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THE ACTIVE STRUCTURE OF LOCAL ANESTHETICS

EFFECTS ON ELECTRICAL AND CHOLINESTERASE ACTIVITY

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SUMMARY

The potency of several local anesthetics as a function of pH has been tested both as to their inhibitory effect on purified acetylcholinesterase (EC 3.1.1.7) prepared from electric tissue, and their blocking action on the electrical activity of the isolated single electroplax. The inhibition of acetylcholinesterase in solution by the tertiary nitrogen derivatives procaine, dibucaine and tetracaine and the quaternary analogue of tetracaine decreases with an increase in pH from 6 to 9. When, however, the pH is raised from 7.5 to 9 the inhibiting effect of the tertiary compounds procaine and tetracaine decreases more strongly than that of the quaternary tetracaine. Carbamylcholine and quaternary tetracaine block electrical activity of the electroplax about equally well at pH 6 or 9. In contrast the tertiary tetracaine is more potent at the acid pH. The results provide further evidence that the cationic form is the more active structure of tertiary nitrogen-containing local anesthetics and support the view of the presence of an anionic group in the active site of the receptor comparable to that existing in the esterase.

INTRODUCTION

The chemical structure of most local anesthetics is similar to that of acetylcholine and their effect on electrical activity of nerve have been attributed by NACHMANSOHN[1,2] to a competition with ACh for receptors in the membrane. The nature of the action has recently been tested with the monocellular electroplax preparation and the antagonism found indicated competition for the same membrane receptor[3]. ACh has a limited number of forces capable of interacting with a macromolecule. One of them is the cationic nitrogen group. It has been shown that ACh-esterase (EC 3.1.1.7) has an anionic group in the active site which attracts this group by coulombic forces[4–6]. It may be assumed that an ACh receptor protein would have a similar negatively charged site. Indeed, it has been reported that the binding of the cationic form of tetracaine to an isolated receptor protein in solution was greater than that of the uncharged base[7], suggesting the presence of an anionic group in the active site of

Abbreviation: ACh, acetylcholine.

the receptor. Most local anesthetics are tertiary nitrogen compounds with pK's usually between 8 and 9, and therefore exist at neutral pH partially in cationic and partially in uncharged form. If the suggested mechanism of action of local anesthetics is correct, one would expect them to be more potent both in affecting electrical activity and in inhibiting ACh-esterase when they are present in their charged form. Since the proteins reacting with ACh will probably have similar active sites it is not surprising that local anesthetics inhibit ACh-esterase even though block of conduction by these agents at the low concentrations at which they act is not due to this effect[8,9].

In contrast to the above noted expectation observations were reported in which local anesthetics were more potent on biological preparations at an alkaline pH, where they exist mainly as the uncharged base[10–15]. This may, however, be due to permeability barriers since tertiary amines penetrate tissue more readily than do quaternary ammonium ions[16–21]. At the nodes of Ranvier where the barriers covering the conducting membrane are relatively small, local anesthetics are not more potent at an alkaline pH[22]. With other preparations the cationic form of local anesthetics was also found to be the active form[23,24]. It appeared, however, surprising that procaine and tropacocaine were found in solution to be more potent inhibitors of ACh-esterase in their uncharged form while the results expected by theory and opposite to the above were found with tetracaine and physostigmine[25].

In order to clarify further the points discussed above, it appeared desirable to compare a local anesthetic containing a tertiary nitrogen grouping with its corresponding methylated quaternary analogue. We have, therefore, tested at varying pH the ability of tetracaine and its quaternary analogue, methyltetracaine, to inhibit ACh-esterase and to block electrical activity of the monocellular electroplax preparation.

MATERIALS AND METHODS

Measurement of ACh-esterase activity. Partially purified ACh-esterase from electric tissue of electric eel was used as the source of enzyme in these experiments. The activity was such that at 30°, pH 7.1, 1 mg of protein hydrolyzed 963 mg of ACh chloride per h. This was diluted in our experiments to give a final activity of 0.4 mg ACh hydrolyzed per ml incubation mixture per h.

