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ELECTRICAL EXCITABILITY OF ARTIFICIAL ENZYME MEMBRANES

II. ELECTROCHEMICAL AND ENZYME PROPERTIES OF IMMOBILIZED ACETYLCHOLINESTERASE MEMBRANES

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This paper deals with aspects of the reciprocal interaction between enzyme activity and the microenvironment or the potential difference in artificial proteinaceous membranes bearing cross-linked acetylcholinesterase. The potential difference resulting from asymmetric substrate injection into the system is recorded as a function of time. The influence of the membrane charge density on both enzyme activity and potential difference is studied by varying the external solution pH. The enzyme specific potential is initiated by local change of pH at the membrane level and the dependence on the buffer strength is studied. The recorded potential difference appears to be the result of the reciprocal interaction between enzyme reaction and the diffusion of substrate or products.

1. Introduction

Most of the kinetic work on enzymes has been carried out with both the substrate and enzyme in solution. This is surprising, since the great majority of enzymes are attached to membrane structures or contained in cell organelles, with complex structured environments. So, it is essential to understand the important influence of the local microenvironment on the mode of action of enzymes, and to take into account modifications of the local microenvironment by the enzymatic reaction itself.

Artificially immobilized enzymes allow the study of the interaction between enzyme activity and the microenvironment in a well defined context. Model systems are of use in studying the effect of a single microenvironmental parameter such as the influence of: (i) a polyelectrolyte environment [1,2]; (ii) an electric field [3–5]; (iii) local concentrations on mono-enzymatic [6,7] or multi-enzymatic systems [8–10]; (iv) a hydrophobic medium [11,12].

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) is a widely distributed enzyme in excitable membranes of nerve and muscle. Its molecular properties are of particular interest because of its involvement in nerve impulse transmission [13]. The enzyme catalyses the hydrolysis of acetylcholine to choline and acetate by means of a two-step process involving the formation of an acyl-enzyme intermediate [14].

With few exceptions, the extraction of acetylcholinesterase from any tissue source releases various molecular forms of the enzyme. The basis for such heterogeneity is not fully understood, but in part may relate to cellular location. It is widely suggested that the three main molecular forms correspond to three locations: intracellular, membrane bound and immobilized on basal lamina [15].

The goal of this paper is to study the reciprocal

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interaction between acetylcholinesterase activity and membrane potential. The artificial membrane configuration is particularly convenient for an analysis of such an interaction.

2. Materials and methods

2.1. Enzyme membrane production

The artificial acetylcholinesterase membranes were prepared with the co-cross-linking method previously described by Thomas and Broun [16], using an inactive protein as support. For albumin and gelatin membranes, acetylcholinesterase from electric eels (Sigma, St. Louis) was added to the inactive protein solution before spreading on the glass surface. The basic physico-chemical data of artificial proteinaceous membranes obtained are given in table 1.

2.2 Enzyme activity measurement

Enzyme activity was tested in 1 mM phosphate buffer solution, by adding 0.01 N NaOH with a pH stat (Metrohm). The experiments were performed at 25°C.

2.3. Potential difference measurements

Potential difference measurements were performed in a diffusion cell where the membrane separated two 25-ml compartments. Identical phosphate buffer solutions were circulated continuously in both compartments. Acetylcholine chloride (Sigma, St. Louis) was injected into one compartment and the potential difference was then measured using a vibrating-reed electrometer (Cary 401, Varian) with calomel reference electrodes. The pH on each side of the membrane was regulated with pH stats.

3. Experimental results

The kinetic study of both immobilized and soluble acetylcholinesterase has been done by using a potentiometric method. The results obtained for the immobilized enzyme activity as a function of the substrate concentration at pH 7.5 and of buffer pH are shown in fig. 1. The behaviour of the native enzyme exhibits a bell-shaped curve as a function of pH, which is not influenced by substrate concentration. After insolubilization in a membrane, the pH-activity profiles are displaced toward more acid pH values. The lower the substrate concentration, the lower the apparent optimum pH value.

The acetylcholinesterase reaction produces choline and acetate with a net increase in H^+ concentration. The local production of H^+ decreases the intramembranal pH, and due to the amphoteric properties of the proteinaceous membrane, one might expect, by using the technical device previously described, to measure acetylcholinesterase-specific membrane potential differences. The artificial acetylcholinesterase membrane separated two compartments containing phosphate buffer solutions. Asymmetry was introduced into the system by injecting acetylcho-

Table 1

Basic physico-chemical data of artificial proteinaceous membranes

Values represent the mean ± S.E. of four determinations.

