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SULFUR AND SELENIUM ISOLOGS RELATED TO ACETYLCHOLINE AND CHOLINE IV. ACTIVITY IN THE ELECTROPLAX PREPARATION*†

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Abstract—Studies using the isolated single cell electroplax preparation indicated that the hydrolysis products of acetylthiocholine and acetylselenocholine, cholinethiol and cholineselenol, are powerful depolarizing agents. The activity of cholinethiol was greater at pH 6 than at pH 9. Methylation to form methylthiocholine and methylselenocholine increased activity further. Choline disulfide and choline diselenide were antidepolarizing blocking agents. Differences between the active site of the depolarizing receptor and that of acetylcholinesterase are discussed.

IT HAS been suggested[1] that a configurational change in the receptor protein, induced by the attachment of acetylcholine, may be an essential factor in the permeability changes of excitable membranes during electrical activity. Such a view is supported by studies of the depolarizing activity of acetylcholine and related compounds in the electroplax preparation.[2] Similarly, configurational changes in a receptor protein have been implicated[3, 4] as taking place in the enzymic hydrolysis of acetylcholine by acetylcholinesterase as well as in the synthesis of this ester by choline acetylase. In all cases, replacement of one of the methyl groups attached to the quaternary nitrogen by hydrogen greatly reduced activity. Moreover, it was found[4] that the relatively rapid rate of enzymic hydrolysis of acetylcholine compared to that of 2-dimethylaminoethylacetate could be ascribed to the entropy of activation in the reaction of the quaternary nitrogen compound being more favorable than for its tertiary amine analog.

It has been reported that muscarinic receptors and acetylcholinesterase display similar stereospecificity when presented with stereoisomers of muscarine analogs or of substituted trimethylammoniummethyl-l, 3-dioxolanes.[5] This similarity was used to suggest "the near identity of these two bioreceptors for acetylcholine"[5] and the "near identity of the two proteins"[6]. This claim is incompatible with

* For earlier papers see Refs. 10, 11, and 15.

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the observation[7] that in the single-cell electroplax, some compounds will have marked effect on electrical activity in concentrations that do not inhibit enzymic activity, while carbamylcholine and related compounds can induce depolarization in this preparation after acetylcholinesterase has been blocked completely.[8] Furthermore, it has been found recently[9] that the relative affinity of a series of benzoquinonium and ambenonium analogs for the cholinergic receptor of the electroplax differs markedly from the relative affinity of the same compounds for electric eel acetylcholinesterase. In any case, it seems unlikely that identical receptors could be involved in both processes, since the attachment of acetylcholine to a cholinergic receptor presumably induces a configurational change altering permeability to sodium and potassium ions, while attachment of acetylcholine to acetylcholinesterase would be expected to induce a configurational change in the protein, favoring hydrolysis of the substrate.

To throw additional light on this problem, the comparative study of acetylcholine, acetylthiocholine, acetylselenocholine,[10] choline, cholinethiol, cholineselenol,[11] choline disulfide, and choline diselenide[10] was undertaken.

In oxygen, sulfur, and selenium isologs of this type, molecular size is very similar, so that ability to fit receptor sites should not be affected appreciably. On the other hand, electron distribution in esters, thiolesters, and selenolesters may be rather different as evidenced by kinetic,[12–14] spectroscopic, and dipole moment measurements. Therefore, it may be expected that isosteric oxygen, sulfur, and selenium isologs should differ in their abilities to bind to receptor sites or in inducing conformational changes in receptor proteins.

It was noted recently[15] that acetylcholine, acetylthiocholine, and acetylselenocholine all exerted cholinergic effects on the guinea pig ileum and frog rectus abdominis preparations. While the addition of inhibitors of cholinesterase greatly enhanced response to acetylcholine, response to acetylthiocholine and acetylselenocholine was reduced. This effect was attributed to the fact that the thiolester and selenolester, which undergo enzymic hydrolysis at a rate very similar to that of their oxygen isolog,[16] yield cholinethiol and cholineselenol, compounds with depolarizing activities greater than those of their acetyl esters. On the other hand, choline, the hydrolysis product of acetylcholine, is essentially inert. Cholinethiol and cholineselenol were readily converted by oxidation to choline disulfide and choline diselenide, which had little activity in the above systems. The structures of these compounds are summarized on the facing page.

