Electrical Phenomena Associated with the Activity of the Membrane-Bound Acetylcholinesterase

Abstract. *Treatment of isolated electroplax with physiological solutions supplemented with either 1 molar sodium chloride, 2 molar urea, or 2 molar sucrose renders the cell insensitive to carbamylcholine, phenyltrimethylammonium, or decamethonium even at high concentrations. The treated cells have a residual resting potential of* -20 ± 10 *millivolts (negative inside) and are depolarized by acetylcholine at concentrations larger than* 10^{-3} *mole per liter. This response is not affected by* d-*tubocurarine but is blocked by physostigmine, diisopropylphosphorofluoridate, or strong buffers and thus depends on the catalytic activity of the membrane-bound acetylcholinesterase.*

The monocellular electroplax from the electric organ of *Electrophorus electricus* constitutes one of the more reliable and simple preparations of chemically excitable membranes. A single cell is isolated from the bundle of Sachs and mounted between two chambers in such a way that the cell separates two pools of fluid, one of them bathing the innervated membrane, and the other bathing the noninnervated one; membrane potentials are recorded with intracellular microelectrodes filled with 3*M* KCl (*1*). In the normal cell, perfusion of the innervated side by solutions of compounds referred to as "receptor activators," like carbamylcholine (CCh), decamethonium (Dk), phenyltrimethylammonium (PTA), or acetylcholine (ACh) in association with physostigmine causes a decrease of the membrane potential from -75 ± 15 mv in the resting cell to a steady-state value which depends on the nature and the concentration of the compound, but is never lower than -15 ± 5 mv at saturating concentrations of activator. The action of receptor activators is antagonized by compounds, such as *d*-tubocurarine or flaxedil, referred to as "receptor inhibitors" (*2*).

In the course of studies with the isolated electroplax we observed that when the dissected cells are subjected in vitro to several treatments they become insensitive to a number of receptor activators although they still exhibit a resting potential and respond to high concentrations of ACh. The following treatments render the normal cells completely insensitive to CCh, PTA, and Dk: (i) incubation for about 1 hour in eel's physiological saline solution (*3*) supplemented with either 1 mole of NaCl, 2 mole of sucrose, or 2 mole of urea per liter; (ii) freezing in eel's physiological solution containing 5 percent glycerol followed by rapid thawing; (iii) aging in eel's physiological solution for 24 hours at 4°C. A reliable procedure is incubation for 1 hour in a solution of 1*M* NaCl in eel's physiological solution followed by an extensive washing (3 hours) in the normal physiological solution. The whole operation is carried out at room temperature.

A cell treated in such a manner (which will be referred to as a "modified" cell or M-cell) is no longer electrically excitable, but a potential of -10 to -30 mv (negative inside) with an average value of -20 mv can be recorded with standard intracellular microelectrodes filled with 3*M* KCl. This potential decreases to zero in the presence of physiological solutions containing 0.5 mole of NaCl or 0.5 mole of KCl per liter. Neither this potential nor the membrane resistance changes when the innervated side of an M-cell is perfused with a $10^{-2}M$ solution of CCh, PTA or Dk, although in a normal cell half maximum depolarization would have occurred in $3 \times 10^{-5}M$ CCh, $10^{-5}M$ PTA, or $10^{-6}M$ Dk. However, the M-cells are still depolarized when ACh is applied on the innervated side at concentrations greater than $10^{-3}M$. This response differs strikingly from that of the normal electroplax to ACh in that: (i) The action of ACh is not potentiated by inhibitors of acetylcholinesterase (AChE) like physostigmine ($5 \times 10^{-5}M$) and diisopropyl phosphorofluoridate (DFP) ($3.6 \times 10^{-4}M$); neither is the effect blocked by receptor inhibitors like *d*-tubocurarine and flaxedil ($10^{-3}M$) (Fig. 1). (ii) The membrane resistance is not modified by the presence of ACh, and there is no apparent limit for the change of potential when the ACh concentration is increased. The potential measured in the presence of ACh can be abolished, and in several experiments a reversal of the sign of the potential is observed (up to + 30 mv) (Fig. 1). These observations strongly suggest that in the M-cells the membrane structures are altered in such a way that the main site of action of the receptor activators seems to be either no longer functional or greatly modified, although these cells still preserve a residual resting potential and a residual response to ACh.