To determine the inhibitory potency of the local anesthetics on ACh-esterase they were added to 5 mM ACh chloride in a Ringer's solution usually used in work with electroplax[26]. The solution was buffered at pH 6 with 0.1 M phosphate and at pH 7.5 and 9 with 0.1 M Tris. At zero time a concentrated solution of ACh-esterase was added and enzyme activity was determined at 22–24° by the colorimetric method of HESTRIN[27]. The hydrolysis of ACh during the time period tested was less than 40 %. All results were corrected for spontaneous hydrolysis of ACh which was substantial only for the experiments at pH 9. Experiments at a pH more alkaline than 9 were not performed because of the large spontaneous hydrolysis of ACh and because of the relative insolubility of tetracaine and dibucaine at alkaline pH. No spontaneous hydrolysis of the local anesthetics was observed, nor were any of them hydrolyzed by the enzyme preparation used. All absorbancy readings were corrected for the color developed by procaine, tetracaine and methyltetracaine.

The control ACh-esterase activity was determined at a particular pH. The per cent inhibition of ACh-esterase at this same pH by several concentrations of

a local anesthetic was determined and from this the I_{50} concentration (that concentration of local anesthetic causing 50% inhibition of ACh-esterase) was estimated. This was done for every pH tested and for all the anesthetics used.

Measurement of effects on electrical activity. The isolated single electroplax developed by SCHOFFENIELS[28,29] was used as the biological test object for determining the potency of tetracaine, methyltetracaine and carbamylcholine at different pH values. This preparation can either be stimulated directly by way of the conducting membrane or indirectly by way of neural innervation. The criterion for the effect on the end plate was the disappearance of the postsynaptic potential[30]. The effects of methyltetracaine were determined with the improved recording technique using intracellular electrodes[31]. Extracellular electrodes were used for most of the pH studies. At pH 9 the usual phosphate buffer in Ringer's solution was replaced by 1.5 mM Tris. All agents tested were applied in the solution bathing the innervated membrane of the electroplax. The pH was altered in both the pools, on the innervated and non-innervated membranes. All cells were immersed in Ringer's solution of the desired pH at least 10 min prior to the application of the test solutions. At the end of the experiments the pH values in the pools were about the same as at the beginning.

Preparation of methyltetracaine iodide. Methyltetracaine iodide was synthesized by Dr. S. GINSBURG as follows: an ice-cold solution of 6.6 g (25 mM) tetracaine base in 60 ml acetone was treated with an excess (5 ml) of methyl iodide. After standing in ice for 4 h, the salt was filtered and recrystallized from a mixture of ethanol and ether. Yield was 7.7 g (76% of theoretical). Another recrystallization from isopropanol gave pure, almost colorless, crystals (m.p. 141–142°). (Found: C, 47.59 (47.36); H, 6.62 (6.74); N, 6.76 (6.98); I, 31.22 (31.49). $C_{14}H_{27}N_2O_2I$ (mol. wt. 406.3) requires C, 47.27; H, 6.65; N, 6.89; I, 31.26%.)

RESULTS

The approximate I_{50} concentration for each of the local anesthetics is shown in Fig. 1. ACh was used as substrate in a concentration of 5 mM in all experiments because it was found to be an optimum concentration at pH 6 and only slightly greater than optimal at pH 7.5 and 9. The control ACh-esterase activity at pH 7.5 and 9 was approximately equal whereas the activity at pH 6 was considerably lower which agrees with earlier results[32]. The pH values in all tubes were within 0.1 pH unit of the mean values shown in the figure. Each of the points shown in Fig. 1 was calculated from the mean esterase activities in 2–4 series of experiments, with 5 or 6 concentrations of local anesthetic used in each series. The inhibition by dibucaine at pH 9 could not be determined because of its very limited solubility at that pH.

The effects of carbamylcholine, tetracaine and methyltetracaine on the electrical activity of the isolated single electroplax are shown in Table I. Carbamylcholine depolarizes the synaptic regions of the membrane[31], thereby short circuiting the entire conducting membrane[33], so that both the direct and indirect responses are blocked simultaneously. Therefore, no values are shown in Table I for time required for carbamylcholine to block the indirect response. The periods of time required for blocking electrical activity by carbamylcholine and methyltetracaine are similar within the pH range of 6–9. The tertiary tetracaine appears more potent at pH 6 than at pH 9 although because of relatively large variations in response the times noted at pH 6

are significantly different from those at pH 9 only to the $P < 0.10$ level except for the effect of 0.1 mM tetracaine on the direct response where the difference is significant to the $P < 0.05$ level. However, in experiments where the same cell was exposed to tetracaine at pH 6 and 9 the difference in potency was obvious. For example see Fig. 2.