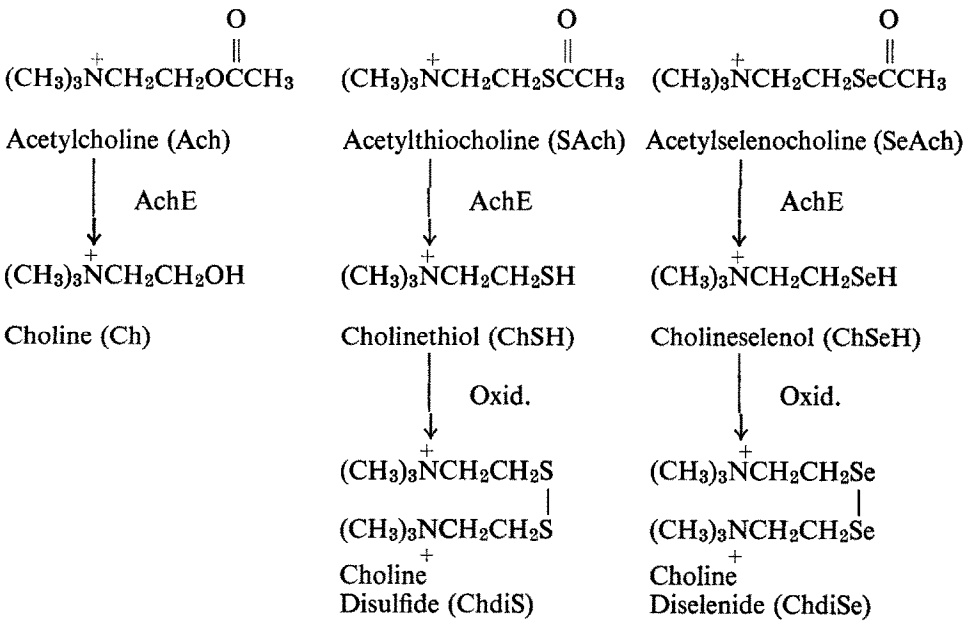
For an attempt to obtain more precise information about the actions of the above compounds, use of the improved isolated single-cell electroplax preparation[17, 18] appeared to provide a most favorable tool. This sensitive preparation, that has the added advantage of being active over a wide pH range, has been found to be extremely useful for analyzing structure–action relationships in the past.[2, 9, 17, 18]

MATERIALS AND METHODS

The sulfur and selenium analogs of choline and acetylcholine were synthesized[10, 11] by Mr. B. H. Yeaton with the guidance of Dr. W. H. H. Günther.

Single electroplax cells from the organ of Sachs of *Electrophorus electricus* were isolated and mounted in a chamber as described previously.[17, 19, 20] The cells were

impaled with a microelectrode, permitting the determination of the depolarizing effects of varying concentrations of the compounds being tested by continuous recording of the potential across the innervated membrane. Postsynaptic and direct and indirect action potentials were photographed on an oscilloscope, permitting the evaluation of the relative effectiveness of anti-depolarizing blocking agents.[21] All determinations were carried out on three or more cells derived from at least two different eels.



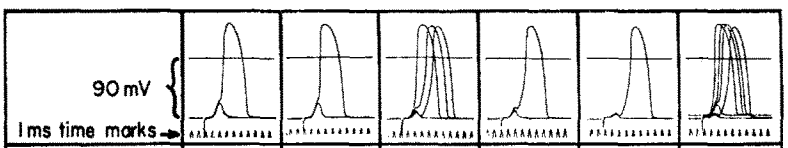
A Ringer's solution of the following composition was used: 160 mM NaCl, 5 mM KCl, 0·3 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1·2 mM Na_2HPO_4, 10 mM glucose; this solution had a pH of 7. When a pH of 9 had to be achieved, solutions were buffered with Tris instead of phosphate, to prevent the precipitation of divalent cations. Solutions on the innervated side of the electroplax were changed as described previously.[9]

RESULTS AND DISCUSSION

The anti-depolarizing blocking actions of choline disulfide, choline diselenide, and hexamethonium, summarized in Table 1, were ascertained by the effects of these compounds on the indirect action potential. An example of this blocking action is shown in Fig. 1. The postsynaptic and indirect action potentials may not disappear completely when block occurs, because some of the synapses are outside the area of the window against which the innervated side of the cell is pressed and thus are not exposed to the drug solution.[21] It can be seen in Fig. 1 that the postsynaptic potential is reduced reversibly to one half the initial value by exposure to a $2{\cdot}5 \times 10^{-4}$ M solution of choline disulfide and even further by a more concentrated solution. Picture 6 (Fig. 1) indicates that a 2-min recovery in Ringer's solution not only restores

TABLE 1. EFFECT OF ANTI-DEPOLARIZING AGENTS ON ELECTROPLAX PREPARATION

Compound	No. of cells tested	M conc. required to block indirect action potential
Choline disulfide	6	3×10^{-4}
Choline diselenide	3	2×10^{-4}
Hexamethonium	4	5×10^{-4}



picture no.	1	2	3	4	5	6
M conc. of choline disulfide	0	10^{-4}	2.5×10^{-4}	2.5×10^{-4}	5×10^{-4}	0
time in min. in solution	-	14	7	14	7	2
settings in V on Grass S4E Stimulator	25, 28	32, 35	30, 33, 40, 50	34, 37	42, 45	26, 33, 40, 45*

*The base line is elevated slightly and these four tracings are superimposed on the two tracings from picture no. 5.