To proceed further in the identification of the site of action of ACh in the M-cells we tested various cholinesters. Of the four compounds assayed, acetylcholine, acetyl β-methyl choline, butyrylcholine, and carbamylcholine, only the first two show an effect (Fig. 1). Only these two are hydrolyzed at an appreciable rate by the membrane-bound acetylcholinesterase (*4*). An involvement of AChE in the depolarization of the M-cells by ACh is further suggested by the effect of specific AChE inhibitors. Physostigmine ($5 \times 10^{-5}M$)

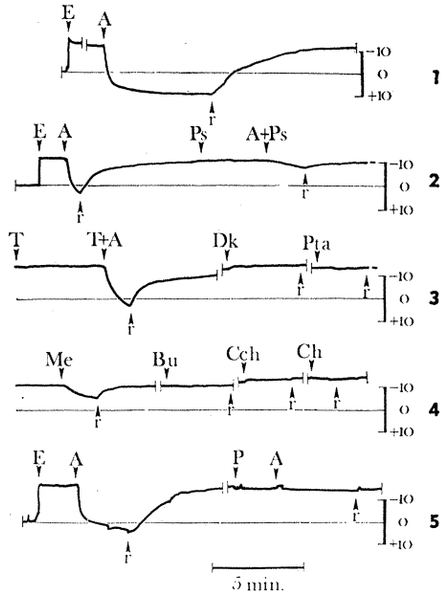

Fig. 1. Pharmacological properties of M-cells. Cells treated with 1*M* NaCl as indicated in the text. Abscissa, time; ordinate, potentials in millivolts. (1) Typical response to acetylcholine bromide (ACh): (*E*) empalement; (*A*) perfusion of the innervated side of the cell with $10^{-2}M$ ACh; (*r*) rinsing with eel's physiological solution. (2) Inhibition of the response to ACh by physostigmine; *Ps*, $5 \times 10^{-5}M$ physostigmine salicylate; *A* + *Ps*, $10^{-2}M$ ACh and $5 \times 10^{-5}M$ physostigmine. (3, 4) Effect of several receptor activators and inhibitors: *T*, $10^{-5}M$ *d*-tubocurarine chloride; *T* + *A*, $10^{-5}M$ *d*-tubocurarine and $10^{-2}M$ ACh; *Dk*, $10^{-2}M$ decamethonium bromide; *Pta*, $10^{-2}M$ phenyltrimethylammonium chloride; *Me*, $10^{-2}M$ acetyl β-methylcholine bromide; *Bu*, $10^{-2}M$ *n*-butyrylcholine iodide; *CCh*, $10^{-2}M$ carbamylcholine chloride; *Ch*, $10^{-2}M$ choline chloride. (5) Effect of phosphate buffer on the response to ACh. Beginning at *P*, both sides of the cell are perfused with eel's physiological solution containing $5 \times 10^{-2}M$ sodium phosphate buffered at *p*H 6.9; then a solution of $10^{-2}M$ ACh in the same medium (*A*) is added to the innervated side of the cell.

strongly inhibits the response of the M-cells to ACh (Fig. 1). Similarly, a 5-minute treatment of an M-cell with $3.6 \times 10^{-4}M$ DFP completely abolishes the sensitivity to ACh. Moreover, compounds like pyridine 2-aldoxime methiodide (2-PAM) which reactivate AChE inhibited by DFP (*5*), fully restore the sensitivity to ACh of M-cells previously incubated with DFP (Fig. 2). The depolarization of the M-cells by ACh is therefore associated with the catalytic activity of AChE.