	Bovine serum albumin	Ossein gelatin	Pig skin gelatin
Isoelectric pH	5.0 ± 0.1	4.9 ± 0.1	7.4 ± 0.2
Membrane thickness (μm)	50 ±10	150 ±25	100 ±20
Protein concentration (g/cm³)	0.45 ± 0.09	0.19 ± 0.03	0.25 ± 0.03
Water content [a] (w/w, dry membrane)	1.2 ± 0.2	5.6 ± 0.6	3.2 ± 0.4

[a] Measurements were performed in 10 mM phosphate buffer, pH 6.0.

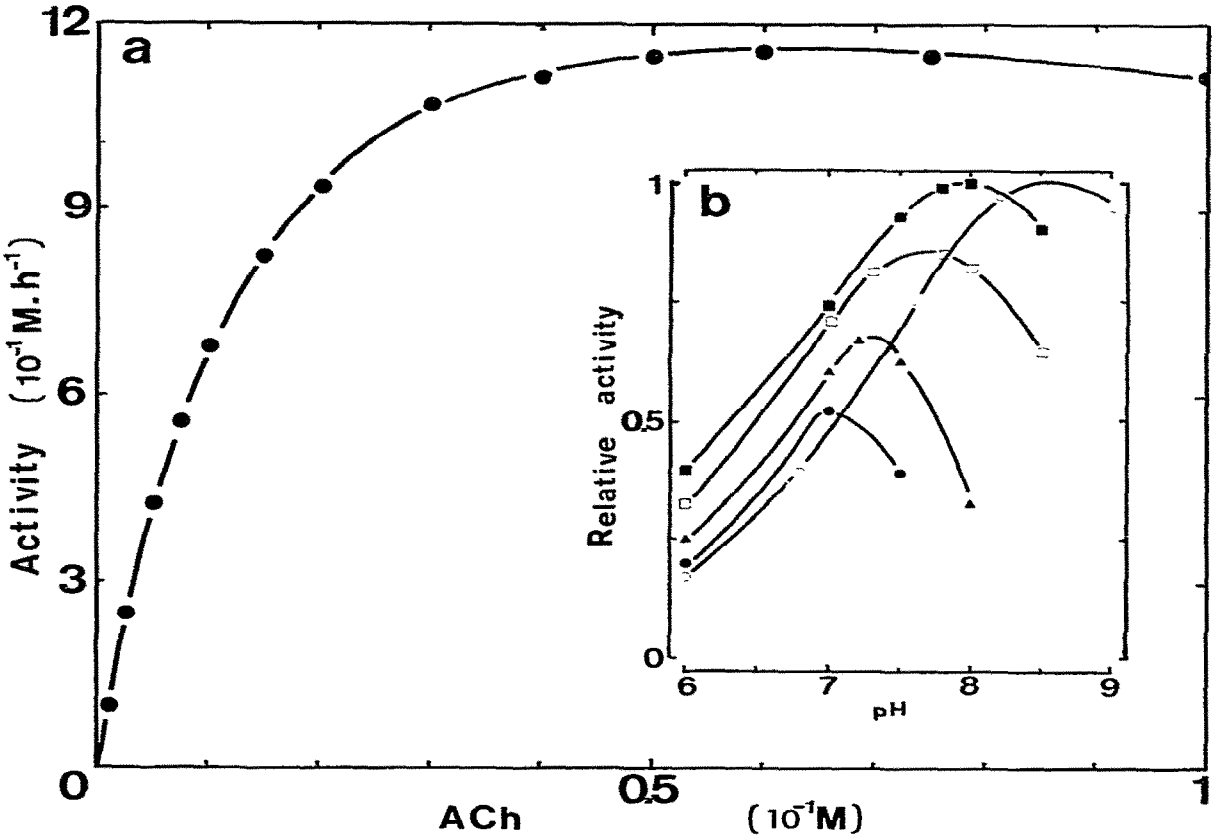

Fig. 1. Bovine serum albumin/acetylcholinesterase membranes. (a) Acetylcholinesterase activity in M/h as a function of acetylcholine (ACh) concentration in 10 mM phosphate buffer, pH 7.5. (b) The pH dependence of soluble (○) and immobilized electric eel acetylcholinesterase activity in the presence of 5 (●), 10 (△), 20 (□) and 40 mM (■) acetylcholine chloride. Membranes were produced with 10 I.U. acetylcholinesterase/cm^2 (1 unit hydrolyzes in solution 1 μM acetylcholine/min at pH 8.0 at 37°C).

