Acetylcholine Action: Biochemical Aspects

Two major approaches to understanding the mechanism of action of acetylcholine are examined.

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Knowledge of the anatomical structures and the physiological events associated with neural transmission mediated by acetylcholine (ACh) has progressed considerably since ACh was first implicated as a chemical transmitter in the 1920's by Loewi and Dale (*1*). The bulk of evidence now available indicates that ACh exerts its effect on the receptor membrane by increasing the permeability of this membrane (*2*). Several mechanisms have been proposed to account for this effect; one involves the reversible interaction of ACh with a constituent of the receptor membrane, producing conformational changes which result in increased permeability. As proposed by Nachmansohn (*3*), this theory provides for ACh's being bound to a macromolecule in the resting state (possibly a protein or conjugated protein). Stimulation (for example, by an electric current) releases the ester, which then reacts with a receptor producing the conformational changes and increasing the membrane permeability. He notes that proteins are the most versatile substances and that ACh is formed by a protein and hydrolyzed by a protein, acetylcholine esterase (AChE). He suggests that the reaction with the receptor is likely to be another reaction with a specific protein rather than with some other class of compounds.

More recently this concept has been extended by Karlin (*4*) and by Changeux and Podleski (*5*). The latter have used the transmembrane potential of the isolated monocellular electroplax to quantitate the response to a variety of ligands. They have confirmed earlier studies showing a sigmoid shape of the dose response curve, which suggests cooperativity. Further evidence for cooperativity is provided by the observation that when two activators (carbamylcholine and decamethonium) are used together, the presence of one alters the shape of the dose response curve of the other. To explain the cooperativity, they have applied the model for allosteric transitions, formulated by Monod, Wyman, and Changeux for regulatory enzymes (*6*), to the interaction of ACh and its receptor (AChR).

Using this allosteric model, they explain the cooperativity of ACh's effects by postulating two states for a hypothetical protomer (*7*) ($P \rightleftharpoons D$), where P represents the state of the protomer when the membrane is polarized and D represents the protomer when the membrane is depolarized. The changes of membrane potential are determined by the fraction of protomers which undergo the transition to the D state. Antagonism between activators and inhibitors is then described in terms of differential stabilization of either the D or the P conformation. The cooperative response seen with activators is due to the transition of any one protomer favoring identical transitions in neighboring protomers. The relatively low degree of cooperativity demonstrated (Hill coefficient 1.8) may be explained either by strong interactions restricted to small clusters of protomers or by weak interaction over a larger field of protomers.

Another extension of the conformational theories has been proposed by Watkins (*8*). He cites the similarities in structure and charge distribution of the polar head portions of several lipids and possible neurotransmitters, such as phosphatidylcholine-acetylcholine, phosphatidylethanolamine-γ-aminobutyric acid, and phosphatidylserine-glutamine. He first considers the evidence that biological membranes have breaks in the nonpolar lipid layer. To show how these may control permeability, he hypothesizes that, at restricted regions of greater vulnerability or "polar discontinuities," these transmitters replace the polar head portion of the corresponding lipid by combining with a lipid-protein complex, thus causing a change in permeability. When he applies this model to ACh action (see Fig. 1) the proposed membrane structure involves (i) electrostatic binding of the quaternary ammonium group of the lecithin to an anionic protein side chain, (ii) a divalent metal ion bridge between the lipid phosphate group and a second anionic group of the protein, and (iii) coordinate bonding between the double-bonded oxygen atom of the phosphate group and a peptide group of the protein chain.

Acetylcholine then competes with phosphatidylcholine for two of these sites (sites 1 and 3), and a conformational change takes place in the protein as it binds with the ACh. This weakens the binding to the membrane of the divalent cation, which can then be displaced by a univalent cation. If this phosphatidylcholine-protein complex controlled the ionic permeability of that region, the "conformational changes and liberation of anionic groups on both lipid and protein could easily open up cation-selective channels and increase the permeability of the region to sodium and potassium ions." Differing effects with analogs could depend upon whether they block the new channel themselves when binding. Ehrenpreis proposes that the protein undergoing the conformational change in the model is in fact AChE (*9*). There is not general agreement, however, that AChE and AChR are the same, for not all of the properties examined are identical (*9, 10*).