Acetylcholinesterase catalyzes the hydrolysis of ACh to choline and acetic acid. Choline ($10^{-2}M$) has no effect on the potential recorded with the M-cells. On the other hand, since the pK of the carboxyl group of acetic acid is 4.75, hydrogen ions should be generated

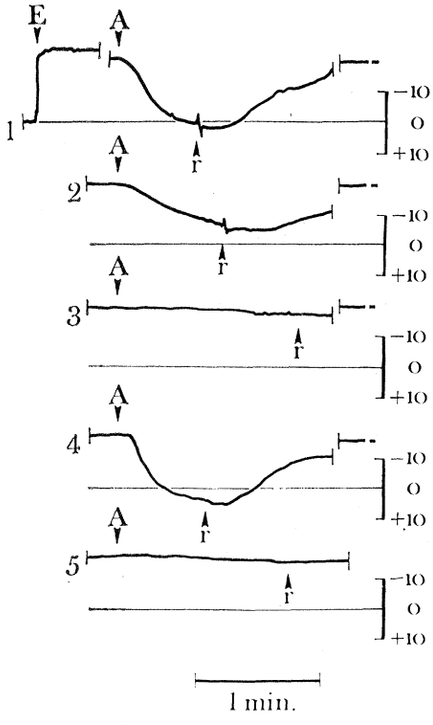

Fig. 2. Effect of diisopropylphosphorofluoridate (DFP) and pyridine 2-aldoxime methiodide (2-PAM) on the response of an M-cell to ACh. (1) Typical response to $10^{-2}M$ ACh; same abbreviations as Fig. 1. (2) The innervated side of the cell is incubated with $6 \times 10^{-6}M$ DFP for 5 minutes and then washed with eel's physiological solution for 5 minutes; then $10^{-2}M$ ACh is added (*A*). (3) Same experiment as in 2, but with $3.6 \times 10^{-4}M$ DFP. (4) Immediately after experiment 3, the innervated side of the cell is incubated with $10^{-3}M$ 2-PAM for 15 minutes, rinsed 5 minutes with eel's physiological solution; at *A* $10^{-2}M$ ACh is added. (5) Control: incubated in $3.6 \times 10^{-4}M$ DFP for 5 minutes and in eel's physiological solution for 30 minutes; (*A*) $10^{-2}M$ ACh is added. The experiment is performed with the same cell and with a single empalement.

by the liberation of acetic acid in our experimental conditions. Therefore we investigated whether the action of ACh on the M-cells is caused by a change in pH. No appreciable alteration of the membrane potential is recorded when the pH of the eel's physiological solution is lowered to pH 5.5. However, at pH 5.3 the potential starts to decrease at a rate of about 2.2 mv/min and reaches a steady value about 6 mv lower than the resting potential; at pH 3.0 the rate of depolarization becomes as large as 300 mv/min, and the steady potential obtained differs by about 30 mv from the potential measured at pH 6.9. In all these instances the inside of the cell becomes more positive as the external pH is lowered. These changes are reversible, and the normal potential can be restored by perfusion with physiological solution at pH 6.9.

A simple explanation of the depolarization of the M-cells by ACh would be that the pH of the perfusing solution drops below 5.0 as a result of AChE activity. This interpretation would seem unlikely considering the large volumes of solution applied to the cell (10 to 30 ml), the small size of the accessible cell surface (2 to 3 mm^2) and the rapidity of the measurements (few minutes). Nevertheless, the pH of the effluent solution that had been in contact with the cell was measured and was not found to differ from the pH of the initial solution (pH 6.9).

An alternative explanation would be that a pH change occurs locally at the membrane level or even inside the cell and that this change of pH directly or indirectly causes the decrease in potential. An excellent model of such an effect is offered by the artificial membranes made of papain molecules cross-linked on a collodion matrix (*6*). When the papain membranes are incubated with benzoyl-L-arginine methyl ester, a papain substrate, the internal pH of the membrane drops as a result of the steady accumulation of hydrogen ions liberated by the substrate hydrolysis. A characteristic property of these membranes is that the local change in pH can be overcome by concentrated buffer solutions. These observations, recently extended by H. I. Silman and A. Karlin (*7*) on the basis of enzymatic studies to preparations of membrane-bound AChE, prompted us to study the effect of concentrated buffers on the depolarization of the M-cells by ACh. In the presence of eel's physiological solution (*3*) where an equivalent amount of NaCl has been replaced by

