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INTERACTIONS OF CALCIUM WITH PURIFIED AND INTACT CELL ACETYLCHOLINESTERASE OF *ELECTROPHORUS ELECTRICUS*

JUDITH K. MARQUIS* and GEORGE D. WEBB

Department of Physiology and Biophysics, University of Vermont College of Medicine, Burlington, Vt. 05401, U.S.A.

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Abstract—The effects of calcium on the activity of purified preparations of acetylcholinesterase (AChE) from the electric organ of *Electrophorus electricus* and the activity of the AChE in intact electroplaques from the same species were studied. The activity of the purified AChE was measured using an automatic pH-stat. The AChE activity of a single intact electroplaque was measured by a radiometric assay technique in which ^{14}C-labeled acetylcholine (ACh) was applied to the innervated membrane only. The activity of purified AChE was markedly increased by 10 and 20 mM Ca^{2+} at all substrate concentrations tested. These same concentrations of Ca^{2+} increased the AChE activity of intact electroplaques at low substrate concentrations and inhibited the enzyme at ACh concentrations greater than 10^{-3} M. The differences observed between the purified and intact cell enzyme are discussed in terms of electrostatic neighboring-group interactions.

MANY STUDIES have been made of the hydrolytic activity of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; AChE) utilizing purified preparations of the enzyme from the electric organ of the electric eel, *Electrophorus electricus*.[1–5] Other studies have been made of the activity of AChE *in situ*.[6–9] The findings from these two groups of studies are not entirely consistent.

Measurements of the AChE activity of intact tissues may be distorted by the presence of diffusion barriers, by charged regions of the membrane which attract or repel a charged substrate, and by differences in conformation between membrane-bound enzyme and enzyme in solution.[10] Other problems may include the presence of nonspecific cholinesterase (ChE) and changes in the local pH as the hydrolysis of acetycholine (ACh) proceeds.[7]

In spite of the numerous difficulties inherent in the interpretation of membrane-bound enzyme activity, the very fact that these difficulties exist requires that enzyme studies be carried out, insofar as possible, on both a soluble enzyme preparation and on intact cells or tissues known to contain large quantities of that enzyme. In the specific case of AChE, the need for measuring the activity in intact cells (e.g. electroplaques) is reinforced by recent evidence which suggests that the moderately purified preparations of AChE which are commercially available may actually be globular aggregates of two or more native enzyme species.[11] It also appears that, during the preparation of highly purified enzyme, the native forms of AChE may undergo proteolysis, resulting in a nonaggregating form of the enzyme.[5]

* Present address: Department of Biochemistry and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Mass. 02111, U.S.A.

It has been frequently pointed out that calcium ions play a very important role in the production and regulation of cholinergic transmission and of electrogenic membrane actions.[12–14] The activity of Ca^{2+} at presynaptic nerve terminals and, in particular, in the mechanism of ACh release is well documented.[12] It has been suggested that Ca^{2+} may also be a key link between the binding of ACh to its postsynaptic receptor site and the changes in ion permeabilities subsequent to ACh–receptor interaction.[15, 16] An important step in this process may be that responsible for the destruction of ACh, that is, hydrolysis by AChE. It is difficult, however, to find sufficient evidence for a coherent model relating the activity of Ca^{2+} ions and the activity of AChE. There are numerous early examples of ChE activation by Ca^{2+}, Mg^{2+} and Mn^{2+},[17, 18] but most of these studies were conducted on crude enzyme preparations of low specific activity. In addition, many of these early experiments employed horse serum ChE, which has since been described as a "pseudo-cholinesterase".[19]

Despite many earlier[20–22] and more recent[4, 23–27] investigations of the effect of divalent cations on AChE activity, to our knowledge no comprehensive study has yet been made to compare the effects of cations on purified enzyme in solution with the effects on membrane-bound enzyme from intact cells of the same tissues. Since such a study might help to elucidate the normal functioning of AChE, as well as the postsynaptic sites of action of Ca^{2+} in cholinergic transmission, we have investigated the effects of Ca^{2+} on both partially purified AChE and on the AChE of intact electroplaques. An abstract of this work has been published.[28]

METHODS

AChE preparations partially purified from the electric organ of *Electrophorus electricus* were obtained from Sigma Chemical Co. Specific activities of 50–150 m-moles ACh hydrolyzed/hr/mg of protein were obtained.