A major difficulty with the models presented above is that they have not readily lent themselves to experimental validation. We are now proposing an entirely different theory for the action of ACh; our theory is based on the postulate that ACh alters membrane permeability by enhancing the enzymatic hydrolysis of one or more of the phospholipids present in these membranes. This hypothesis is in accord

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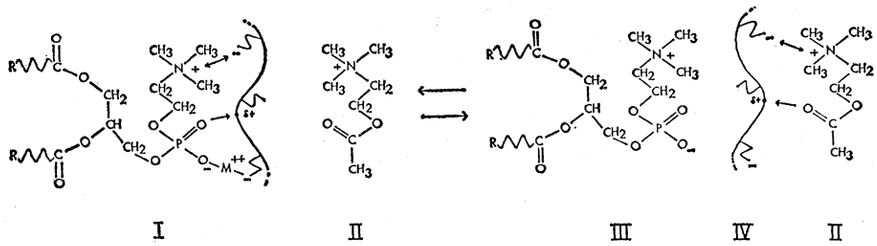
with a number of biochemical effects of ACh on glandular and neural tissues, and it also has considerable heuristic value.

Fifteen years ago Hokin and Hokin demonstrated that ACh stimulates the incorporation of radioactive inorganic orthophosphate ($^{32}P_i$) into the phospholipids of pancreas slices (*11*). Studies on slices of a number of other ACh-sensitive glands and on several preparations of neural tissue have shown similar effects (*12–14*). Phosphatidylinositol and phosphatidic acid, which is a precursor in the biosynthetic pathway of the former (*15*), are the phospholipids whose synthesis is most consistently stimulated. Phosphatidylcholine synthesis may also be stimulated in the superior cervical ganglion (*16*), but this is not a consistent finding in all tissues (*13, 17*). The turnover of the glycerol moiety of these lipids is not stimulated by ACh to the same extent as the phosphate; the response of the inositol moiety of phosphatidylinositol, however, parallels the changes in the phosphate (*14*). The relative stability of the diglyceride portion suggests that it may remain bound to the membrane throughout the changes in the phosphoryl group.

Although it had been suggested (*18*) that the increased incorporation of $^{32}P_i$ into phosphatidic acid and phosphatidylinositol was the result of a generalized stimulation of phospholipid synthesis, there is increasing evidence of a greater degree of specificity (*16, 17*). By comparing chronically denervated and normal superior cervical ganglia, Hokin (*16*) showed that the ACh effect on phosphatidylcholine and part of its effect on phosphatidic acid are presynaptic, but that upon phosphatidylinositol is postsynaptic—and thus is unaltered by the degeneration of the nerve terminals, which results from the chronic denervation. Several other phospholipids examined show no significant changes with ACh.

The demonstration of specific stimulation of $^{32}P_i$ incorporation into phosphatidylinositol in sympathetic ganglia during electrical stimulation (*17*) supports the idea that these changes have physiologic significance. Moreover, our own studies on the ACh-stimulated incorporation of $^{32}P_i$ into phosphatidic acid in brain homogenates have shown that this effect is observed maximally in the brain subcellular fraction containing a high concentration of "synaptosomes" (*19*), corroborating a possible relation to synaptic transmission (*20*).



I II III IV II

Fig. 1. Dissociation of a hypothetical phosphatidylcholine-protein complex (I) by acetylcholine (II). The interaction with ACh induces a conformational change in the protein which weakens the binding of the divalent metal ion and facilitates its displacement by univalent ions. (III) Phosphatidylcholine; (IV) unstable acetylcholine protein complex. [Used with the permission of Academic Press] (*8*).