FIG. 1. Effect of choline disulfide on the postsynaptic and neurally evoked action potentials. Pictures 1, 2, 4, and 5 show the maximal height of the postsynaptic potential obtained with subthreshold stimulation and the action potential with threshold stimulation. Pictures 3 and 6 show the same and in addition superimposed action potentials obtained with increased stimulus strengths as indicated. Temp. 25°, pH 7·0.

the postsynaptic potential but also decreases the latency of the action potential. Probably the action potential seen in picture 5 (Fig. 1) originated outside the area of the window, accounting for the greater latency.

It can be seen in Table 2 that depolarizing activity in the electroplax preparation decreases as the oxygen of acetylcholine is replaced progressively with sulfur and selenium, provided that hydrolysis of the esters is inhibited by eserine. It should be noted that acetylselenocholine in the presence of physostigmine cannot induce complete depolarization at any concentration.

While choline is essentially devoid of depolarizing activity, its sulfur analog, cholinethiol, as noted previously,[15] is at least as active as its acetyl ester. Unfortunately, cholineselenol is oxidized so rapidly to choline diselenide, a bis-onium compound, that quantitation of the depolarizing activity of the selenol proved impossible; however, it is apparently more potent than is acetylselenocholine, as is indicated by the behavior of the latter compound in the presence and absence of eserine.

Since cholinethiol possesses a pK_a of 7·7,[22] while cholineselenol has a pK_a of 4·7,[11] it had been postulated[15] that the relatively high depolarizing activities of cholinethiol and cholineselenol might be due to the relatively high tendencies of the

TABLE 2. EFFECT OF DEPOLARIZING AGENTS ON ELECTROPLAX PREPARATION

Compound	No. of cells tested	S.E.*	Average M conc. required to depolarize to 45 mV	Equipotent molar ratios (ACh = 1)
ACh (with eserine)†	6	0·3	3×10^{-6}	1
SACh (with eserine)	7	0·6	5×10^{-5}	17
SACh (without eserine)	6	0·9	5×10^{-5}	17
SeACh (with eserine)	6		does not depolarize to 45 mV	
SeACh (without eserine)	4	0·2	1×10^{-4}	33
Choline	3		1×10^{-1} (only to 65 mV)	33,000
Cholinethiol	6	1·2	5×10^{-5}	17
Cholineselenol	5		variable rapid oxidation	?
Methoxycholine	3		2×10^{-4}	67
Methylthiocholine	3		2×10^{-5}	7
Methylselenocholine	4	0·2	1×10^{-5}	3
Dimethylaminoethanethiol	3		does not depolarize at all	
Decamethonium	3		2×10^{-6}	0·7

* Standard error $= \sqrt{[\Sigma d^2/n(n-1)]}$.

† ACh without eserine at a concentration of 5×10^{-3} M is inactive; at a higher concentration ACh without eserine does induce effects, which will be described in a forthcoming paper by Eva Bartels.

The eserine concentrations used with the ACh isologs was 5×10^{-5} M. The 1×10^{-1} M choline solution contained half the normal amount of sodium.

anions of these compounds to be bound to the "esteratic site" of the depolarizing receptor. If this were so, it would be expected that cholinethiol would be more active at pH 9 than it is at pH 6·2, since, of course, this compound would be ionized to a much greater extent at the former than at the latter pH. The electroplax preparation is uniquely useful for work of this kind, since it can be used to measure depolarization over a very wide pH range,[18] as was shown by the observation[18] that the ability of carbamylcholine to induce depolarization remained constant as pH was increased from 6 to 9. When a study of the depolarizing activity of cholinethiol over a pH range of 6·2 to 9 was carried out, it was found that, contrary to expectation, this compound was more active at the lower pH (3·1% ionized) than at the higher pH (95% ionized). That this difference in depolarizing activity is not due to oxidation being faster at the higher pH could be demonstrated by changing the pH of the cholinethiol solution in the electroplax preparation reversibly, as shown in Fig. 2. It can be seen that depolarizing activity always increased as pH was lowered, indicating that this compound is more active in the mercaptan than in the mercaptide form.

