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Effects of Mg^{2+} and Ca^{2+} on soluble and membrane-bound acetylcholinesterase from *Electrophorus electricus*

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NUMEROUS investigators have studied the effects of monovalent and divalent cations on acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7; AChE).[1–7] Most of the enzyme preparations used for such studies have been either soluble[1–4,7] or some undefined mixture of soluble and membrane-bound AChE.[5,6] Fries,[2] Nachmansohn[1] and others[3–8] have reported that $CaCl_2$ and $MgCl_2$ increase the maximal rate of hydrolysis of acetylcholine (ACh) by AChE. The extent of increase in the maximal rate, however, is not a consistent finding among investigators;[1–8] the reported variations are most probably due to the different sources of AChE (red blood cell membranes and electric tissue of *Torpedo marmorata* and *Electrophorus electricus*) used, as well as to the different assay conditions and methods. No comparative study has been made of the effect of $MgCl_2$, $CaCl_2$ and NaCl on membrane-bound and soluble AChE obtained from the same source.

We have prepared both membrane-bound and soluble AChE from the electroplaque of *Electrophorus electricus*. The K_m for ACh using membrane-bound AChE is nearly twice as great, and the amount of inhibition by excess substrate, half that of the soluble enzyme.[9] We report here the effects of $MgCl_2$, $CaCl_2$ and NaCl on the kinetics of membrane-bound and soluble AChE.

The two states of the enzyme respond differently with respect to at least one of the kinetic parameters studied (K_m, V_{max} and inhibition by excess substrate) when the concentration of any one of the above inorganic ions is varied.

Preparation of soluble and membrane-bound AChE. Membrane-bound and soluble AChE were prepared as previously described.[9] Electric organ from live eels, *Electrophorus electricus*, was homogenized at 4° for 15 sec in a Sorvall Omni-mixer and then again with ten up and down strokes of a Potter–Elvehjem apparatus operating at 5700 rev/min in a solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1·5 mM $MgCl_2$, pH 7·2. The homogenate was filtered through a stainless steel sieve (96 μm diameter openings), rehomogenized and centrifuged at 20,000 *g* for 30 min. The pellet was washed three times with the above salt solution; the third wash had less than 1 per cent of the AChE activity of the pellet. The pellet was resuspended in a volume of the salt solution equal to that of the original wet weight of the tissue; the suspension was stored at 4° for 48 hr and then centrifuged at 20,000 *g* for 30 min. The pellet was resuspended in the salt solution described above and this suspension will be referred to as the membrane-bound enzyme preparation; its supernatant was centrifuged at 100,000 *g* for 2 hr and the enzyme activity in the resulting nondialyzed supernatant will be called soluble AChE. The ratio of soluble AChE activity to soluble plus membrane-bound AChE activity, the per cent solubilization, reaches 50 per cent after storing the membrane preparation for 48 hr at 4° from the time of initial preparation and remains constant for at least another 7 days.[9]

Assay of AChE. AChE activity was determined by measuring the rate of hydrolysis of AChI by a pH-stat (Radiometer Corp.). Assays were done under a nitrogen atmosphere at 30° in 1·0 ml of a salt solution