Methyltetracaine acts on the conducting membrane as well as on the synaptic junction. As seen in Table I its effects on the synaptic junction (time required to block the indirect response) are 2–4 times as potent as its effects on the conducting membrane (time required to block the direct response). As seen in Figs. 3 and 4 quaternary tetracaine does not depolarize the electroplax membrane. It may be noted in Fig. 3

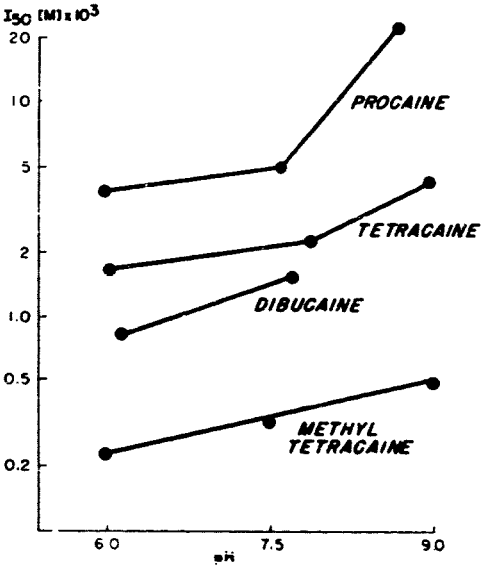


Fig. 1. Concentrations of procaine, tetracaine, dibucaine and methyltetracaine required for 50% inhibition (I_{50}) of purified ACh-esterase prepared from electric tissue as a function of pH.

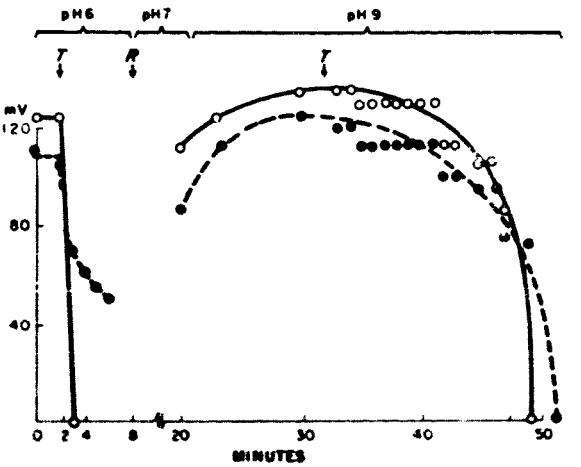


Fig. 2. Effect of 0.1 mM tetracaine (T) on the indirectly (x — x) and directly evoked (O — O) spikes of the isolated single electroplax at pH 6 and 9. R indicates return to Ringer's solution.

TABLE I

BLOCK OF ELECTRICAL ACTIVITY OF ISOLATED SINGLE ELECTROPLAX BY CARBAMYLCHOLINE, TETRACAINE AND METHYLTETRACAINE AS A FUNCTION OF pH

The period of time required for blocking electrical activity is given in the last 2 columns. All results are recorded as mean ± S.E. of the mean.

Compound	Concn. (mM)	pH	No. of Expts.	Time for block of conduction (min)	
				Indirect	Direct
Carbamylcholine	$5 \cdot 10^{-2}$	6.0	2	—	2 ± 0.5
		7.3	2	—	2 ± 0.0
		8.9	2	—	2 ± 0.5
	$2.5 \cdot 10^{-2}$	5.9	2	—	7 ± 1
		7.1	2	—	4 ± 2
		8.8	2	—	5 ± 1
Tetracaine	0.1	6.1	4	2 ± 0.4	4 ± 2
		7.6	3	3 ± 1	7 ± 3
		9.1	6	12 ± 4	16 ± 3
	$5 \cdot 10^{-2}$	6.1	3	4 ± 1	4 ± 2
		8.8	5	14 ± 4	19 ± 5
Methyltetracaine	$5 \cdot 10^{-2}$	7.0	2	> 30	> 30
	0.1	7.0	2	17 ± 6	> 30
	0.25	7.0	2	5 ± 1	15 ± 2
	0.5	7.0	2	2 ± 1	10 ± 0
	1	6.0	1	2	5
		7.0	1	2	5
		9.0	1	2	5
	0.5	6.1	5	3 ± 1	7 ± 1
		7.4	4	3 ± 1	9 ± 1
		9.0	4	5 ± 2	10 ± 2

