 2.0 $\mu$M acetyl ([$^{14}$C] *N*-methyl) choline chloride (57.7 mCi/mmol, Amersham-Searle Corp.). The iris was homogenized with 10 strokes. Two 10-$\mu$l aliquots were transferred to scintillation vials, to which 0.2 ml Protosol (New England Nuclear, Boston, Mass.) was added, and kept at 60°C for 10 min. Liquifluor (New England Nuclear) toluene scintillation fluid was added to the samples which were then counted in a Beckman LS-8000 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.).

The remainder of the homogenate was centrifuged 10 min at 3000 rpm in a Sorvall refrigerated centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). 60 $\mu$l of the supernate was applied to Whatman 3 MM paper (Whatman, Inc., Clifton, N.J.) and dried. The samples were electrophoresed at 50 V/cm for 1 h using the Savant FP 22b system (Savant Instruments, Inc., Hicksville, N.Y.) with a 1.5 M acetate, 0.75 M formate buffer at pH 2 (Potter and Murphy, 1967). Ch and ACh had electrophoretic mobilities of 29 and 24 cm/h, respectively, with $< 2\%$ cross-contamination. The compounds were visualized with iodine vapor; the ACh spot was cut out and ACh was eluted with 50 $\mu$M HCl in methanol. The elutants were dried and reconstituted in 1 ml methanol and 10 ml of Liquifluor toluene scintillation fluid and then counted. The degree of recovery was determined by using the counts present as [$^{14}$C]ACh. The values reported for Ch uptake represent the total amount of tritiated compounds retained.

### *Solutions and Chemicals*

Normal Tyrode's solution consisted of 150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$,1 mM $MgCl_2$, 12.2 mM glucose, 10 mM Tris adjusted to pH 7.33–7.39. For high-$K^+$ Tyrode's, KCl was raised to 55 mM and NaCl was reduced to 98 mM. For $Na^+$-free solutions, NaCl was replaced with LiCl or sucrose, as indicated, to maintain osmolarity. Other modified solutions were normal Tyrode's except for the changes noted in Results. All solutions were kept oxygenated during the experiments.

Ouabain octahydrate, 4-aminopyridine, valinomycin, and unlabelled ACh chloride were obtained from Sigma Chemical Co., St. Louis, Mo.; tetraethylammonium chloride from J. T. Baker Chemical Co., Phillipsburg, N. J.; veratridine from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and tetrodotoxin (TTX) from Sankyo, Tokyo, Japan. D600 hydrochloride was the generous gift of Drs. Oberdorf and Kleinsorge of Knoll AG, Ludwigshafen, West Germany. A28695A, mixed Na, K salt, was the generous gift of Dr. Robert Hamill of Eli Lilly and Co., Indianapolis, Ind. A28695A and valinomycin were dissolved in ethanol before use. The final concentration of ethanol in Tyrode's solution did not exceed 0.5%; at this concentration it was without detectable effect on Ch uptake or ACh synthesis. All other chemicals were reagent grade. Solutions were made with double glass-distilled water.

Statistical significance was evaluated using the two-tailed Student's $t$-test. All lines were fitted by the method of least squares.

## RESULTS

### *Ch Uptake and ACh Synthesis during Electrical Nerve Stimulation*

When the ciliary nerve was stimulated at frequencies of 30 Hz or less, a tonic contracture of the iris (Pilar and Vaughan, 1971) was fully maintained for the entire 8-min incubation. At 40 or 50 Hz, there was often a small decrement in the magnitude of contracture over the period of stimulation, whereas at higher frequencies the contracture often rapidly decreased to the point where none was visible. It has not been determined whether the decrement in tonic contracture at stimulation frequencies greater than 30 Hz was due to presynaptic nerve conduction block or to a pre- or postsynaptic failure in transmission. It can be seen in Fig. 1 that when the ciliary nerve was stimulated repetitively during the entire period in which it was incubated in labelled Ch, there was a large, parallel increase of Ch uptake (●) and ACh synthesis (▲) with increasing frequencies of stimulation up to 30 Hz. It must be emphasized that these values

represent lower limits on the increase in uptake and synthesis, as it is expected that some of the newly synthesized [$^3$H]ACh was released by stimulation (Collier, 1969; Potter, 1970) and [$^3$H]Ch diluted by unlabelled Ch from hydrolyzed ACh (Collier and Katz, 1974). When $Na^+$ was replaced in the incubation medium by $Li^+$ and repetitive stimulation at 30 Hz was applied, no increase in either Ch uptake (○) or ACh synthesis (△) was observed, even though muscle contracture was still elicited.

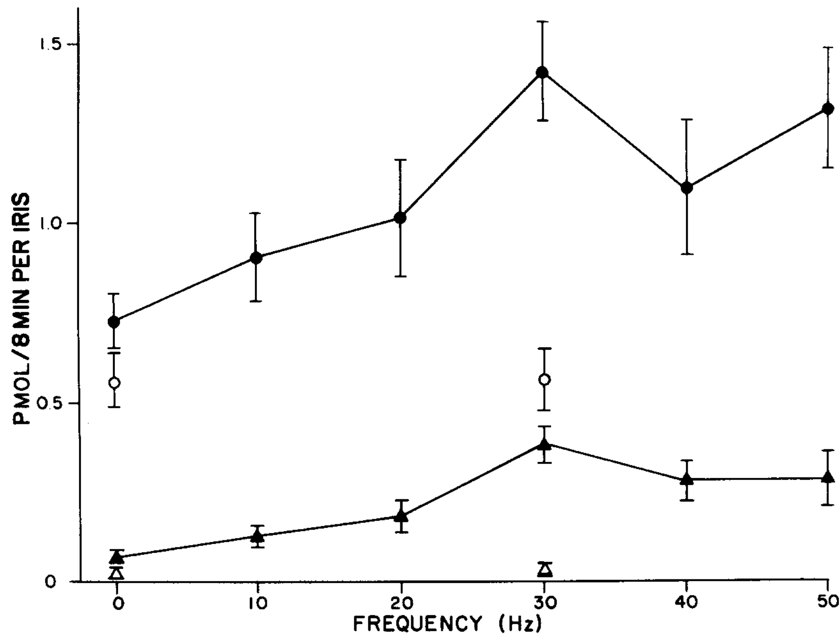


FIGURE 1. Effect of electrical stimulation on [$^3$H]Ch uptake and [$^3$H]ACh synthesis. The ciliary nerve was stimulated repetitively with bipolar pulses of 0.2 ms duration at the indicated frequency during the entire incubation in Tyrode's solution containing 0.6 $\mu$M [$^3$H]Ch. Normal Tyrode's: (●) [$^3$H]Ch uptake, (▲) [$^3$H]ACh synthesis. $Na^+$-free Tyrode's, $Li^+$ replacement: (○) [$^3$H]Ch uptake, (△) [$^3$H]ACh synthesis. The increase Ch uptake and ACh synthesis observed in normal Tyrode's during nerve stimulation are not elicited when $Li^+$ replaces $Na^+$ at 0 and 30 Hz. Each point represents the mean ± SE of at least six experiments.

The difference between values in the presence and absence of $Na^+$ (when substituted by $Li^+$, Fig. 1) may be used to estimate minimum values for $Na^+$-dependent uptake and synthesis. 30-Hz stimulation resulted in at least a four fold increase in $Na^+$-dependent Ch uptake and at least a 7.5-fold increase in $Na^+$-dependent ACh synthesis. Similarly, electrical stimulation of the cat superior cervical ganglion has been reported to have a relatively greater effect on acetylation, relative to uptake, of homocholine and triethylcholine (Collier et al., 1977; Ilson et al., 1977).