To prove that a negative charge on sulfur and selenium is not implicated in the relatively high depolarizing activities of cholinethiol and cholineselenol, methoxycholine, methylthiocholine, and methylselenocholine were synthesized:

$(CH_3)_3\overset{+}{N}CH_2CH_2OCH_3$ $(CH_3)_3\overset{+}{N}CH_2CH_2SCH_3$ $(CH_3)_3\overset{+}{N}CH_2CH_2SeCH_3$

In these compounds a negative charge cannot be accommodated on oxygen, sulfur, or selenium; however, in all three cases increased depolarizing activity was observed. Decamethonium, which is devoid of ester groupings, is a well-known depolarizing neuromuscular blocking agent which was also found to be highly active in the electroplax preparation (Table 2). Similarly, trimethylbutyl ammonium and trimethylpentyl

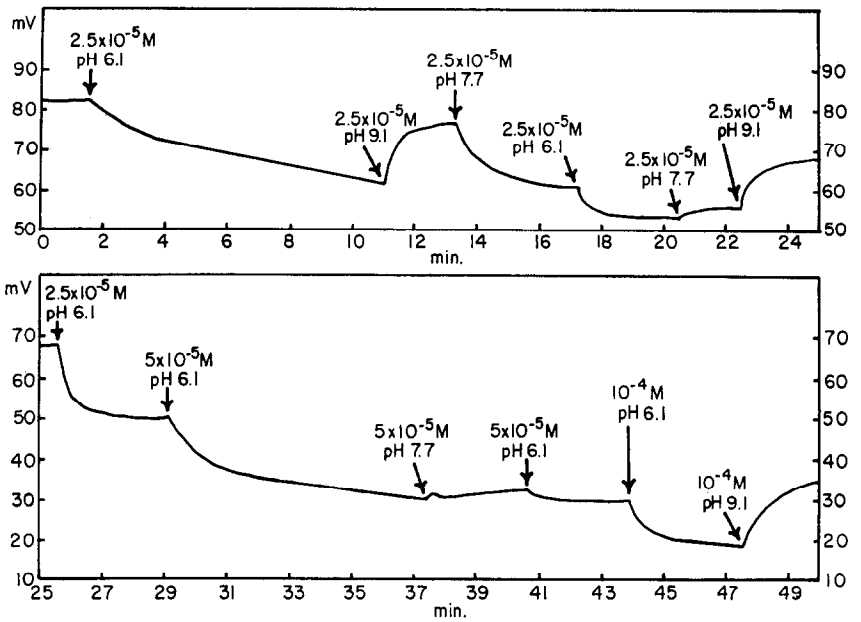


FIG. 2. Direct tracing of a single experiment showing the effect of pH on the steady-state membrane potentials produced by various concentrations of cholinethiol. Arrows indicate pH changes. The cell was incubated for 10 min in normal Ringer's solution of pH 6·1 before application of the first test solution. Temp. 25°.

ammonium were effective depolarizing agents in this system;[23] the latter compound is also quite active in the guinea pig ileum.[24]

The lack of depolarizing activity of the dimethyl analog of cholinethiol indicates the requirement for an onium nitrogen for attachment to an "anionic site" on the receptor, similar to the requirement for a quaternary nitrogen seen in acetylcholinesterase[4] and choline acetylase[3] action. It had been noted previously that the dimethyl analog of acetylcholine has only slight activity in the monocellular electroplax preparation.[2] However, while there appears to be a common requirement for an anionic site in the receptor of the electroplax preparation and the active site of electric eel acetylcholinesterase, the data presented here are incompatible with a requirement for an "esteratic site" in both proteins. Thus, acetylcholine, acetylthiocholine, and acetylselenocholine are hydrolyzed by acetylcholinesterase at very similar rates[16] but have widely different depolarizing activities. Furthermore, molecules devoid of anything resembling ester groupings were found to be powerful depolarizing agents. In view of these findings, it seems tempting to speculate that the membrane receptor may possess a "hydrophobic bonding site" in lieu of the esteratic site of acetylcholinesterase, while both receptors must have anionic sites for conformational changes to be induced. In view of the high activity of so many quaternary nitrogen compounds not closely related to acetylcholine, the almost total lack of depolarizing activity of choline is striking and physiologically just as important as the high depolarizing activity of acetylcholine. That the lack of depolarizing activity of choline compared to its acetyl ester is highly specific is emphasized by the observation that homocholine and acetylhomocholine have fairly similar depolarizing activities.[25]

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