Recent experiments in our laboratory suggest that the observed effects of ACh on the synthesis of phosphatidic acid and phosphatidylinositol in brain homogenates may be secondary to effects on the hydrolysis of phosphoinositides and perhaps other phospholipids. This led to the proposal of a reaction sequence which explains the effect of ACh on phosphatidic acid and phosphatidylinositol by assuming that it is secondary to the effects on the hydrolysis of phospholipids. This reaction sequence also helps to clarify some heretofore perplexing aspects of the relative concentration dependencies of the effects on phosphatidic acid and phosphatidylinositol. First we will present the scheme and the evidence for ACh's modulation of this hydrolysis. Then we will discuss the physiological consequences of this model.

Hypothetical Reaction Sequence

To account for many of the observed biochemical effects of ACh we have proposed the reaction sequence shown (*21*). It is assumed that the diglyceride moiety is stable and is bound to the membrane throughout the sequence. Reactions 3 and 4 are the generally accepted pathways for the synthesis of phosphatidylinositol (*13, 22*). Reaction 2 is one of the three alternative pathways known for the synthesis of phosphatidic acid in brain (*23*). Reaction 1 is a general statement representing phosphodiesteric cleavage of a phosphatide.

Central to the proposed mechanism is the assumption that, in the intact excitable membrane, reaction 1 is rate limiting and is stimulated by ACh. If it is further assumed that reaction 2 is rapid as compared to reactions 3 and 4, a number of previous observations can be explained. Under conditions of minimum stimulation of reaction 1, it would remain the rate-limiting reaction; and newly synthesized ^{32}P-labeled phosphatidic acid would not accumulate, but it would be converted to ^{32}P-labeled phosphatidylinositol (if we assume also that there is no significant accumulation of the nucleotide intermediate). Low concentrations of ACh would therefore result primarily in increased incorporation of ^{32}P into phosphatidylinositol, with only small changes in ^{32}P-labeled phosphatidic acid—due perhaps to changes in the steady-state concentration of a rapidly turning over fraction of the acid.

At higher concentrations of ACh,

Hypothetical Reaction Sequence

1. Diglyceride–P–X $\xrightarrow{\text{ACh-stimulated}}$ Diglyceride + P–X
2. Diglyceride + $AT^{32}P$ $\longrightarrow$ Diglyceride–^{32}P + ADP
3. Diglyceride–^{32}P + CTP $\longrightarrow$ Diglyceride–^{32}P–P–C + PP
4. Diglyceride–^{32}P–P–C + In $\longrightarrow$ Diglyceride–^{32}P–In + CMP

Diglyceride–P–X represents any phosphatide except phosphatidic acid; diglyceride–^{32}P represents ^{32}P–phosphatidic acid; diglyceride–^{32}P–P–C represents beta ^{32}P–CDP–diglyceride; In represents L-myoinositol; and diglyceride–^{32}P–In represents ^{32}P–phosphatidylinositol. ADP and ATP, adenosine di- and triphosphates; CMP, CDP, and CTP, cytidine mono-, di-, and triphosphates.

reaction 1 may be accelerated sufficiently so that reactions 3 and 4 become rate limiting, and ^{32}P-labeled phosphatidic acid would accumulate. These conclusions are consistent with observations on salt gland slices (*24, 25*) and brain homogenates (*26*). In these tissues, low concentrations of ACh stimulate primarily the synthesis of ^{32}P-phosphatidylinositol, while at higher concentrations of ACh the amount of $^{32}P_i$ incorporated into phosphatidic acid can far exceed the amount incorporated into the inositol.

Substrate for Phosphodiesterase

Considering the kinetic arguments presented above, any phosphatide except phosphatidic acid itself would be a suitable substrate, since it is proposed that the concentration of the membrane-bound diglyceride limits the overall rate of phosphatidylinositol synthesis. Phosphatidic acid could not be the substrate for reaction 1, if this reaction sequence is to explain the observation that synthesis of phosphatidylinositol appears to be stimulated at lower concentrations of ACh than those required to stimulate synthesis of phosphatidic acid. Indeed, if the acid were the substrate for reaction 1, the ratio of ^{32}P-labeled acid to inositol would be greatest at the lowest concentration of ACh.