Single electroplaques, dissected from the organ of Sachs of *Electrophorus electricus*,[29] were used as the source of intact cell AChE. All of the compartment membrane and most of the gelatinous connective tissue were removed from the innervated side of the cells. As most of the AChE activity is on the innervated side of the cell,[30–33] a single cell was mounted in a small Lucite chamber (Fig. 1) with the innervated side facing outward and covered with a Lucite window to isolate the AChE-rich half of the cell. The resulting "sandwich" was held together by a stainless steel clip and sealed with petroleum jelly. Niemi[33] has demonstrated that the use of this chamber largely eliminates the non-specific ChE activity of the non-innervated side of the cell and the surrounding connective tissue.[6] Using similar single electroplaque holders and tetraisopropyl pyrophosphoramide to inhibit selectively the nonspecific ChE,[34] he found that the innervated side of the cells contains nearly exclusively "true" AChE activity.

Solutions for all experiments were made up in standard physiological saline for *E. electricus* (188 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM Tris, adjusted to pH 7·4 with HCl).[35] To maintain isosmolarity, the amount of NaCl was appropriately adjusted in the Ca^{2+}-free and 10 or 20 mM Ca^{2+} solutions. In addition, for experiments with intact cells, 5 mM glucose and 12·2 mM sodium acetate were substituted for an equal amount of NaCl. The latter was added to reduce the cellular uptake of acetate produced during ACh hydrolysis. (Dawson and Crone[27] have shown that the nature of the anions in AChE solutions has very little effect on

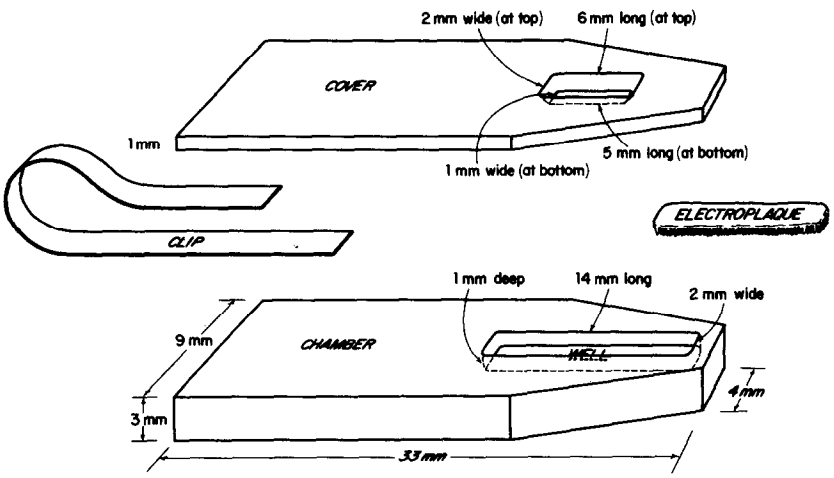

FIG. 1. Diagram of the small Lucite holder used in the measurement of the AChE activity of the exposed innervated face of a single electroplaque. The edges of the window are beveled to facilitate the flow of solution and reduce the possibility of unstirred layer formation.

enzyme activity.) To obtain Ca^{2+}-free solutions, the cation was simply eliminated from the physiological saline; no chelating agents were added.

The pH-stat automatic titrator technique used in this investigation to measure the hydrolytic activity of purified AChE is described in detail elsewhere.[36] The rate of hydrolysis of 0·1 to 10 mM ACh was measured. A final enzyme concentration of 2×10^{-5} mg protein/ml in a total volume of 10 ml was used in all the reaction solutions. The temperature was maintained at 25° and the pH-stat was set for pH 7·4.

To measure the AChE activity of intact electroplaques, three or four of the Lucite chambers described above were fastened onto the inside wall of a 10-ml beaker. The innervated face of several cells was thus exposed to 5 ml of external bathing medium to which substrate and other ligands could be added. The bathing solution was continuously stirred, temperature was maintained at 25°, and pH was set at 7·4.

The radiometric technique chosen to follow the hydrolysis of ^{14}C-labeled ACh by intact cells has been described in detail for rat diaphragm[37] and for guinea-pig ileum.[38, 39] The method utilizes cation-exchange columns to trap the labeled substrate in aliquots of the bathing medium, allowing the labeled acetate ions to pass through the columns into vials for liquid scintillation counting.

Acetyl-1-^{14}C-choline chloride (sp. act., 23 mCi/m-mole) was purchased from New England Nuclear Corp. Substrate concentrations ranged from 10^{-6} to 10^{-2} M ACh. The amount of labeled ACh was adjusted to give about 20,000 dis/min/ml in the final solution.