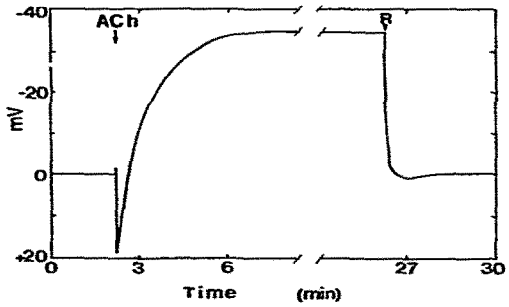

Fig. 2. Membrane potential vs. time after acetylcholine (ACh) injection in one compartment and rinsing (R), for acetylcholinesterase/ossein gelatin membrane. The buffer solution was 1 mM sodium phosphate, pH 7.5.

line chloride, the substrate, into only one compartment. The potential difference between the two sides of an artificial enzyme/gelatin membrane was recorded after asymmetric addition of the substrate (fig. 2). 6 min after acetylcholine injection, the system reaches a non-equilibrium steady state.

Measurements of potential were carried out under steady-state conditions as a function of injected substrate concentration, with and without acetylcholinesterase in ossein gelatin (fig. 3a), bovine serum albumin (fig. 3b) and pig skin gelatin (fig. 3c) membranes. The difference between the steady-state potential values for artificial mem-

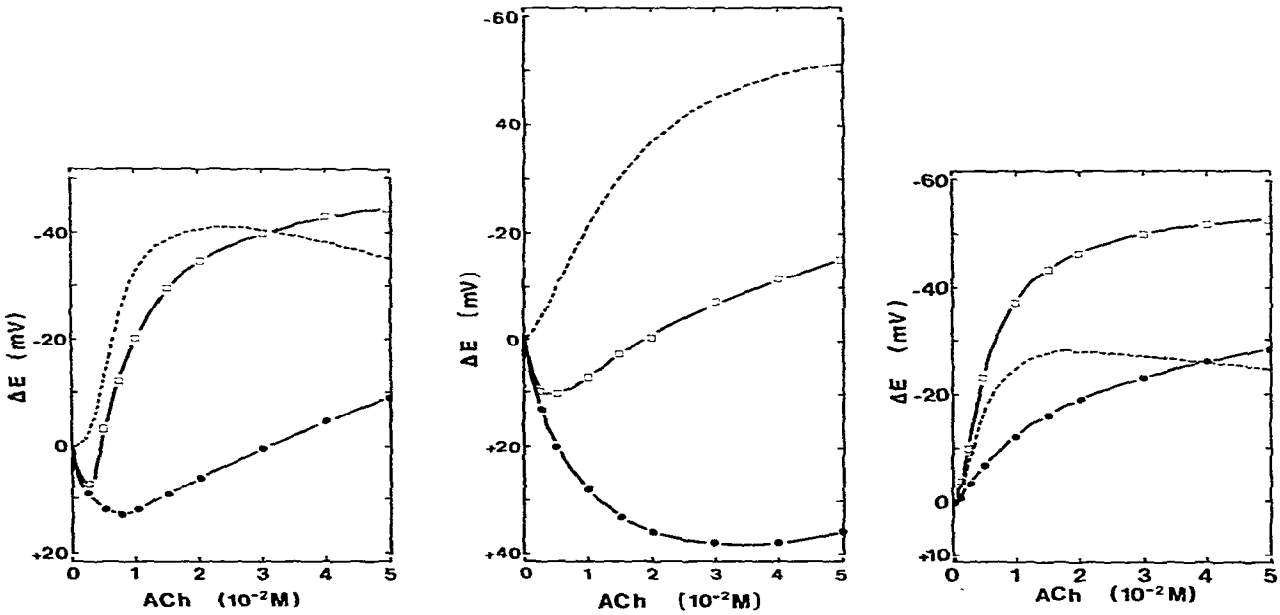

Fig. 3. Membrane potentials under steady-state conditions vs. acetylcholine concentration for active (□) and inactive (●) membranes. The curve due to the enzyme activity obtained by difference is given by the dashed lines. The buffer solution was 1 mM sodium phosphate, pH 7.5. Membranes were produced with ossein gelatin (a), bovine serum albumin (b) and pig skin gelatin (c).