When conditioning trains of stimuli at 30–50 Hz were applied for 4–5 min before a 3-min incubation with [$^3$H]Ch, no significant effect on uptake or ACh

synthesis could be detected (not shown). It was not practical to use shorter incubation times because equilibration with the extracellular space takes ~ 1min (Beach et al.[1]). Thus, activation of Ch uptake and ACh synthesis by electrical activity diminished to nonmeasurable levels in no more than 2-3 min. This may be due in part to a rapid and efficient reaccumulation of Ch from hydrolyzed ACh released by stimulation.

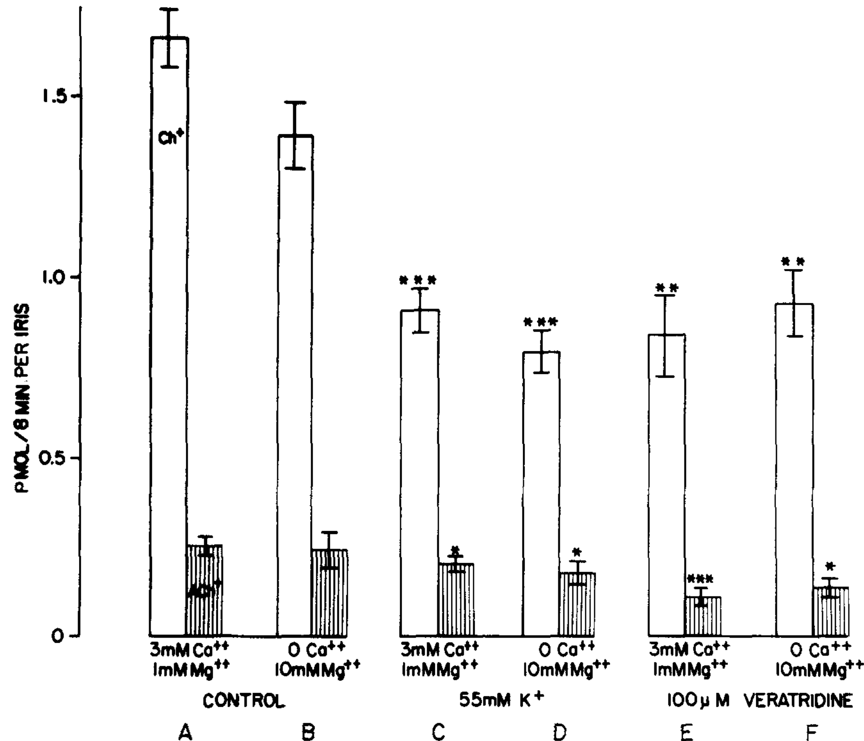


FIGURE 2. Modification of [$^3$H]Ch uptake and [$^3$H]ACh synthesis by depolarization. High [$K^+$] (C, D) and veratridine (E, F), present only during the period of [$^3$H]Ch uptake, reduced Ch uptake, and ACh synthesis when compared to controls (A, B). High [$Mg^+$] and omission of $Ca^{++}$ (D, F) did not alter these effects. [$^3$H]Ch = 0.65 $\mu$M. Each bar represents mean ± SE ($n$ = 4). Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. Clear bars indicate [$^3$H]Ch uptake; shaded bars indicate [$^3$H]ACh synthesis. A similar convention was used in Figs. 8, 9, and 10.

### *Driving Force for Ch Uptake: Effect of Membrane Potential*

To evaluate how electrical activity leads to an increase in $Na^+$-dependent Ch uptake and ACh synthesis, it is necessary to understand the forces that drive the uptake of Ch. The dependence of Ch uptake on membrane potential was investigated by elevating medium [$K^+$] or by adding veratridine, a depolarizing agent which acts on the action potential $Na^+$ channel (Ulbricht, 1969). In Fig. 2 (and in subsequent similar figures), the paired bars represent Ch uptake (clear) and ACh synthesis (shaded) of isolated irises under the same incubation

conditions. Comparing (Fig. 2 A) the control values with (Fig. 2 C) the 55 mM $K^+$ and (Fig. 2 E) 100 $\mu$M veratridine values, it is clear that there was a 45–50% reduction in total Ch uptake and a smaller, though significant, decrease in ACh synthesis. Neither high $[K^+]$ nor veratridine had any effect on low affinity, $Na^+$-independent Ch transport (Beach et al.[1] and Vaca[2]). By definition, $Na^+$-independent uptake is the low-affinity uptake observed in the absence of $Na^+$ ($Na^+$ being substituted by $Li^+$ or sucrose). Depolarization with elevated $[K^+]$ or veratridine also reduced the Ch uptake into isolated synaptosomes (Murrin and Kuhar, 1976), and it may be the rule for the $Na^+$-dependent uptake of other neurotransmitters (Holz and Coyle, 1974; Blaustein and King, 1977).

For most excitable tissues, where $K^+$ permeability is high relative to $Na^+$ permeability, a nearly linear relationship between membrane potential and

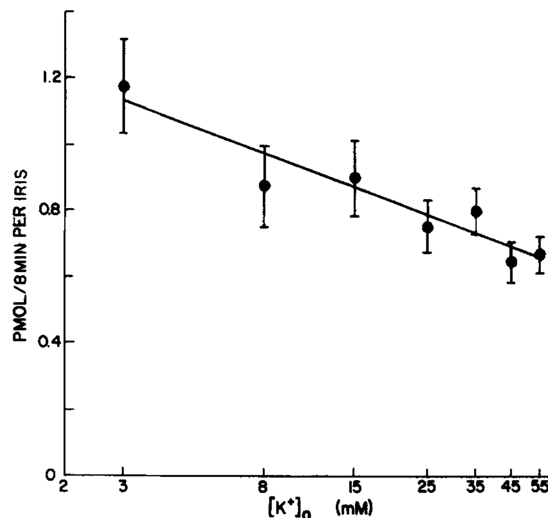


FIGURE 3. $[^3H]$Ch influx as a function of $[K^+]_o$. Note that the abcissa, log $[K^+]_o$, should be inversely proportional to membrane potential, as has been demonstrated for many excitable tissues. $[Na^+]_o = 98$ mM, $[K^+]_o + [Li^+]_o = 55$ mM. $Li^+$ was used to maintain tonicity at constant $[Na^+]_o$. $[^3H]$Ch = 0.6 $\mu$M. Each point represents the mean ± SE ($n$=4).

log $[K^+]_o$ is obtained as predicted by the Goldman equation. There was an inverse linear relationship between Ch transport and log$[K^+]_o$ (Fig. 3), which suggests that Ch transport was a linear function of membrane potential. Furthermore, the effect of depolarization on transport was not secondary to an increase in the release of newly synthesized $[^3H]$ACh, because when similar $[K^+]$ and veratridine challenges were applied with O $Ca^{++}$ and 10 mM $Mg^{++}$, conditions which inhibit transmitter release, the inhibitory effect of depolarization was not significantly modified (Fig. 2 D and F). Although depolarization may increase Ch efflux, it is clear that the net effect is a decrease in Ch influx.

[2] Vaca, K. Unpublished observations.