RESULTS AND DISCUSSION

The effects of varying Ca^{2+} concentrations on the hydrolysis of ACh by purified AChE are shown in Fig. 2. The activity of purified AChE in Ca^{2+}-free solution was less than the 2 mM Ca^{2+} control values at all substrate concentrations except 10^{-3} M ACh, the optimal concentration. Increasing the amount of Ca^{2+} to 10 and 20 mM

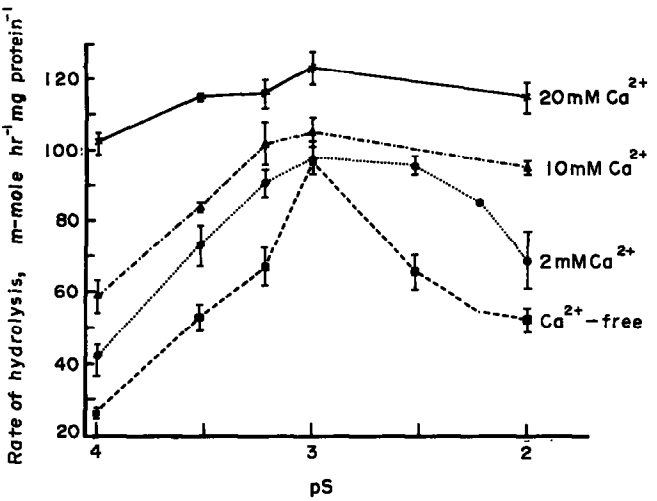

FIG. 2. Effect of varying the Ca^{2+} concentration of the physiological saline on the activity of purified AChE. S = ACh. Determinations were made at molar concentrations of ACh corresponding to pS = 4 (10^{-4} M), pS = 3·52 (3×10^{-4} M), pS = 3·22 (6×10^{-4} M), pS = 3 (10^{-3} M), pS = 2·52 (3×10^{-3} M), pS = 2·22 (6×10^{-3} M) and pS = 2 (10^{-2} M). Values obtained were measured for the initial 5 min of the reaction. The data labeled 2 mM Ca^{2+} were obtained by measuring the rate of hydrolysis of ACh in normal physiological saline. This curve might be considered the control curve. Daily controls demonstrated no significant variations in the specific activity of the enzyme over the period of use. Each point represents the mean of between three and five determinations. Vertical bars are used to indicate the range of the mean $\pm$ the standard error, except where the values lie within the area of the symbol used to denote a point. The standard error is $\sqrt{[\Sigma(X_i - \bar{X})^2/n(n-1)}$.]

resulted in a corresponding increase in enzyme activity at all substrate concentrations. Preincubation of the enzyme in a high (20 mM) Ca^{2+} solution did not alter this effect (data not shown).

According to the model suggested by Wilson,[3] there is only one anionic site in the active center of AChE. If this were so, and if Ca^{2+} were bound only to the active center, then Ca^{2+} should competitively inhibit the enzyme rather than activate it, as was found here. It can be seen in Fig. 2 that raising the Ca^{2+} concentration from 2 to 20 mM increased the rate of hydrolysis of ACh by purified AChE almost independently of ACh concentration, suggesting that there was no competition between Ca^{2+} and ACh. Thus, Ca^{2+} may bind to AChE simultaneously with the substrate, perhaps at noncatalytic peripheral anionic sites.[9, 40] This agrees with the recent work of Roufogalis and Quist,[41] who found similar results with Ca^{2+}-activation of partially purified erythrocyte AChE and postulated an allosteric anionic site separate from the catalytic anionic site.

It is of interest that the per cent activation of Ca^{2+} was least at the optimal substrate concentration of approximately 10^{-3} M. Also, in Ca^{2+}-free solutions, enzyme activity was decreased at all ACh concentrations except 10^{-3} M. This may have some physiological significance, as it has been calculated that during a normal endplate potential the ACh concentration near the postjunctional membrane reaches 10^{-3} M.[42]

In contrast to the above findings were those using membrane-bound AChE of intact cells. The rate of hydrolysis of ^{14}C-ACh by intact electroplaques at several sub-