Though not required by the above kinetic arguments, from the physiological point of view it seems attractive to postulate that the proposed reaction sequence is part of a closed cycle. If phosphatidylinositol were the substrate of reaction 1, the four reactions would themselves constitute a closed cycle. If a polyphosphoinositide [for example, phosphatidylinositolphosphate (PIP) or phosphatidylinositoldiphosphate (PIPP)] were the substrate, completion of the cycle would require one or two phosphorylation steps after reaction 4. The relatively high concentrations of the above-mentioned polyphosphoinositides in neural tissue and the rapid increase in specific radioactivity of PIP when a crude synaptosome-rich fraction is incubated with ^{32}P-labeled adenosine triphosphate (*27*) suggest that these compounds may be involved. Finally, electric convulsions increase the labeling of a phospholipid in rabbit brain which is thought to be phosphatidylinositol, while decreasing the labeling in another phospholipid resembling PIPP (*28*).

Experimental Results

To investigate this further, we directed experiments in our laboratory toward finding direct evidence for ACh-stimulated hydrolysis of one or more phospholipids. Since the inositides are most strongly implicated, we began by studying them. These experiments measure the liberation of ^{3}H-inositol phosphates from the endogenously labeled inositol phosphatides of a crude mitochondrial fraction, during a 3-minute incubation with and without ACh. Eserine, an inhibitor of acetylcholine esterase, was also added to minimize the hydrolysis of the ACh.

Table 1 shows the results of an illustrative experiment. The values in the first column reflect the radioactivity in the barium precipitable phosphate esters at the end of the 3-minute incubation. The "net" values reflect the increase that occurred during the incubation period, which can be attributed principally to the hydrolysis of the previously labeled inositol phospholipids. Acetylcholine increases the hydrolysis 64 percent. A series of seven similar experiments was done, and the results were normalized by setting the average number of counts per minute after incubation of the controls equal to 100 for each experiment. The average stimulation was 46.2 ± 13.1 percent (computed S.E.M.), $.01 < P < .02$ (*29*). We have investigated the pharmacological specificity of ACh in several experiments. At about equimolar concentration, atropine, which abolishes the muscarinic action of ACh, blocks the ACh stimulation (Table 2). Choline alone cannot substitute for ACh in causing stimulation, although it also has a quaternary nitrogen (Table 3).

We are now trying to determine whether the effect of ACh on phospholipid hydrolysis is limited to phosphatidylinositol. In one experiment phosphatidylcholine was endogenously labeled by choline-^{14}C, and ACh caused a decrease in the release of labeled phosphocholine (Table 4). When the actual amounts of lipid hydrolyzed are computed (if the specific activity of the lipid hydrolyzed is the same as the lipid extracted from the brain of other animals similarly injected), they are very similar to those obtained for phosphatidylinositol hydrolysis. A similar experiment with endogenously labeled phosphatidylethanolamine showed no liberation of phosphoethanolamine during the incubation with or without ACh, but the quantity of labeled phosphoethanolamine in the zero time tubes is so great that changes of the same order of magnitude as are seen with phosphatidylinositol and phosphatidylcholine would not be detectable in our system.

Physiological Implications

There is no general agreement concerning the physiological role of the effects of ACh on phospholipids. One of the early hypotheses of Hokin and Hokin was a possible relation to secretion of proteins and hydrophilic substances, particularly sodium ions, via a phosphatidic acid cycle (*13, 24*). This hypothesis that a phosphatidic acid cycle is involved in sodium ion transport has been seriously questioned by several investigators (*30*). These authors have proposed instead that the membrane permeability to sodium and potassium ions is controlled through a mechanism which involves phosphorylation of phosphatidylinositol to PIP and PIPP and degradation of the polyphosphoinositides by phosphomonoesterases. This mechanism cannot explain ACh's effects on phosphatidic acid or phosphatidylinositol. In contrast, we propose that the effects of ACh on permeability are secondary to the stimulation of a phospholipid diesterase. If this reaction occurred in the postsynaptic membrane, the release of the phosphorylated moiety, especially if it were anionic, might result directly in an increase in permeability to cations and a resultant depolarization. If there was a differential susceptibility to diesterase action among phosphatidylinositol, PIP, and PIPP, the excitability could be modulated by interconversions involving monoesterases and kinases.