*Initial Velocities of Ch Uptake and ACh Synthesis*

It has been demonstrated that, under control conditions, Ch uptake and ACh synthesis are linear for 16 min (Beach et al.[1]). Inasmuch as proper interpretation of the present experiments requires the ability to distinguish effects on initial velocity from changes in the steady state, it seemed advisable to confirm the linearity of uptake during depolarization with high [$K^+$] and also under

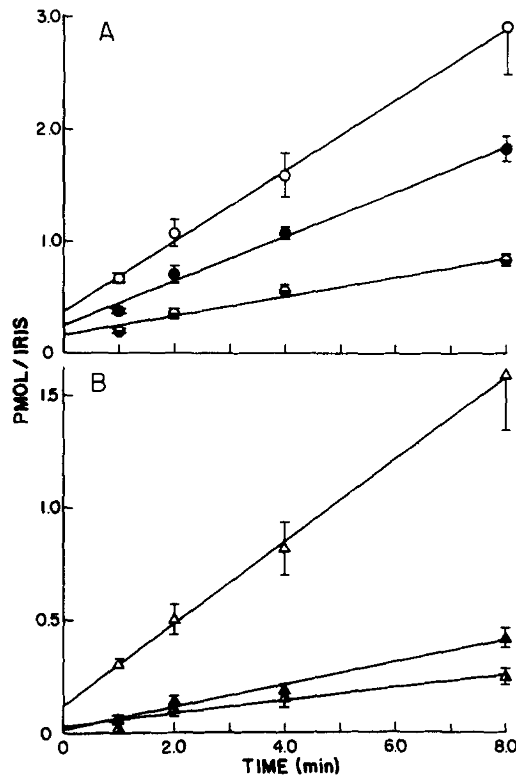


FIGURE 4. Time-course of (A) [$^3$H]Ch uptake and (B) [$^3$H]ACh synthesis. Control, incubation (with [$^3$H]Ch) in normal Tyrode's (●, ▲). Incubation in 55 mM $K^+$ Tyrode's (◒, ⧌). 6-min preincubation in 55 mM $K^+$ Tyrode's, incubation in normal Tyrode's (○, △). Extrapolated values at zero time represent nonspecific binding plus uptake and synthesis during 10-ml stop-wash with cold Tyrode's. [$^3$H]Ch = 0.7 $\mu$M. Each point represents mean ± SE ($n$ = 4).

conditions where uptake is activated. An analysis of the initial 8-min time-course for Ch uptake and ACh synthesis is shown in Fig. 4 A and B, respectively. The linearity is maintained in the control conditions for Ch uptake (●) and ACh synthesis (▲) and in high [$K^+$] Tyrode's (◐ for Ch and ⧌ for ACh). Note that the initial velocity of ACh synthesis (Fig. 4 B) was decreased less markedly than Ch uptake (Fig. 4 A) by membrane depolarization. It needs to be emphasized that this effect on the initial velocities of Ch uptake and ACh synthesis does not contradict observations of increased ACh synthesis during prolonged depolar-

izations (such as 1 h or more), where it is expected that some steady-state relation between synthesis and release may be reached such that Ch influx increases to compensate for ACh depletion (i.e., Grewaal and Quastel, 1973).

In Fig. 4 it is also shown that, even during maximal activation of Ch uptake A (○) and ACh synthesis B (△), the time-courses were still linear (see Discussion for interpretation of this activation due to prior depolarization.) Thus, under conditions where the velocity of transport is either accelerated or depressed, a unidirectional flux was measured.

*Role of the $Na^+$ Gradient*

In addition to the role that the membrane potential seems to play as a driving force for Ch transport, the energy provided by the $Na^+$ gradient has been shown to contribute to the $Na^+$-coupled uptake of many solutes (Schultz and Curran, 1970). These authors have suggested that the inhibitory effect of ouabain on transport systems for organic solutes is due to a decline in the $Na^+$ gradient. If part of the driving force for $Na^+$-dependent Ch uptake were contingent upon a $Na^+$ gradient, when Na,K-ATPase (E.C.3.6.1.3) is inhibited with ouabain, the gradient should decay with first-order kinetics and there should be a concomitant decrease in $Na^+$-dependent Ch uptake. Experiments where 0.1 mM ouabain was introduced at various intervals before and during an 8-min incubation with [$^3$H]Ch yielded results consistent with this hypothesis (graph not shown because it is similar to the one obtained with 3-min incubation presented immediately below). There was a gradual decline in rate of uptake with time which could indeed be fitted with an exponential. Therefore, this inhibition could be attributed at least in part to a dissipation of the $Na^+$ gradient. However, extrapolation of the curve describing the temporal dependence of ouabain inhibition to zero time yielded values for uptake and synthesis considerably lower than the uninhibited control, indicating the presence of a rapid phase of inhibition. An attempt was made to resolve this rapid phase of inhibition by reducing the [$^3$H]Ch incubation time to 3 min, indicated in Fig. 5 A by the shaded area. In this experiment the irises were preincubated with ouabain for progressively longer intervals in increments of 3 min. The experimental curve suggests that the time constant for the rapid phase of inhibition is $< 3$ min. This is consistent with the values obtained for squid axon by Baker and Willis (1972) where 0.1–1.0 mM ouabain inhibited the $Na^+$ pump with a half-time of 10–13 s. Evidently this very rapid initial effect of Na, K-ATPase inhibition cannot be explained in terms of a dissipation of the $Na^+$ gradient. Inasmuch as the $Na^+$ pump appears to be electrogenic in almost all nervous tissues (Thomas, 1972), a simple explanation for the rapid effect of $Na^+$ pump inhibition on Ch uptake (●) would be a direct reduction in membrane potential. That the effects of ouabain on Ch uptake were due to a block of Na,K-ATPase activity is supported by a similar experiment in which both the rapid phase of inhibition and the slow exponential decline were observed if $K^+$-free Tyrode's solution was used as an inhibitor (Fig. 5 B). We have shown previously that the effects of inhibition of the $Na^+$ pump were limited to the $Na^+$-dependent component of Ch uptake (Beach et al.[1]).

A corollary of the sodium gradient hypothesis for the transport of organic

solutes (Schultz and Curran, 1970) is that $Na^+$ ions should be cotransported stoichiometrically with the solute, in this case Ch. In the ciliary nerve iris muscle preparation, it is difficult to demonstrate directly cotransport of $Na^+$, which has many alternative pathways into muscle and connective tissue as well as nerve terminals. However, it is possible to determine the order of the uptake reaction using the Hill equation. If one plots the log of velocity of the reaction as a

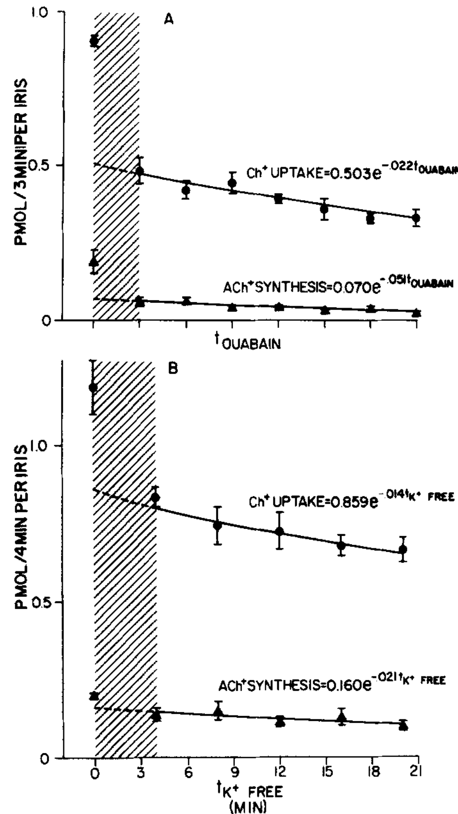


FIGURE 5. Time-dependent effect of Na,K-ATPase inhibition on [$^3$H]Ch uptake (●) and [$^3$H]ACh synthesis (▲). The shaded areas represent the period of incubation with 0.7 $\mu$M [$^3$H]Ch. The equations for the best exponential curve fit of the data (excluding the zero time control values) are given. Each point represents the mean ± SE ($n$ = 4). (A) Inhibition with 1.0 mM ouabain. (B) Inhibition with $K^+$-free Tyrode's solution.

function of the log of the substrate concentration, the slope is $n$, the stoichiometric coefficient. For $Na^+$-coupled Ch transport, $\log J_{Ch \sim Na} = n \log[Na^+] + k$, where $J_{Ch \sim Na}$ is the velocity of transport and $k$ is a constant. The data illustrated in Fig. 6 (●) yielded a value of 0.92 for $n$, suggestive of a 1:1 stoichiometry between $Na^+$ and Ch. Saturation occurs at a little more than 100 mM [$Na^+$]. However, the data did not readily fit Michaelis-Menten kinetics. This is probably due to a complex interaction of $Na^+$ ions with Na,K-ATPase, membrane potential, and the Ch transport system. For this reason, the stoichiometric

coefficient must also be viewed with some caution. An apparent affinity constant for $Na^+$ binding (the [$Na^+$] at which $V_{max}/2$ is obtained) to the Ch transport system can be estimated to be ~50 mM, in agreement with Haga and Noda (1973). The stoichiometric coefficient for $Na^+$: ACh synthesis, 0.82 (Fig. 6, ▲), is in reasonably close agreement with the coefficient for transport.