that the postsynaptic potential is reduced within 3 min exposure to 1 mM methyltetracaine and blocked completely after 6 min. The direct response would be blocked only later. The effects on the indirect response were completely and easily reversible. The effects of 0.5 mM methyltetracaine on the conducting membrane are seen in Fig. 4. The neurally evoked potential is blocked after 2 min, while 10 min after the application of the compound only a small graded local response is left in place of the

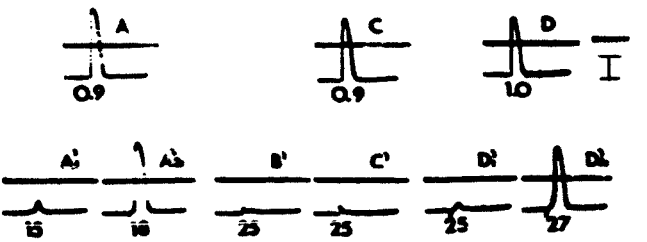


Fig. 3. Reversible effect of methyltetracaine on the ACh endplate potential. A–D directly, A_1'–D_2' indirectly elicited action potentials. A–A_2' controls in Ringer's solution, B' and C–C' 3 and 6 min after exposure to 1 mM methyltetracaine. D–D_2' 16 min after return to Ringer's solution. Calibration: 50 mV and 1000 cycles/sec. Stimulating voltages are shown below each frame of the figure.

normal direct response. Block of the directly elicited action potential by methyltetracaine was not reversible even after rinsing in Ringer's solution for up to 1 h.

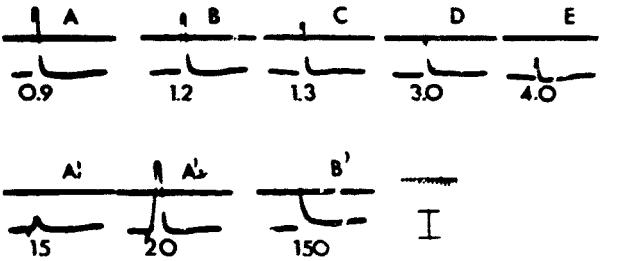
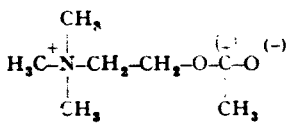


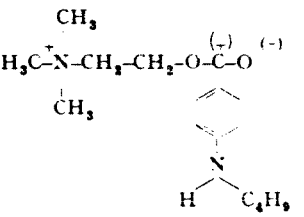
Fig. 4. Effect of methyltetracaine on the membrane action potential. A–E directly and A'–B' indirectly elicited action potential. A–A_2' control in Ringer's solution; B and B', 2 min; C, 5 min; D, 8 min; and E, 10 min after addition of 0.5 mM methyltetracaine. After return to Ringer's solution for 20 min there was no recovery of the action potential. Calibration: 50 mV and 1000 cycles/sec. Stimulating voltages are shown below each frame of the figure.

DISCUSSION

All the local anesthetics tested including methyltetracaine are more potent inhibitors of ACh-esterase at an acid than at an alkaline pH. With procaine and tetracaine, however, the potency declines more rapidly from pH 7.5 to 9 than is observed with methyltetracaine.



Acetylcholine



Methyltetracaine

Since the quaternary nitrogen of methyltetracaine exists only as a cation the effects of pH must be attributed to changes in ACh-esterase. The aniline nitrogen could not be involved since when its pK was tested it was found to be below 3, so that in the pH range studied it would exist well over 99% in the uncharged form.

The anionic group in the active site of ACh-esterase apparently does not change charge in the pH range of 6.5–10, as shown by BERGMANN AND SHIMONI[24]. Increasing the pH of a solution of the enzyme may, however, increase the number of nonspecific negatively charged sites of which there are probably more than there are specific anionic sites, although only the latter are important for the activity of the enzyme. Increasing the pH might, therefore, increase the nonspecific binding of methyltetracaine allowing less of it to reach the active sites of the enzyme which may contribute to the observed decrease in potency of this compound with increasing pH. Below pH 6.5 the anionic groups in the active site become progressively inactivated[24], so that had we decreased the pH in our experiments below 6 we might have observed a decrease in ACh-esterase inhibition by the local anesthetics.