If PIP or PIPP, or both, were the substrate for reaction 1, this mechanism could also result in a transient local release of calcium ions, since both PIP and PIPP are chelators of calcium and are thought to be important perhaps in controlling calcium ion concentration (*30*). It may be that this initiates the depolarization, for it has been postulated that calcium ions block the sodium ion "pores" in the resting state (*2*).

Brain does have the enzymes necessary for all of these reactions (*31–33*). Phosphatidylinositol inositolphosphohydrolase is primarily a soluble enzyme, but a significant fraction is bound in the

Table 1. Hydrolysis of inositol phosphatides. The values for the counts per minute are given as the arithmetic mean ± the computed standard error for the three tubes in each group. Inositol-2-^{3}H (200 μc) was injected intracisternally 10 hours before the crude mitochondrial fraction was prepared; under these conditions the endogenous phosphatidylinositol has about 700 count/min per nanomole (efficiency 19 percent). The concentrations of ACh and eserine were $10^{-4}M$ when added. The reaction mixture included hexokinase and 2-deoxyglucose to trap the adenosine triphosphate formed and thus prevent phosphorylation of free inositol-2-^{3}H during the incubation (*29*).

Additions	Radioactivity in phosphatidylinositol		
	Total (count/min)	Net (count/min)	Percent of control
ACh + eserine	2697 ± 49*	1302	164
Eserine	2083 ± 50	688	86
None	2190 ± 136	795	100

* Differs from control by $.02 < P < .05$, and from eserine alone by $P < .01$ by *t*-test.

particulate fraction (*34*). There is no evidence for ACh sensitivity when the activity of this enzyme is assayed with a purified phosphatidylinositol preparation as substrate (*35*). Both PIPP, PIP phosphodiesterases, and both PIPP and PIP phosphomonoesterases, are activated by divalent cations or "cationic amphipathic substances," but they are not definitely known to be activated by ACh or other substances thought to be neural transmitters. Both phosphatidylinositol kinase and PIP kinase are activated by ACh however (*32*).

It is of interest to note that similar effects on phospholipid metabolism can be produced in other tissues by specific compounds. Thyroid stimulating hormone stimulates the incorporation of $^{32}P_i$ into the phospholipids of thyroid slices, as does ACh (*36*). The effect was most marked on phosphatidylinositol. Epinephrine stimulates $^{32}P_i$ incorporation into the phosphatidylethanolamine and inositol phosphatides in rat liver slices (*37*). It may be that the effect of ACh on phospholipids in neural tissues is a specialized expression of a general mechanism which controls alterations in membrane permeability by a wide variety of substances.

Table 2. Effect of atropine on ACh stimulation (*29*). The ratio of atropine to ACh varied from 1 : 2 to 2 : 1. The results express the net incorporation (count/min).

Additions	No. of samples	Incorporation (count/min)	*P*
None	4	23 ± 24.3	
ACh + eserine	4	46 ± 10.3	
ACh + eserine + atropine	4	22 ± 9.7	$.01 < P < .02$

Hokin and Hokin have studied salt gland slices (*24, 25*). The kinetics of incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol have led them to propose that the activation of specific glandular sites involves the conversion of the inositol to the acid through the intermediate formation of digylceride; return to the resting state is accompanied by resynthesis of the inositol. Though similar chemical reactions are suggested, it differs from the mechanism proposed above in that the cycle is not continuous, but of an on–off character. The authors believe that ACh activates the gland by depolarization at a receptor site *distinct* from the site of conversion of phosphatidylinositol to phosphatidic acid (*38*). To account for all the ^{32}P-incorporation phenomena, they propose, in addition, a continuous rapid turnover of phosphatidic acid through the action of phosphatidic acid phosphatase and rephosphorylation of diglyceride. Alternatively, as suggested above, the data can be completely explained by the scheme we suggest if one assumes that, at high concentrations of ACh, the rate constant for the hydrolysis of a small active fraction of phosphatidylinositol (reaction 1) exceeds the rate constant for its synthesis (reactions 3 and 4). The action of phosphatidic acid phosphatase is not required.