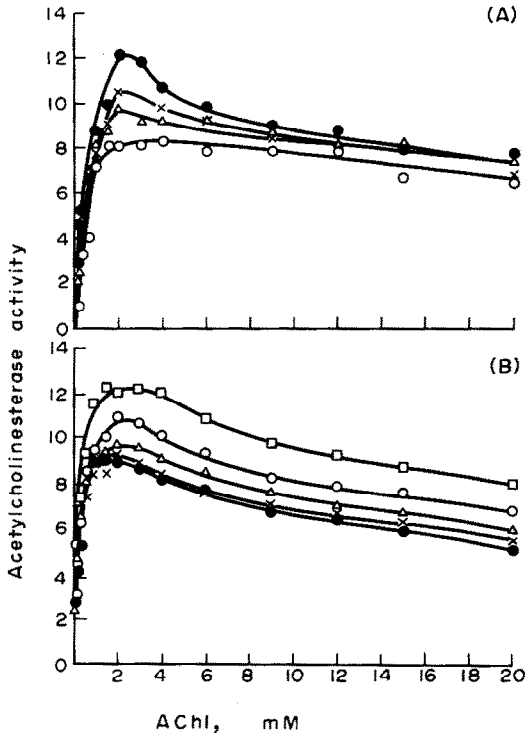

FIG. 1. Effects of various concentrations of $MgCl_2$ on the rate of hydrolysis of ACh by soluble and membrane-bound AChE. AChE activity is expressed in terms of μmoles of acid formed/min/g wet weight of tissue. The abscissa represents the concentration of substrate (AChI) used in each assay. All assays were done with a pH-stat. The upper panel (A) shows the rate of hydrolysis of ACh by membrane-bound AChE and the lower panel (B) the rate of hydrolysis of ACh by soluble AChE. The concentrations of $MgCl_2$ are ●—●, no $MgCl_2$; ×—×, 0·1 mM $MgCl_2$; △—△, 1·5 mM $MgCl_2$; ○—○, 10 mM $MgCl_2$; □—□, 50 mM $MgCl_2$.

containing 5 mM KCl, 2·0 mM phosphate, pH 7·20, as well as varying amounts of $CaCl_2$, $MgCl_2$ and NaCl; the concentrations of the last three salts are described in the legends of the figure and tables. Experiments in which $CaCl_2$ concentrations were changed were done using 2·0 mM imidazole instead of 2·0 mM phosphate buffer. Silman and Karlin[10] have established that the effects of imidazole in membrane-bound as well as soluble AChE are quite similar to those of phosphate.

Varying the concentration of divalent cation ($MgCl_2$ or $CaCl_2$) alters the kinetics of membrane-bound AChE differently from those of the soluble enzyme when assayed under identical conditions. The effects of $MgCl_2$ on membrane-bound AChE are shown in Fig. 1A. Two observations can be made from this family of curves: (1) the apparent maximal velocity decreases as the $MgCl_2$ concentration is changed from no $MgCl_2$ to 10 mM $MgCl_2$; and (2) the per cent substrate inhibition (%SI)* decreases with increasing concentration of $MgCl_2$.

In contrast to these observations, with soluble AChE, the presence of increasing concentrations of $MgCl_2$ results in an increase in the maximal rate of hydrolysis of ACh while the %SI remains unchanged (Fig. 1B). Fitting the data shown in Fig. 1, A and B, to a rectangular hyperbola, by computer analysis,[11] using only the data points before substrate inhibition becomes apparent, gives values for K_m and V_{max} which are shown in Table 1. Four observations can be made from this table: (1) the V_{max} of the membrane-bound enzyme decreases by 31 per cent with the addition of 10 mM $MgCl_2$, while the V_{max} of soluble

* %SI has been previously defined[9] as $R_2 - R_{20}/R_2$ where R is the rate of hydrolysis of ACh expressed as μmoles of acid formed/min/g wet weight of tissue and the subscripts 2 and 20 refer to the concentration (mM) of AChI.

TABLE 1. EFFECTS OF $MgCl_2$ ON THE V_{max}, K_m AND %SI OF MEMBRANE-BOUND AND SOLUBLE AChE FROM *Electrophorus electricus**

$MgCl_2$ concn (mM)	V_{max}† (± S.E.M.) Membrane-bound	Soluble	K_m (μM ± S.E.M.) Membrane-bound	Soluble	%SI‡ Membrane-bound	Soluble
0·0	100 ± 5·7	100 ± 7·0	499 ± 91	281 ± 69	37	39
0·1	86 ± 7·1	103 ± 4·0	524 ± 130	289 ± 43	36	40
1·5	81 ± 5·0	109 ± 4·0	435 ± 82	251 ± 38	18	38
10·0	69 ± 5·7	134 ± 4·5	485 ± 97	334 ± 58	19	38
50·0		132 ± 5·0		281 ± 30		36

* The assays were done at 30°, under a nitrogen atmosphere, with a pH-stat in a medium containing 180 mM NaCl, 5 mM KCl, 2 mM phosphate at pH 7·20.

† The V_{max} is expressed as a percentage of the V_{max} in the absence of $MgCl_2$ and the standard errors (S.E.M.) as well as the K_m values and their S.E.M. are obtained from the computer analysis of Cleland[11] (see text).

‡ %SI refers to the per cent substrate inhibition which is defined in the text.