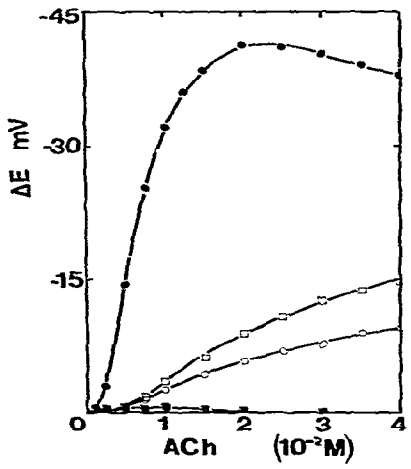

Fig. 4. Membrane potentials due to acetylcholinesterase activity under steady-state conditions as a function of acetylcholine concentration with different phosphate buffer concentrations (pH 7.5): (●) 10^{-3}, (□) 5×10^{-3}, (○) 10^{-2} and (■) 5×10^{-2} M.

branes with and without acetylcholinesterase gives the contribution of acetylcholinesterase activity to the potential difference as a function of the amount of injected substrate. In all cases, the contribution of the enzyme activity gives rise to a sigmoid shape with a decrease in the potential difference at high acetylcholine concentrations.

If the potential difference due to acetylcholinesterase is induced by a local pH change produced by acetylcholine hydrolysis, the use of concentrated buffer solutions might affect the membrane potential. Fig. 4 shows the potential difference due to enzyme activity for different phosphate buffer concentrations. The potential was strongly dependent on buffer strength; in strong buffers, the phenomenon became negligible.

Due to the pH dependence of acetylcholinesterase activity and to amphoteric properties of the proteinaceous membranes, the pH of external buffer solutions might also strongly affect the membrane potential. Serum albumin membranes

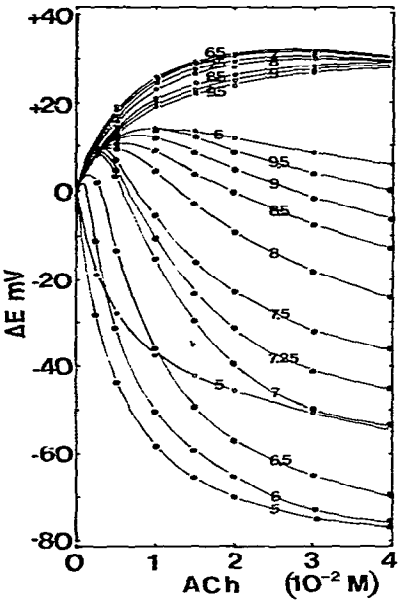

Fig. 5. Serum albumin membrane potentials under steady-state conditions as a function of acetylcholine concentration for active (●) and inactive (○) membranes. The pH values of external phosphate buffer (10^{-3} M) solution are indicated on the different curves.

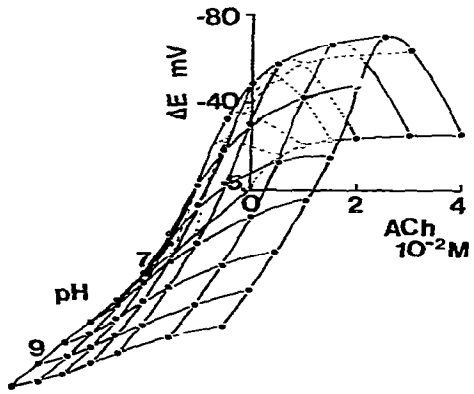

Fig. 6. Membrane potential due to enzyme activity as a function of both acetylcholine concentration and pH. Membranes were produced with bovine serum albumin and the buffer solutions were 1 mM sodium phosphate.

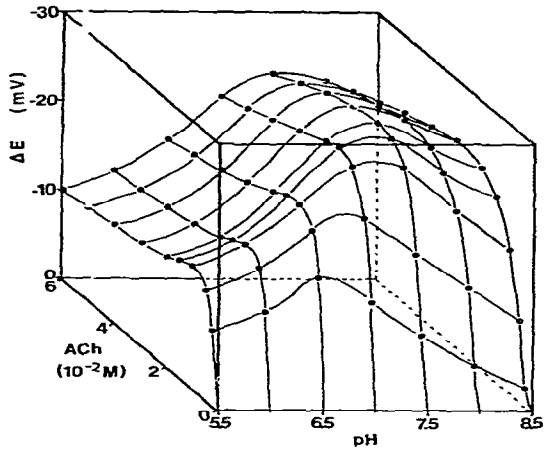

Fig. 7. Pig skin gelatin membranes. Membrane potential due to acetylcholinesterase activity as a function of both acetylcholine concentration and pH in 1 mM phosphate buffer.

with and without immobilized acetylcholinesterase were studied with 10^{-3} M buffer solutions at different pH values (fig. 5). The potential difference due to acetylcholinesterase activity as a function of both external pH and substrate concentration is shown in fig. 6. The apparent optimum pH value is 6 for low substrate concentrations and 6.5 for higher concentrations. The amphoteric properties of the inactive protein used may affect the potential difference. Membrane potentials due to enzyme activity as a function of both pH and acetylcholine concentration are shown in fig. 7 with pig skin gelatin/acetylcholinesterase membranes (isoelectric point approx. 7). In this case, the apparent optimum pH value is 6.5 for low substrate concentrations and 7.5 for higher ones. In both cases, the responses are sigmoid shaped at alkaline pH values but lose their sigmoidicity at low pH values.