*Activation of Ch Uptake after Depolarization*

It was hypothesized that a prolonged period of depolarization in the ciliary nerve-iris preparation might lead to a relatively prolonged activation of Ch uptake and ACh synthesis upon repolarization of the nerve terminals, as was shown before in synaptosome preparations (Murrin and Kuhar, 1976; Barker,

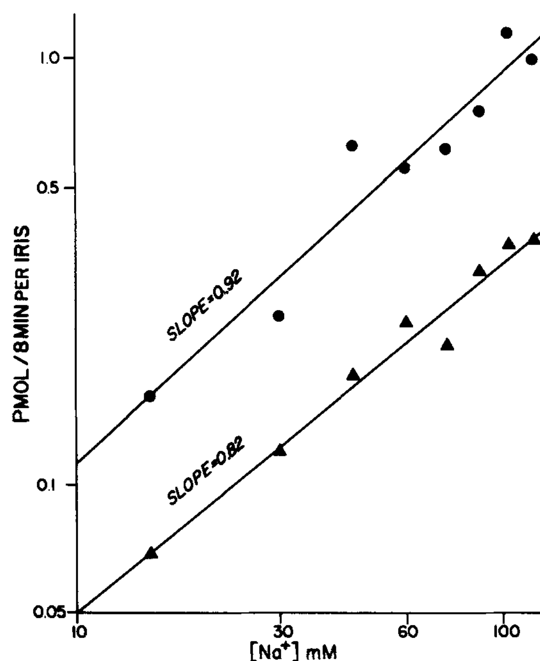


FIGURE 6. Log-log plot of sodium dependence of [$^3$H]Ch uptake (●) and [$^3$H]ACh synthesis (▲). [$Na^+$] + [$Li^+$] = 150 mM. The mean value in $Na^+$-free Tyrode's solution has been subtracted out from the mean value at each [$Na^+$], ($n$ = 4). [$^3$H]Ch = 0.7 $\mu$M.

1976). We intended to mimic the effect of repetitive nerve stimulation with a single depolarization-repolarization cycle, which would permit study of the ionic dependence of the uptake activation process dissociated from direct membrane potential changes.

Irises were exposed to high-$K^+$ Tyrode's solution for 10 min, then returned to normal Tyrode's for 2 min before incubation in normal Tyrode's containing 0.7 $\mu$M [$^3$H]Ch. Control irises preincubated in normal Tyrode's took up 2.04 ± 0.15 pmol $^3$H-Ch/8 min per iris, of which 0.30 ± 0.06 pmol were converted to ACh; those preincubated in high $[K^+]_o$ took up 3.95 ± 0.17 pmol $^3$H-Ch/8 min per iris, of which 1.76 ± 0.10 pmol were converted to ACh. The magnitude of these

effects was independent of the duration of depolarization over the range tested, 5–20 min. When $Na^+$ was replaced by sucrose during the [$^3$H]Ch incubation (not shown), there was no effect of preincubation on uptake or synthesis, which indicates that the effect of prior depolarization is limited to SDHACU. The Hill coefficient for $Na^+$ remained ~1 with respect to both uptake and synthesis after depolarization with elevated $[K^+]_o$.[2] Depolarization with veratridine (20 $\mu$M) was also an effective activator provided that tetrodotoxin (0.5 $\mu$M) was introduced upon return to normal medium. Veratridine preincubation (8 min) increased Ch uptake over control from 2.17 ± 0.12 to 2.99 ± 0.18 pmol 8 min per iris ($P <$ 0.01) and ACh synthesis from 0.56 ± 0.07 to 0.88 ± 0.05 pmol/8 min per iris ($P$ $<$ 0.02).

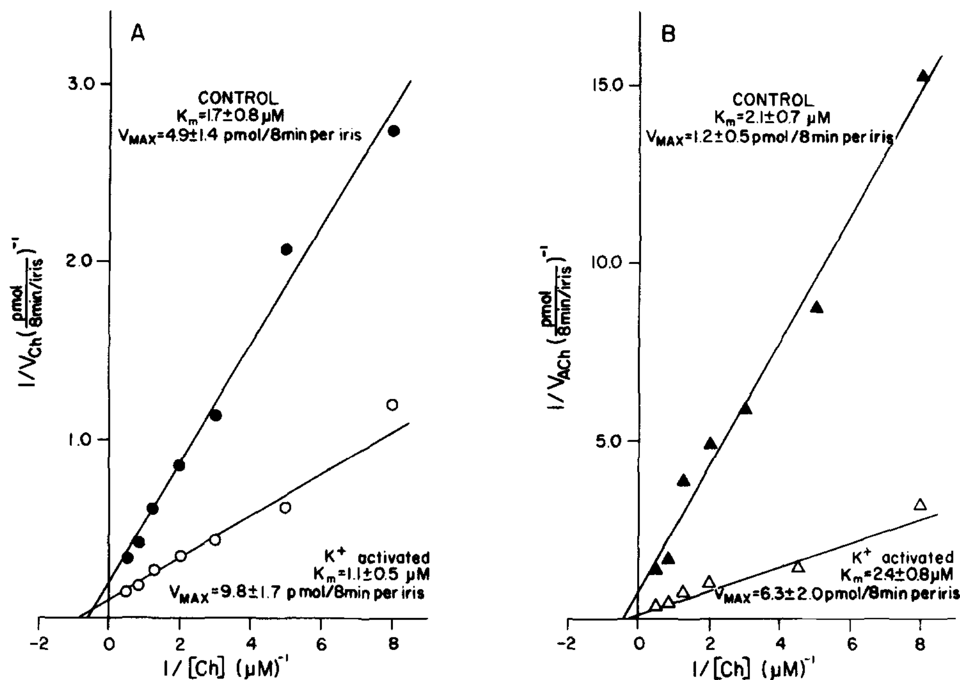


FIGURE 7. Double reciprocal plot of the effect of preincubation in 55 mM $K^+$ Tyrode's solution on the kinetic parameters of (A) [$^3$H]Ch uptake and (B) [$^3$H]ACh synthesis. Control (●, ▲); 10 min preincubation in high-[$K^+$] Tyrode's (○, △). Each point represents the mean of four experiments.

When the activation after depolarization was analyzed kinetically, it was revealed that the $K_m$ values were not significantly changed but rather that the increases in Ch uptake and ACh synthesis were accounted for by an increase in $V_{max}$. Fig. 7 A is a Lineweaver-Burke analysis of high affinity uptake into irises from controls (●) and after 55 mM $K^+$ depolarization (○). Although the $K_m$ was likely unchanged, 1.7 ± 0.8 $\mu$M (mean ± SE) for controls and 1.1 ± 0.5 $\mu$M for $K^+$-activated, the $V_{max}$ was increased from 4.9 ± 1.4 to 9.8 ± 1.7 pmol/8 min per iris. Similar results were obtained by Murrin and Kuhar (1976) for hippocampal synaptosomes. Fig. 7 B shows a similar kinetic analysis of ACh synthesis. Here,

also there was no significant change in $K_m$ (~2 $\mu$M), but the $V_{max}$ for ACh synthesis was increased by high $[K^+]_o$ preincubation from 1.2 ± 0.5 for control to 6.3 ± 2.0 pmol/8 min per iris. Analysis by direct linear plot (Eisenthal and Cornish-Bowden, 1974) yielded similar kinetic values for both uptake and synthesis. It appears that, as with electrical stimulation, prior depolarization had a greater relative effect on ACh synthesis than on high affinity Ch uptake, although the absolute magnitude of each of the increases were about the same.