AChE increases by 34 per cent with the same concentration of $MgCl_2$; (2) at 10 mM $MgCl_2$ the %SI in membrane-bound AChE is about 50 per cent that in the absence of added $MgCl_2$, while in soluble AChE there is no significant change in %SI with changes in $MgCl_2$ concentration; (3) the K_m values of ACh for either membrane-bound or soluble AChE are unchanged when the $MgCl_2$ concentration is changed from 0 to 10 mM; and (4) the K_m of ACh for soluble AChE is consistently lower than that for the membrane-bound enzyme.[9]

Table 2 shows the data obtained from experiments similar to those described in Table 1, with the exception that $CaCl_2$ rather than $MgCl_2$ concentrations were altered. The results are similar to those described in Table 1. Increasing the concentration of $CaCl_2$ from 0·1 to 50 mM results in a decrease in the V_{max} and in the %SI of membrane-bound AChE. Similar changes in the concentration of $CaCl_2$ result in an increase in the V_{max} without affecting the %SI of the soluble enzyme. The K_m values of ACh for both soluble and membrane-bound AChE remain constant as the $CaCl_2$ concentrations are altered from 0·1 to 50 mM.

Since the range of the concentrations of $MgCl_2$ and $CaCl_2$ used was large, there was a possibility that the effects described in Tables 1 and 2 and Fig. 1 were due to changes in ionic strength. We therefore investigated whether such effects could be produced by increasing the concentration of NaCl. The results shown in Table 3 indicate that none of the changes produced by $MgCl_2$ or $CaCl_2$ shown in Tables 1 and 2 on either membrane-bound or soluble AChE could be reproduced by increasing the NaCl concentration. Neither the V_{max} nor the %SI was significantly affected by increasing NaCl from 180 to 1000 mM. It is interesting to note, however, that changing the NaCl concentration from 180 to 1000 mM produced a marked increase in the K_m of ACh for soluble AChE, while the K_m of ACh for membrane-bound AChE was not affected.

TABLE 2. EFFECTS OF $CaCl_2$ ON THE V_{max}, K_m AND %SI OF MEMBRANE-BOUND AND SOLUBLE AChE FROM *Electrophorus electricus**

$CaCl_2$ concn (mM)	V_{max} (± S.E.M.) Membrane-bound	Soluble	K_m (μM ± S.E.M.) Membrane-bound	Soluble	%SI Membrane-bound	Soluble
0·1		100 ± 2·9		303 ± 42		32
1·0	100 ± 5·2	112 ± 6·3	600 ± 94	285 ± 89	33	38
6·0	84 ± 3·1	121 ± 2·3	501 ± 66	253 ± 41	24	36
20·0	72 ± 3·8	122 ± 5·0	418 ± 66	364 ± 61	18	36
50·0	73 ± 4·4	125 ± 4·1	421 ± 88	367 ± 49	16	31

* The assays were done in a medium containing 180 mM NaCl, 5 mM KCl, 1·5 mM $MgCl_2$, 2·0 mM imidazole, pH 7·20. All other conditions and the definition of terms are as in Table 1.

TABLE 3. EFFECTS OF NaCl ON THE V_{max}, K_m AND %SI OF MEMBRANE-BOUND AND SOLUBLE AChE FROM *Electrophorus electricus**

NaCl concn (mM)	V_{max} (± S.E.M.) Membrane-bound	V_{max} (± S.E.M.) Soluble	K_m (μM ± S.E.M.) Membrane-bound	K_m (μM ± S.E.M.) Soluble	%SI Membrane-bound	%SI Soluble
90		100 ± 1·9		115 ± 14		29
180	100 ± 4·0	101 ± 0·9	392 ± 40	181 ± 10	20	33
500	104 ± 2·5	101 ± 1·7	368 ± 25	227 ± 21	17	27
1000	97 ± 4·6	91 ± 3·2	375 ± 46	258 ± 17	16	29

* The assay medium contained 5 mM KCl, 6 mM $CaCl_2$, 1·5 mM $MgCl_2$, 2 mM phosphate at pH 7·20. All other conditions and the definition of terms are as in Table 1.

Both $CaCl_2$ and $MgCl_2$ cause an increase in the maximum rate of hydrolysis of ACh by soluble AChE. These ions, however, produce a decrease in the maximum rate of hydrolysis when the enzyme is in the membrane-bound form. Most published reports indicate that these divalent cations increase the activity of AChE.

Nachmansohn[1] was the first to show that the rate of hydrolysis of ACh by soluble AChE from *Electrophorus electricus* could be increased by $MgCl_2$, $CaCl_2$ and other divalent cations. Wilson and Cabib[12] confirmed this observation and established that AChE is not a metallo enzyme. There have been other reports[2–8] of these effects of $CaCl_2$ and $MgCl_2$ on soluble forms of AChE prepared from *Electrophorus electricus*, *Torpedo marmorata* and human and bovine erythrocytes. However, there have been few published reports of the effects of $MgCl_2$, $CaCl_2$ and NaCl on membrane-bound AChE. The study of Heller and Hanahan,[6] on erythrocyte preparation, also showed that divalent cations increased the rate of hydrolysis of ACh by AChE, but the nature of the kinetics in this study is hard to determine, since only one or two concentrations of substrate were used and the age of the preparation was not specified.