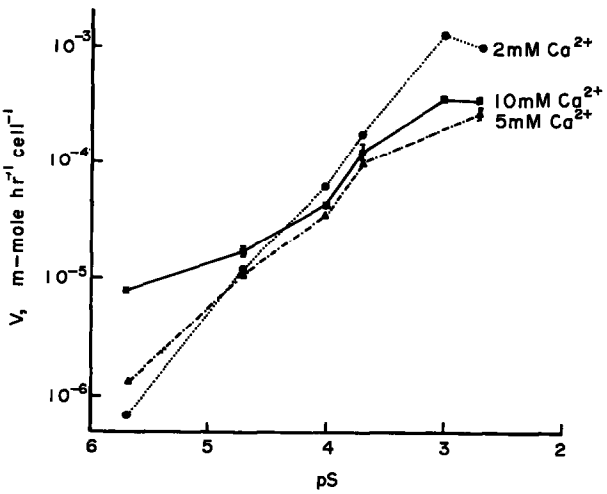

FIG. 3. Effect of varying Ca^{2+} concentrations on the rate of hydrolysis of ACh by intact electroplaques. Determinations were carried out at pS = 5·7 (2×10^{-6} M), pS = 4·7 (2×10^{-5} M), pS = 4 (10^{-4} M), pS = 3·7 (2×10^{-4} M), pS = 3 (10^{-3} M) and pS = 2·7 (2×10^{-3} M). Each point represents the mean of three experiments, except pS = 5·7 which represents only two experiments. Standard errors are indicated as in Fig. 2. In this case, most of the errors are smaller than the size of the symbols because the reproducibility was very good and because the logarithmic ordinate scale is compressed relative to the linear scale in Fig. 2.

strate and Ca^{2+} concentrations is shown in Fig. 3. The effects of Ca^{2+} were highly dependent on substrate concentration. Preincubation of the cells in a high Ca^{2+} solution did not alter these effects. The observed activation of intact cell AChE by Ca^{2+} at low substrate concentrations and inhibition at high substrate concentrations is typical of allosteric effectors,[43] suggesting the possibility of a regulatory role for Ca^{2+}. Thus, there may be a functional relationship between Ca^{2+} and membrane-bound enzyme. The data agree with those of Wombacher and Wolf,[44] who found similar results with a membrane-bound AChE preparation from red blood cells.

The differences in the effects which Ca^{2+} exerts upon purified AChE in solution as compared to the membrane-bound AChE activity of intact electroplaques could be attributed to a number of factors. Although high purity is often desirable for measuring enzyme kinetics and for studying the molecular properties of an enzyme, the enzyme *in vivo* is part of a membrane containing charged residues which interact to define the properties *in vivo*. Schoffeniels[40] suggested that AChE loses some of its regulatory properties in the process of purification. Thus, some of the differences observed in this study may be due to the fact that, *in vivo*, AChE interacts in a co-operative manner with other macromolecules in the membrane. Changes in the conformation of the enzyme as a consequence of purification might also be expected.

It has been suggested that an enzyme may alter its microenvironment as a result of its own activity.[10] For example, changes may occur in local pH values within an enzyme membrane in which acid or base is formed as a result of the enzyme reaction. If the pH in the microenvironment of the membrane-bound AChE falls during ACh hydrolysis, as suggested by Silman and Karlin,[7] then the additional H^+ produced at higher ACh concentrations may produce a decrease in the total number of negatively charged sites in the surrounding matrix which are available for interaction with

Ca^{2+}. This might explain why, in the intact cell experiments, Ca^{2+} exerted a positive or activating effect on AChE at low substrate concentrations but exerted the reverse (negative or inhibitory) effect at higher ACh concentrations.

The attachment of an enzyme to a membrane may alter its properties either by a specific interaction with some membrane component or as the result of a more general effect such as that exerted by the electrostatic field created by the charged constituents of membranes. Little is known about the effect of electrostatic fields on enzymes in natural membranes, but the effect of an electrostatic field produced by a highly charged matrix on the behavior of artificially immobilized enzymes has been thoroughly investigated and is discussed in detail by Katchalski *et al.*[10] Enzymes adsorbed onto a polyanionic matrix exhibit a marked shift in their pH-activity profiles toward higher pH values. This effect is attributed to an increase in H^+ concentration at the matrix surface relative to that in the surrounding medium.[45, 46]

It has been shown (G. D. Webb and R. L. Johnson, unpublished results) that the optimal bath pH for the AChE activity of intact cells is much higher than the optimal pH for AChE in solution (pH 10 for the former, regardless of buffer concentration, and pH 7 for the latter[47]). Such a shift in the pH-activity profile of the enzyme suggests that the AChE of the intact cell may be imbedded in a negatively charged matrix, a hypothesis consistent with the results described above, for if this were so, one would expect to observe differences in the binding and activity of Ca^{2+} when AChE is in solution as opposed to when the enzyme is membrane-bound. Surrounding negative charges have previously been attributed important functions in determining the character of electrostatic interactions at the active center of enzymes, including AChE.[18,21]

Thus, Ca^{2+} activity in cholinergic systems may be represented as a perturbation of the membrane system which can assume several forms; for example: a change in the quaternary structure of the protein polymer, alterations of the tertiary protein structure (conformational change) or changes in the character of electrostatic interactions at the protein surface. At present, none of these can be ruled out as a possible mechanism of action for the specific effects of Ca^{2+} on AChE activity or for the more general role of Ca^{2+} in regulating cholinergic transmission.

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