*Influence of Cation Fluxes on Ch Transport*

If membrane depolarization reduces the initial velocity for Ch uptake and ACh synthesis (Fig. 2 A, B), the increase in uptake and synthesis during electrical stimulation cannot be the direct effect of the depolarization phase of the action potential; it is perhaps the result of ionic fluxes accompanying the spike. The following experiments were designed to test whether the activation of Ch uptake and ACh synthesis could be due to the influx of $Na^+$ of $Ca^{++}$ or efflux of $K^+$ during depolarization. If the NaCl in the 55 mM $K^+$ preincubation solution was replaced with either LiCl (Fig. 8 D) or sucrose (Fig. 8 C), the extent of the subsequent activation was substantially reduced when compared to Fig. 8 B, although both Ch uptake and ACh synthesis were significantly higher than the controls (Fig. 8 A) which had not first been depolarized. Most or all of the action of $Na^+$ must be due to its influx via the tetrodotoxin-(TTX-) sensitive $Na^+$ channel because addition of 0.5 $\mu$M TTX during the high-$[K^+]_o$ preincubation (Fig. 8 E) reduced the activation of uptake and synthesis to a similar extent as removal of $Na^+$ ions (Fig. 8 C and D). In all of these three experiments it can be seen that the activation of uptake and synthesis cannot be due solely to $Na^+$ influx. This agrees with the findings of Grewaal and Quastel (1973) that the stimulation of ACh synthesis by $K^+$ in brain slices is due in part to an influx of Na ions. It appears that the effect of $Na^+$ influx in the activation of uptake and ACh synthesis was at least partly due to the activity of the $Na^+$ pump in ciliary nerve terminals, as in the case in brain slices. In experiments where Na,K-ATPase activity was inhibited with ouabain (Fig. 8 F) during the period of Ch uptake subsequent to preincubation with high $[K^+]$, uptake did not increase relative to untreated controls (Fig. 8 A) (although there is an increase in uptake relative to ouabain control, cf. Fig. 5). However, ACh synthesis more than doubled. Thus, the coordinate regulation of Ch uptake and ACh synthesis could be uncoupled under this circumstance. Complementary evidence for uncoupling of uptake and synthesis has been reported by Jope and Jenden (1977). If irises were switched from high-$[K^+]$ to $K^+$-free solution (Fig. 8 G), some activation of uptake still occurred, but stimulation of ACh synthesis was more marked. It is possible that some residual $K^+$, leaking into the extracellular space, prevented complete inhibition of Na,K-ATPase.

Previous investigators have reported tht $Ca^{++}$ must be present during depolarization to stimulate the subsequent uptake of Ch or its analogues (Murrin and Kuhar, 1976; Collier and Ilson, 1977). When $Ca^+$ was removed during the preincubation (Fig. 9 C) or its entry was blocked by D600 (methoxyverapamil, Fig. 9 D), an organic compound which blocks the late $Ca^{++}$ channel (Baker et al., 1973), there was a reduction of the $K^+$-induced stimulation (Fig. 9 B). High

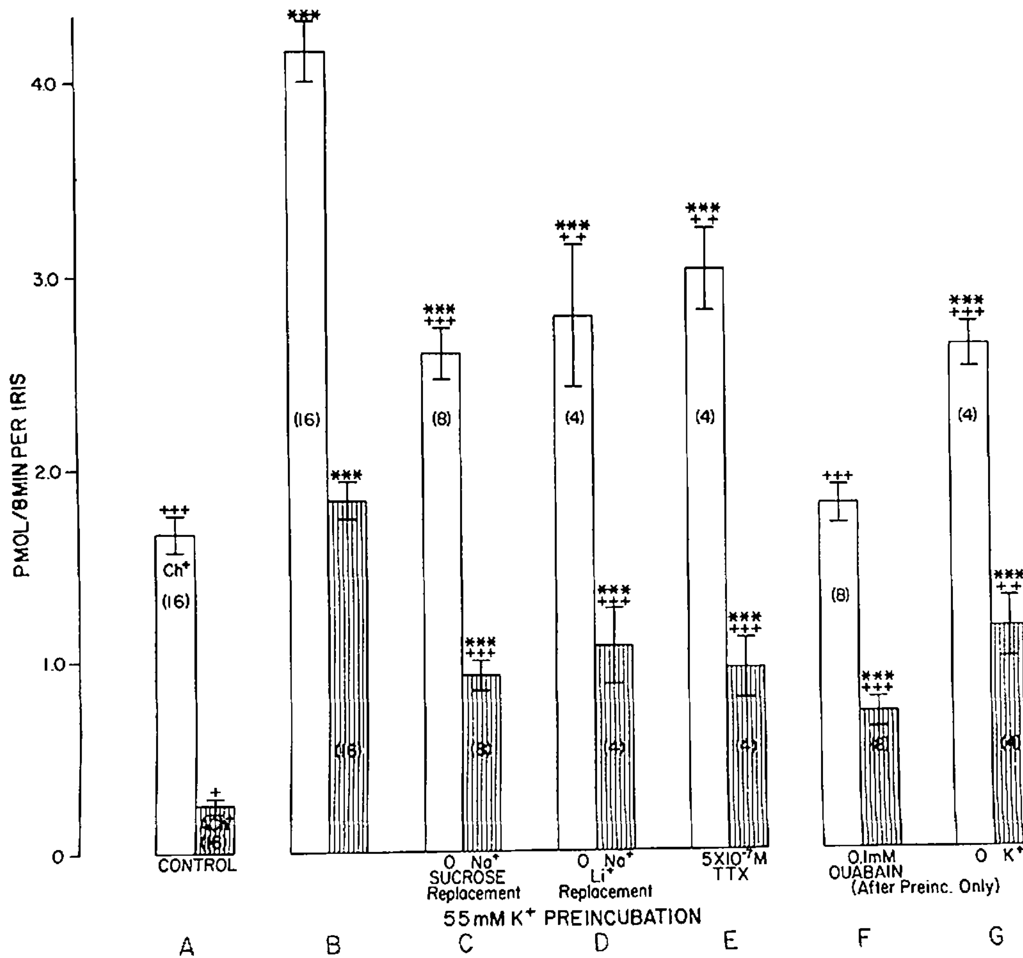


FIGURE 8. Role of $Na^+$ influx in the activation of Ch uptake and ACh synthesis by high-$K^+$ preincubation. Irises were preincubated in 55 mM $K^+$ Tyrode's solution (B) in some cases altered by $Na^+$ replacement by sucrose (C) or Li (D) or addition of TTX (E), for 10 min followed by a 2-min wash in normal Tyrode's solution before incubation with [$^3$H]Ch in normal Tyrode's. In experiments involving ouabain (F) or $K^+$-free medium (G), irises were preincubated in the standard 55 mM $K^+$ Tyrode's solution for 10 min, then switched to Tyrode's containing ouabain or with $K^+$ deleted for a 2-min wash followed by an 8-min incubation with [$^3$H]Ch in a solution of the same composition. [$^3$H]Ch = 0.7 $\mu$M. Each bar represents mean ± SE. Number of experiments in parentheses. Significance levels: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. control (A). $^+P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. standard 55 mM $K^+$ preincubation (B).