Gridelet *et al.*[5] reported that 10 mM $CaCl_2$ increased the rate of hydrolysis of ACh by AChE, obtained from *Torpedo marmorata*, when the concentration of ACh was about 2 mM, but that such a stimulation did not occur when the substrate concentration was raised to or above 10 mM. The enzyme preparation used by these investigators was the supernatant which was collected after centrifuging a homogenate of the electric organ of *Torpedo marmorata* at 17,000 *g* for 45 min. If the AChE from *Torpedo marmorata* has similar properties to that of *Electrophorus electricus*, then the enzyme preparation used by these investigators must have contained both soluble and membrane-bound AChE, since we have shown[9] that 10 per cent of the AChE in the homogenate of the electric organ of *Electrophorus electricus* remains soluble after a 100,000 *g* × 2 hr centrifugation. Furthermore, from the report of Gridelet *et al.*,[5] it is not clear whether AChE was assayed immediately after preparation of the enzyme or at a later time. This information is important, since we have shown that after storage of electroplaque membranes for 5 hr at 4°, 30 per cent of the AChE activity becomes soluble. The possible presence of soluble as well as membrane-bound AChE in the preparation of Gridelet *et al.*[5] makes the interpretation of their kinetic data difficult.

Our results on the effects of $MgCl_2$, $CaCl_2$ and NaCl on the kinetics of ACh hydrolysis by AChE are not inconsistent with previous reports, but the apparent inconsistency may simply be due to the fact that previous workers studied the kinetics of either a soluble or a mixture of soluble and membrane-bound AChE.

The different effects of $MgCl_2$, $CaCl_2$ and NaCl on membrane-bound and soluble AChE obtained from the electric tissue of *Electrophorus electricus* may be due to some change in the conformation of the active site of the enzyme. This change could be due to either one of two mechanisms. First, the release of AChE from membranes alters the conformation of the active site. Second, the release of AChE alters some peripheral (allosteric) site,[5] where these cations produce their effects, and this in turn changes the conformation of the active site. Such sites could have become masked, unmasked or altered by the presumed changes in conformation caused by solubilization.

Several investigators[3,5,13–18] have postulated the existence of peripheral or allosteric binding sites on AChE. Roufogalis and Quist,[16] Belleau and DiTullio,[19] and Iverson[20] have shown the presence of an anionic site on AChE distinct from the active centre of the enzyme. However, the criteria established by Monod *et al.*[21] for proving that an enzyme is allosteric have not been met for AChE. It is therefore preferable, at this stage, to assume that the differences between membrane-bound and soluble AChE from *Electrophorus electricus* are due to some change in the active site of the enzyme caused by solubilization and are not correlated to an allosteric phenomenon.

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Changes in D-glucaric acid excretion in relationship to alterations in the rate of antipyrine metabolism in man

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THE INDUCTION of hepatic microsomal enzymes may be an important factor affecting the rate of metabolism and hence the extent and duration of action of drugs in man. Enzyme induction may be produced not only by a large number of frequently used drugs,[1,2] but also by many chemicals, such as organochlorine pesticides and polycyclic hydrocarbons, which are encountered in man's environment.[3] Recently considerable evidence has been obtained that the urinary excretion of D-glucaric acid provides a quantitative, although indirect, index of microsomal enzyme activity in man.[4–6] We now report further studies of the relationship of changes in D-glucaric acid excretion to variations in the rate of metabolism of antipyrine, a drug which is metabolized in man by hepatic microsomal enzymes.

In an initial study, intersubject variations were eliminated by assessing changes in antipyrine half-life in relation to glucaric acid excretion in the same subjects before and after treatment with enzyme inducing drugs. Twelve normal volunteers (nine female, three male, aged 19–28) took part. An oral dose of antipyrine 18 mg/kg was administered after an overnight fast. Five blood samples were taken at intervals ranging from 3 to 15 hr after ingestion of the drug. Plasma levels of antipyrine were determined by the method of Welch *et al.*[7] At the same time, a complete 24 hr urine collection was made for glucaric acid, which was determined by the method of Marsh,[8] and expressed as μ-moles of glucaro-1,4-lactone. Subjects then