4. Discussion

In the living cell, many enzymes are bound to membranous structures and function in a microenvironment which may differ from that which pertains in solution. Active artificial membranes are

useful for studying the reciprocal effects between an enzyme activity and its microenvironment in a well defined context. Silman and Karlin [17] and Silman [18] showed that the acetylcholinesterase activity of the membrane-bound enzyme exhibits an anomalous dependence on pH compared with the purified, soluble enzyme. Artificial acetylcholinesterase membranes show the same phenomenon. Apart from any direct influences of the carrier or of chemical binding on the enzyme itself, these differences are due to the changed microenvironment of the enzyme [19].

Acetylcholinesterase reactions producing H^+ generate pH gradients across the Nernst diffusion layers and within the matrix. Due to the amphoteric properties of the proteinaceous membrane, the local shift in pH modifies the ion mobilities. So, it is possible under asymmetric conditions to record an acetylcholinesterase-specific potential difference. The steady potential difference due to the enzymic activity vs. substrate concentration is a sigmoid function similar to the behaviour of some biological systems [20]. The coupling between a simple enzymic reaction and a structure is able to explain the 'cooperative' effect observed [4,7].

The hypothesis of the deviation of the local pH from the bulk solution pH was first experimentally supported by Goldman et al. [21] with artificial papain membranes. Silman and Karlin [17], with membrane-bound acetylcholinesterase, and Podleski and Changeux [22], with the depolarization of 'M-cells' bearing acetylcholinesterase, explained the behaviour of their systems by such a local change of pH at the membrane level, after hydrolysis of acetylcholine. A common feature of these studies was that the local change in pH could be overcome by concentrated buffer solutions. We show that the presence of concentrated buffers considerably decreases or even abolishes the potential difference due to the acetylcholinesterase activity.

The proteinaceous membranes offer amphoteric sites with weakly ionizable groups which give rise to ion-exchange properties varying with pH. Due to the cationic nature of the substrate, the apparent diffusion coefficient for acetylcholine is pH dependent. Under steady-state conditions, there is a balance between the reaction rate and the diffusion into or out of the bulksolutions. Both are influenced by the external solution pH and the acetylcholine chloride concentration. When using serum albumin as the inactive protein, the optimum pH value for the acetylcholinesterase-specific potential difference is a function of both external pH and substrate concentration. The contribution of the matrix ion-exchange properties is shown by using pig skin gelatin, whose isoelectric point is approx. 7, as an inactive protein. In this case, optimum pH values are shifted towards higher values.

In all cases the responses exhibit a sigmoid shape at alkaline pH values and become 'Michaelian' for pH values below or close to the inactive protein isoelectric point. This phenomenon can be explained by the coupling between diffusion and enzyme reaction. The increase in the apparent diffusion coefficient as the pH rises from alkaline values to the isoelectric point (data not shown) and the decrease in the reaction rate from pH 8.5 to 5 are due to decreasing diffusional constraints as the pH decreases.

5. Conclusion

This paper deals with aspects of the reciprocal interaction between enzyme activity and the microenvironment or the potential difference in artificial membranes. The study of artificial acetylcholinesterase membranes gives strong experimental evidence for the modulation of membrane potential by an intramembrane enzyme activity.

When acetylcholine was injected onto one side of an active artificial membrane, an enzyme-specific potential might have been recorded. So, an artificial system is chemically excitable in that it responds to the action of a specific molecule by characteristic modifications of the membrane potential.

The potential difference due to the acetylcholinesterase activity vs. substrate concentration is a sigmoid function at physiological pH values, similar to the behaviour of some biological systems [20].

The enzyme-specific potential is initiated by a

local change of pH at the membrane level, caused by hydrolysis of acetylcholine by acetylcholinesterase. This effect is strongly dependent on the buffer strength; in strong buffers the phenomenon became negligible.

The recorded potential difference is the result of the reciprocal interaction between enzyme reaction and the diffusion of substrate or products. By modifying the pH of the bulk solutions and using different inactive proteins, it is possible to modulated qualitatively and quantitatively the enzyme-specific response.

The next step of this work could be the re-introduction, in artificial acetylcholinesterase membranes, of specific molecules interacting with the enzyme in biological systems.

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