[$Mg^{++}$] (Fig. 9 E), which inhibits $Ca^{++}$ influx into nerve terminals (Blaustein, 1975), also reduces the effect of high $[K^+]_o$ preincubation, as previously reported by Barker (1976) for synaptosomes. The $Ca^{++}$-dependent effect of high-$K^+$ preincubation may be related to transmitter release in that a 10-min depolariza-

tion in the presence of normal values of $Ca^{++}$ and $Mg^{++}$ depletes 43% of the endogenous ACh, but no changes were measured with an identical depolarization in $Ca^{++}$-free, high-$Mg^{++}$medium (Pilar and Vaca, 1979). In comparing the three conditions where $Ca^{++}$ influx was blocked (Fig. 9 C,D,E) with the control

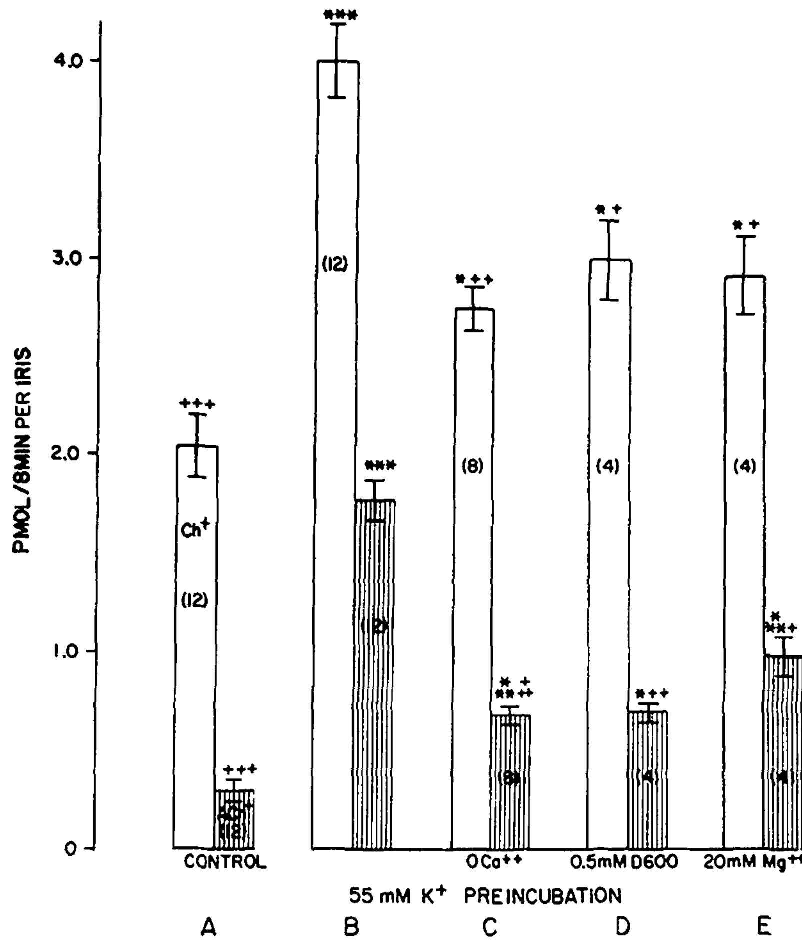


FIGURE 9. Role of $Ca^{++}$ influx in the activation of Ch uptake and ACh synthesis. Irises were preincubated in 55 mM $K^+$ Tyrode's solution (B), with modified $[Ca^{++}]$ (C), D600 (D), or $[Mg^{++}]$ (E) for 10 min, then washed for 2 min before incubation with 0.75 $\mu M[^3H]Ch$ in normal Tyrode's. Each bar represents mean ± SE. Number of experiments in parentheses. Notation as in Fig. 8.

(Fig. 9 A), a small $K^+$-stimulation of Ch uptake and ACh synthesis was visible; this was probably due to $Na^+$ influx, discussed previously.

The role of $Ca^{++}$ was further examined in experiments in which the ciliary nerve was stimulated electrically at the previously determined optimal frequency, 30 Hz (Fig. 10 B). $Ca^{++}$omission (Fig. 10 C) was partially effective in reducing the increase of uptake and synthesis when compared to the stimulated

preparation in normal Tyrode's (Fig. 10 B); however, in the intact ciliary nerve-iris preparation, the large amount of surrounding connective tissue may provide diffusional barriers to loss of $Ca^{++}$. When a $Ca^{++}$-free medium was supplemented with high [$Mg^{+}$] (Fig. 10 D) to antagonize the effects of any residual $Ca^{++}$, the level of ACh synthesis in the stimulated preparation approached that of the control, although there remained a slight increase in Ch uptake relative to the unstimulated control (Fig. 10 A).

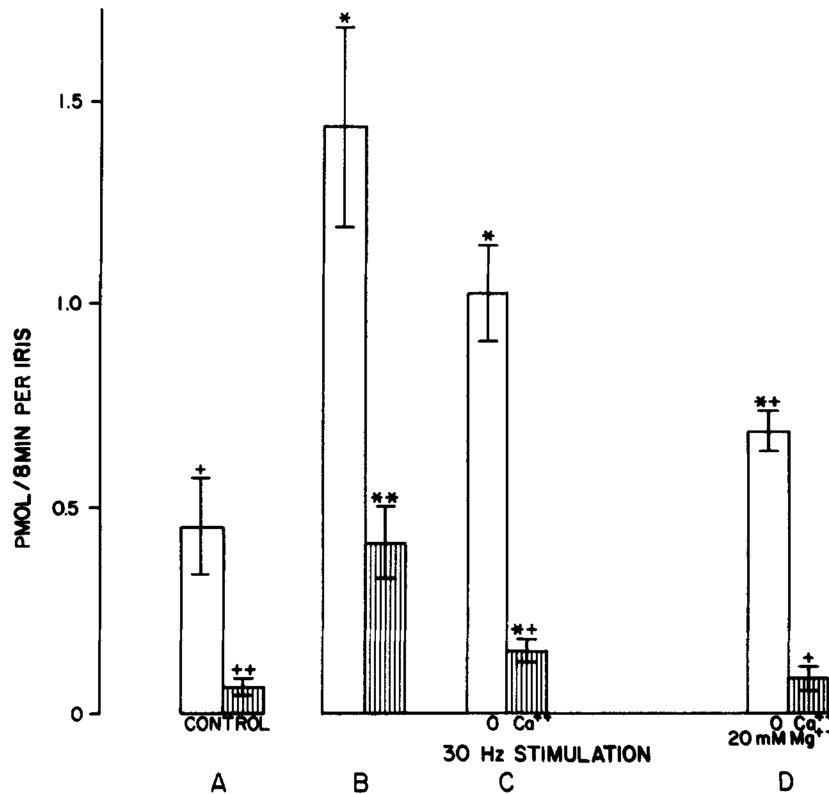


FIGURE 10. $Ca^{++}$-dependent effect of electrical stimulation. Irises were electrically stimulated, as described in Fig. 1, in normal or modified Tyrode's solution. (A) Unstimulated control; (B) control stimulation 30 Hz; (C) $Ca^{++}$-free solution; (D) 20 mM $Mg^{+}$ and O Ca. [$^{3}$H]Ch = 0.55 $\mu$M. Each bar represents the mean ± SE ($n$ = 4). Significance levels: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (A). $^{+}P < 0.05$, $^{++}P < 0.01$ vs. 30-Hz stimulation in normal Tyrode's solution (B).