The decrease in inhibitory strength of procaine and tetracaine from pH 6 to 7.5 can probably be explained as being due to effects on the protein as discussed above. The decreased potency from pH 7.5 to 9, however, is greater than observed with methyltetracaine, indicating that an additional factor is involved besides change in charge of the protein. The change in charge of the local anesthetic is probably the additional factor modifying their inhibitory activity. The aniline nitrogens of tetracaine and procaine were determined to have a pK of less than 3 so that in the pH range of these experiments less than 1 % of these nitrogens is in the charged form. The pK's of the dimethylaminoethyl nitrogen of tetracaine and the diethylaminoethyl nitrogen of procaine are about 8.2 and 8.9, respectively[35]. On the basis of its pK value, and taking into account the decrease in potency of methyltetracaine, the decreased potency of tetracaine from pH 6 to 9 is less than expected. This conclusion, however, depends on the probably incorrect assumption that the uncharged molecule has no inhibitory activity. If it has even weak activity as an inhibitor this could explain why the potency of tetracaine did not decline as rapidly as expected on the basis of its pK value. The change in structure of the protein is also a modifying factor, which was put forth as a possible explanation for the fact that the inhibitory action of tensilon on cholinesterase did not decline as rapidly from pH 8 to 9.5 as would be expected based on its pK value[36]. The quaternary analogue of tetracaine is a more potent inhibitor of ACh-esterase than tetracaine. This parallels the results with the tertiary analogues of phospholine[37] and neostigmine[38] which are weaker inhibitors of ACh-esterase than the quaternary compound. The decrease in inhibitory activity of procaine and tetracaine with an increase in pH agrees with previous results found with physostigmine[4] and amines[39]. With a change of pH no change in potency of neostigmine was observed[4], whereas we found a small change in potency with methyltetracaine. It is difficult to say why the present results with procaine and dibucaine differ from those reported previously[25], since in both studies ACh-esterase from electric tissue of electric eel was used.

The results obtained with tetracaine on the electrical activity of the isolated single electroplax also indicate that the charged molecule is more active than the uncharged form. Carbamylcholine and methyltetracaine which do not change in charge as a function of pH do not change in potency on the isolated electroplax. These observations agree with previous findings that physostigmine and the tertiary analogue of neostigmine are twice as potent at pH 6 as at pH 9 in blocking electrical activity of the electroplax, whereas neostigmine does not vary in potency from pH 6 to 9 (ref. 40). Procaine, tetracaine and dibucaine had previously been found more potent on the electroplax at pH 6 than at pH 9 (ref. 23). The tertiary analogue of ACh is also weaker at an alkaline pH than at an acid pH[26]. There are at least two factors which may minimize the change in potency of tetracaine on electrical activity of electroplax when the pH is increased from 6 to 9. The intramembranous pH has probably not been altered to the same extent as that of the external fluid, and the rate of penetration of the charged molecule is probably lower than that of the uncharged form[20–22].

When one measures the potency of a tertiary nitrogen-containing local anesthetic as a function of pH on a biological preparation, there are at least two competing factors which will determine what results are obtained. In those preparations in which the permeability barriers are relatively small, one observes the greater potency

of the charged form[22-24], however, in other preparations where the barriers are greater one observes an apparently greater potency of the uncharged molecule[10-15,22], which is due to the poor permeability of the charged form[16-21].

It is interesting that quaternary tetracaine does not depolarize the electroplax membrane, since many quaternary nitrogen compounds, with the notable exception of *d*-tubocurarine, have been found to be receptor activators, that is, cause a depolarization of the membrane[1]. Apparently the substituted aniline ring may prevent by steric hindrance the activation of the receptor protein necessary for depolarization.

The results in this paper provide further evidence for previous observations suggesting that ACh-esterase and the receptor of the electroplax have some properties in common. The potency of tertiary compounds as a function of pH is similar for the receptor and for ACh-esterase.

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