In contrast we are suggesting the possibility that ACh induces depolarization at the synapse in neural tissue, and perhaps in other tissue as well, through a direct and continuous effect on the rate of phosphodiesteratic cleavage of one or more phospholipids; we have demonstrated stimulation of hydrolysis of both phospatidylinositol and PIP in cerebral homogenates.

Though speculative, this proposed scheme is consistent with a considerable amount of data and suggests numerous experiments. We are attempting, by experiment, to define more completely the substrate specificity for this system, for it is possible that other phospholipids of the membrane are also hydrolyzed as part of analogous cycles. It would also be of great interest to see whether other compounds proposed as neural transmitters can act similarly. Several experiments suggest that some

Table 3. Comparison of choline and acetylcholine (*29*).

Additions	No. of samples	Incorporation (count/min)	*P*
None	4	81 ± 19.6	
Ch + eserine	4	78 ± 10.6	
ACh + eserine	4	105 ± 14.2	$.01 < P < .02$

(for example, norepinephrine and dihydroxyphenylalanine) can also stimulate phosphatidylinositol hydrolysis (*39*).

Working with the partially purified soluble phosphatidylinositol inositolphosphohydrolase, we could not show any ACh effect on the hydrolysis of purified phosphatidylinositol. Isolated membranes free of phosphatidylinositol inositolphosphohydrolase need to be prepared in order to determine whether ACh can stimulate the hydrolysis of the phosphatidylinositol present in the membrane when the soluble phosphatidylinositol inositolphosphohydrolase is added.

The model we have proposed does not specify the mechanism by which ACh increases the hydrolysis of inositol phosphatides. The rapidity of the effect makes increased synthesis of the enzyme very unlikely. Possibly it alters the lipid itself—as proposed by Thompson and Dawson for the effect of cetyltrimethylammonium bromide on PIPP phosphodiesterases (*33*). Another possibility is a direct effect of ACh on the enzymatic activity of phosphatide phosphodiesterase. The kinetics of ACh activation of the enzyme might demonstrate cooperativity, accounting for the observations of Changeux and Podleski (*5*).

If the membrane has "polar discontinuities," such as Watkins depicts, hydrolysis of a phosphatide (for example, phosphatidylinositol) may lead to a decrease in the binding of divalent cations with a concomitant increase in

Table 4. ^{14}C-Phosphatidylcholine hydrolysis (*29*). Six samples were used in each set.

Addition	Incorporation (count/min)	*P*	Percent of control
ACh + eserine	6.0 ± 5.7	$.01 < P < .02$	44
Control	13.8 ± 2.14		100

permeability to univalent cations. Thus the proposed scheme is also compatible with this concept of the structure of the membrane, but it would provide for specificity of depolarization through enzyme activation rather than by direct competition of the transmitter with a phospholipid for binding sites on the membrane.

Finally, it must be noted that this theory relates only to the function of ACh as a synaptic transmitter. It cannot be assumed that a similar action of ACh is involved in impulse propagation since the latter has no effect on phosphatidylinositol metabolism (*17*) and ACh has no effect on phosphatidylinositol metabolism unless synapses are present (*16*). Unlike the types of theories proposed by Nachmansohn (*3*) and others as well, we are suggesting the possibility that the mechanism of depolarization at the synapse differs from the mechanism of depolarization of the axon. More specifically, we are proposing that chemical events, that is, the hydrolysis of a covalent bond, rather than simply conformational changes of macromolecules, account for the changes in permeability occurring in synaptic transmission.

Summary

Two major approaches to understanding the mechanism of action of ACh have been examined. The theories invoking only a conformational change are enticing in their simplicity but difficult to validate. The salient points of the numerous and sometimes conflicting studies of phospholipid metabolism were reviewed. A unifying hypothesis was presented which accounts for the observed biochemical data, while still being compatible with many of the considerations noted by those proposing models involving only conformational changes. The physiological implications of this model have been discussed.

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