Attempts to determine whether an increase in $K^{+}$ conductance also contributes to the increased rate of Ch transport during electrical activity were inconclusive. The $K^{+}$ conductance blockers, 4-aminopyridine and tetraethylammonium, both inhibited Ch uptake, presumably by depolarizing the membrane (Llinás et al., 1976; Narahashi, 1974), although the latter agent may compete directly with Ch. The antibiotic $K^{+}$ ionophores, valinomycin and A28695A (septamycin), also inhibited uptake, perhaps by uncoupling oxidative phosphorylation (Kessler et al., 1977), and thus indirectly inhibiting Na,K-ATPase (see Table I).

## DISCUSSION

The ciliary nerve-iris preparation was used to study the effect of electrical activity on $Na^+$-dependent Ch uptake and ACh synthesis. In this preparation, SDHACU is exclusively limited to the nerve terminals of a homogenous group of cholinergic neurons (Suszkiw and Pilar, 1976), and none of the treatments described in this paper have any effect on the $Na^+$-independent low-affinity Ch uptake of the iris muscle, the postsynaptic element in these neuromuscular junctions (see also Beach et al.[1]).

In most of the experiments of the present investigations, a close relationship between the transport of Ch and its acetylation was evident. The demonstration that transport is a nonequilibrium reaction (to be discussed later), coupled with the finding that Ch acetylation per se is very close to equilibrium *in situ* (Beach et al.[1]), strongly suggests that Ch transport is a regulatory, rate-limiting reaction step (see also Barker and Mittag, 1975; Simon et al., 1976), although this may not be the case with some Ch analogues (Ilson et al., 1977). Both the rate of Ch transport and its acetylation may in turn be partly limited by the rate of ACh release.

TABLE I

EFFECT OF $K^+$ CONDUCTANCE BLOCKERS AND $K^+$ IONOPHORES ON [$^3$H]Ch UPTAKE AND [$^3$H]ACh SYNTHESIS

| Incubation condition | Observations | [$^3$H]Ch uptake | P | [$^3$H]ACh synthesis | P |
|---|---|---|---|---|---|
| | *n* | *pmol* | | *pmol* | |
| Control | 12 | 1.96±0.10 | | 0.51±0.06 | |
| 4-Aminopyridine, *1 mM* | 4 | 1.03±0.06 | <0.001 | 0.28±0.05 | <0.05 |
| Tetraethylammonium Cl, *1 mM* | 4 | 0.79±0.05 | <0.001 | 0.07±0.01 | <0.001 |
| Valinomycin, *5 μM* | 4 | 1.24±0.09 | <0.01 | 0.37±0.02 | |
| A28695A, *10 μ*g/ml | 4 | 1.46±0.15 | <0.05 | 0.19±0.06 | <0.01 |

Irises were incubated 8 min with 0.7 μM [$^3$H-Ch] at 37°, and the compounds were added at the indicated concentration to the incubating media. Uptake and synthesis values in means ± SE.

These experiments provide conclusive evidence that there is an increase in Ch transport during nerve stimulation via the $Na^+$-dependent, high-affinity uptake system. The frequency dependence (not necessarily linear) of the Ch transport into nerve terminals established in this paper is one effective way to regulate the Ch supply necessary for the synthesis of ACh and thus maintain transmitter stores during its release. This mechanism would allow fine adjustment of the availability of ACh precursor during times of increasing demand.

The increases in Ch uptake and ACh synthesis during electrical activity are not explicable in terms of a single mechanism but appear to be due to at least two separate effects mediated by $Na^+$ and $Ca^{++}$ influxes accompanying the action potential. They in turn modify the electrochemical gradients of Ch and $Na^+$.

### *Dependence of Ch Uptake on Electrochemical Gradient*

The experiments in which Ch uptake was reduced by inhibiting the activity of the $Na^+$ pump with ouabain and with $K^+$-free solutions established the contribution of the $Na^+$ gradient in Ch accumulation. These observations are consistent with the $Na^+$ gradient hypothesis of active transport for a variety of

substances into cells (Schultz and Curran, 1970). A quantitative assessment of the contribution of the $Na^+$ gradient to Ch transport is not possible from these experiments, because inhibition of Na,K-ATPase also results in a loss of $K^+$ from the nerve terminals and thus a slow reduction in membrane potential. In any case, the asymmetric distribution of both $Na^+$ and $K^+$ will provide energy, directly or indirectly, for the translocation of Ch against its concentration gradient. It is therefore not surprising that the plasma membrane of synaptic endings is highly enriched with the enzyme Na,K-ATPase (Cotman and Matthews, 1971); the proposed role of Na,K-ATPase in facilitating the uptake of neurotransmitters and their precursors may provide a teleological explanation for its high concentration in nerve terminals.

The most straightforward explanation for decreased Ch uptake by elevated $[K^+]$, veratridine, and for the rapid effect of $Na^+$ pump inhibition, is that the accumulation is also dependent on energy derived from the membrane potential. A depolarized membrane will reduce the electrical gradient for both $Na^+$ and Ch, and a hyperpolarization will have the opposite effect.

The data suggest that $Na^+$ and Ch are cotransported with a 1:1 stoichiometry. These measurements may be ambiguous because of the complex interaction of Na ions with $Na^+$ pump activity and other membrane functions; such is the probable explanation for the deviation from Michaelis-Menten kinetics. For instance, Yamamura and Snyder (1973) found that $Na^+$ even appeared to inhibit Ch uptake at concentrations above 75 mM. Similar problems may be encountered in the GABA transport system where the stoichiometry with $Na^+$ is a matter of controversy (Martin, 1973; Blaustein and King, 1977). However, the proposed 1:1 ratio for $Na^+$:Ch transport is supported indirectly, for the theoretical Ch equilibrium ratio, $[Ch]_i/[Ch]_o$ assuming a 1:1 stoichiometry is in reasonable agreement with our previous estimate based on steady-state measurements (see below). If a 2:1 $Na^+$:Ch stoichiometry were assumed, the Ch equilibrium ratio would be more than 30 times higher than our experimentally derived estimate. The $Na^+$:ACh stoichiometry also appears to be ~1:1. This is consistent with the notion that the role of $Na^+$ in ACh synthesis is effected exclusively via $Ch^+$ transport, although other actions of $Na^+$ were not ruled out.

The low $Q_{10}$ (30–40°) for SDHACU, ~1.3, tends to rule out that Ch transport is directly coupled to metabolic activity (Beach et al.[1]). In the absence of direct metabolic coupling, it is still thermodynamically feasible to achieve a high intracellular Ch concentration. This can be demonstrated using formalisms for active transport devised by Kedem and Katchalsky (see Katchalsky, 1970). The dissipation function for a closed system,

$$\phi = T\frac{dS}{dt} \geq 0,$$

where $T$ is absolute temperature and $S$ is entropy, has a value equal to the product of the diffusional flows and their conjugate forces. For $Na^+$-dependent Ch uptake,

$$\phi = J_{Ch\sim Na}\,\Delta\tilde{\mu}_{Ch} + J_{Na\sim Ch}\,\Delta\tilde{\mu}_{Na},$$

where

$$\Delta\tilde{\mu}_{Ch} = RT \ln \frac{[Ch]_o}{[Ch]_i} + F(V_o - V_i),$$

and

$$\Delta\tilde{\mu}_{Na} = RT \ln \frac{[Na]_o}{[Na]_i} + F(V_o - V_i);$$

$V_0$ and $V_i$ are the potentials outside and inside the cell; $R$, $T$, $F$ refer to the gas constant, absolute temperature, and Faraday's constant, respectively; $J_{Ch\sim Na}$ and $J_{Na\sim Ch}$ represent the influx of Ch and $Na^+$ respectively, specifically coupled to the other ion—they appear to be equal, as previously discussed. If the activities of these ions are not equivalent inside and outside the cell, the activity coefficients must be included in these equations. At equilibrium, $dS/dt = 0$ and $\Delta\tilde{\mu}_{Ch} = -\Delta\tilde{\mu}_{Na}$. Assuming $[Na^+]_o/[Na^+]_i = 10$ and a membrane potential of ~ −65 mV, the Ch tissue-to-medium ratio for the nerve terminals will be 1,300 at equilibrium. Previous steady-state measurements of Ch uptake combined with morphometric estimates of nerve terminal volume indicate that the presynaptic endings of the iris can concentrate Ch at least 200-fold ovt. the medium (Pilar et al., 1978). It seems likely that there is sufficient energy available from the electrochemical gradients to account for this concentrative uptake without direct metabolic coupling.

In the nonequilibrium state, where $dS/dT > 0$, the same equations apply to transport; however, further constraints must be applied to the flux of these ions. $J_{Ch\sim Na}$ ($=J_{Na\sim Ch}$) will tend to maximize, limited by the number of transport sites available, the mobility of the cotransported ions within the membrane and the magnitude and conformation of the energy barrier(s) they must traverse while crossing the membrane. Although exact equations for the velocity of transport cannot be written without information about the physical nature of the transport system, a qualitative interpretation is possible. When the total energy in the electrochemical gradients ($\Delta\tilde{\mu}_{Ch} + \Delta\tilde{\mu}_{Na}$) is large, relative to the energy barrier, the probability that a $Na^+$ and Ch ion available at the outer face to the transport system will be cotransported is high. As Ch is transported and the inside concentration increases, the energy from the electrochemical gradients becomes smaller relative to the energy barrier and the probability of the ions successfully traversing it decreases; in other words, the system approaches the steady state. For initial velocity measurements, the rate of transport depends on the magnitude of the electrochemical driving force. Thus, when the membrane is hyperpolarized or when cytoplasmic [Ch] is reduced, the velocity of transport is greater, due to a higher probability of traversing the energy barrier.

### *Link between Electrical Activity and ACh Synthesis*

In having arrived at a tentative explanation for the energetics of Ch transport, it is now possible to consider the means by which electrical activity accelerates Ch uptake and ACh synthesis. It was shown that a depolarization decreased Ch uptake and acetylation (Fig. 2). Thus, the observed increase in accumulation

during electrical stimulation could not be due to the spike depolarization but instead is the result of the $Ca^{++}$ and $Na^{+}$ fluxes which accompany it. To determine the contribution of each ionic species, it was desirable to activate the system by depolarization before the incubation in 0.7 $\mu$M [$^3$H]Ch. It was hypothesized that if the synaptic terminals are held in a depolarized state for a prolonged period of time, the system responsible for ACh regulation will be displaced from equilibrium sufficiently for the changes to be detected with biochemical techniques. Although electrical stimulation and $K^{+}$ depolarization may have physiologically diverse effects, both treatments increase $Ca^{++}$ permeability and can be expected to have qualitatively similar consequences if the effects are mediated by the same ionic mechanisms. Indeed, it was shown that after preincubation in high $[K^{+}]_o$, Ch uptake and ACh synthesis were substantially accelerated after repolarization. Because the maximal velocities for both were increased, the phenomenon is relevant at physiological concentrations of extracellular Ch.

The $V_{max}$ for ACh synthesis undergoes a greater relative increase than the $V_{max}$ for Ch uptake and results in an increased percent conversion of Ch to ACh (Fig. 7). A change in the percent conversion of Ch to ACh must be due to a change in the activity of the synthetic or degradative enzymes or in the compartmentation of their substrates or products. Soluble CAT activity from the iris exceeds the maximal velocity of ACh synthesis observed *in situ* by ~ 3 orders of magnitude[2] and is thus unlikely to be an important parameter in regulatory control. Some evidence suggests that ACh may inhibit acetylcholinesterase (E.C.3.1.1.7. AChE) activity (cf. Silver, 1974), yet acceleration of ACh synthesis is correlated with a depletion of ACh (Pilar and Vaca, 1979). If the latter were to remove substrate inhibition of AChE, this would decrease percent conversion of Ch to ACh. Thus, changes in the ACh-hydrolyzing enzyme activity seem unlikely to be of importance in the increased conversion of Ch to ACh. The $Ca^{++}$-dependent nature of the increased conversion to ACh suggests that this effect is related to ACh release from a compartment where it is sequestered from AChE action (i.e., vesicles). It is therefore proposed that the $Ca^{++}$ influx accompanying depolarization (by $[K^{+}]_o$ or action potentials) releases ACh in the nerve ending from the vesicular compartment (see Katz, 1969). In the resting state this compartment is near saturation with respect to ACh concentration, as suggested by experiments with isolated vesicles (Marchbanks, 1968; Whittaker et al., 1972). The desaturation due to release accelerates ACh translocation from the cytoplasm. The fall in cytoplasmic ACh should drive ACh synthesis by mass action, for CAT has a low equilibrium constant (Glover and Potter, 1971; Pieklik and Guynn, 1975). The consequent fall in Ch concentration (and perhaps ACh too) reduces the chemical gradient for Ch and thus increases its influx.[3]

[3] The data presented are consistent with the free diffusion of Ch within the terminal cytoplasm, where it is available for acetylation after transport. In another study (Beach et al.[1]), it was demonstrated that when AChE is inhibited with paraoxon ~90% of the Ch taken up by the high affinity system is acetylated, in close agreement with the equilibrium ratio for CAT (Pieklik and Guynn, 1975). Thus, the constant coupling of acetylation to transport observed in nerve terminals in the resting state (Barker and Mittag, 1975; Suszkiw and Pilar, 1976) is probably a consequence of the rapid equilibration of Ch and ACh (or their analogues) by CAT and cytoplasmic cholinesterases.

The Ch cotransported with $Na^+$ into the terminals, a process modulated by the $Na^+$ electrochemical gradient already discussed, will also result in an increased availability of substrate for ACh synthesis. Beside the role of $Na^+$ being cotransported, $Na^+$ entering the terminals during action potentials may also act indirectly, at the inner surface of the membrane, by stimulating the Na,K-ATPase. This could result in an increase in the rate of $Na^+$ extrusion, and if the pump is the electrogenic, a hyperpolarization (during the interspike interval) which would favor Ch uptake. The participation of such a mechanism in these experiments was suggested by the restraining effect of TTX on the $K^+$-stimulated uptake (Fig. 7 E), and on the decreased activation of uptake when $Na^+$ was replaced by $Li^+$ (Fig. 7 F), even though the $Li^+$ passes through the TTX-sensitive $Na^+$ channel.[4] This latter effect is reminiscent of the failure of $Li^+$ to substitute for $Na^+$ in the generation of posttetanic hyperpolarization elicited in nonmyelinated fibres (Rang and Ritchie, 1968). It is possible that $Na^+$ releases $Ca^{++}$ from intracellular stores; in any case, the intracellular effect of $Na^+$ is probably less important in the regulation of ACh synthesis than that of extracellular $Ca^{++}$ influx.

In summary it is proposed that during electrical activity the ACh synthesis in nerve terminals depends first on the ACh release elicited by $Ca^{++}$ influx accompanying depolarization. This release from the vesicular compartment drives translocation of cytoplasmic ACh into the vesicles. This in turn stimulates ACh synthesis by mass action, with a consequent reduction in cytoplasmic Ch. Therefore, the Ch chemical gradient is reduced across the nerve membrane, and this brings about a further increase in its uptake. Second, $Na^+$ entry results in an activation of Na,K-ATPase, with a consequent $Na^+$ extrusion and probably a membrane hyperpolarization. Thus, the $Na^+$ gradient is maintained, and the potential difference across the terminal membrane may be increased, with an increased driving force for the coupled influx of Ch. Consequently, more intracellular Ch becomes available for ACh synthesis, and CAT converts Ch to ACh, limited by cytoplasmic AChE activity.

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