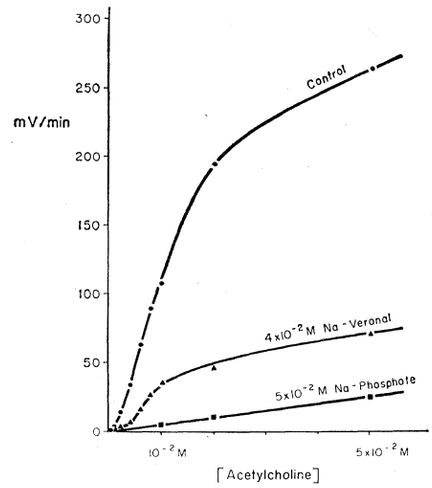

Fig. 3. Effect of buffers on the response of an M-cell to ACh. Maximal rates of depolarization are plotted as a function of acetylcholine concentrations. (●) Eel's physiological saline solution; (▲) same medium with $4 \times 10^{-2}M$ sodium veronal (pH 6.9); (■) same medium with $5 \times 10^{-2}M$ sodium phosphate (pH 6.9).

0.04M sodium veronal buffer (pH 6.9) the effect of ACh on the M-cells is considerably decreased or even completely abolished (Fig. 3). The same result is observed with 0.05M sodium phosphate buffer and 0.05M glycyl-glycine buffer (pH 6.9). None of these buffers, except sodium veronal, antagonizes the effect of CCh or ACh on the intact cell: they affect selectively the action of ACh on the M-cells.

Thus we conclude that (i) the depolarization of the M-cells by ACh is initiated by a local change of pH at the membrane level after hydrolysis of ACh by the membrane-bound AChE; (ii) the change of pH is caused by the accumulation of hydrogen ions within diffusion barriers surrounding the AChE molecules integrated into the membrane structure.

The ionic mechanisms which account for the potential of the M-cells and for its change upon ACh hydrolysis are not yet fully understood. A simple hypothesis is that the microelectrode simply records a pH difference between the inside of the membrane or of the cell and the external medium. The results of the following experiment make this interpretation unlikely. An indifferent electrode is immersed in a physiological solution at pH 6.9 while the pH of the solution surrounding a microelectrode filled with 3M KCl is shifted from 6.9 to 3.1. The change in potential recorded between both electrodes is close to 7 mv and cannot account for the 30- to 60-mv changes in

potential caused by ACh in the M-cells. The hypothesis that a hydrogen-ion gradient established across the membrane is per se the cause of the potential difference cannot be ruled out on the basis of our results. Another interpretation would be that local changes of *p*H trigger indirectly the membrane depolarization by altering the microenvironment and consequently the properties of the macromolecular structures which support the membrane potential.

Several physiological implications of these results can be mentioned: (i) Diffusion barriers are interposed between the external medium and the membrane receptors and might play an important role in the response of cell membranes to their regulatory ligands. (ii) Several sites of action might be involved in the response of a cholinergic membrane to ACh, and the active site of AChE might be one. In this respect the effect of ACh on the M-cells presents several analogies with the response of various conductive membranes to ACh (*8*). (iii) In spite of the rather drastic treatment to which the cells are subjected, they still respond to ACh, and thus some of their constitutive macromolecules are still functional. Studies with alternative treatments and other specific ligands may provide pertinent insight into the molecular mechanisms which control the electrical parameters of these membranes.

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3. Composition of eel's physiological saline solution: 160 mmole of NaCl, 5 mmole of KCl, 0.3 mmole of $NaH_2PO_4 \cdot H_2O$, 1.2 mmole of Na_2HPO_4, 2 mmole of $MgCl_2 \cdot 6H_2O$, 2 mmole of $CaCl_2$, and 10 mmole of glucose per liter, *p*H 6.9.
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Hemoglobin Gun Hill: Deletion of Five Amino Acid Residues and Impaired Heme-Globin Binding

Abstract. *Hemoglobin Gun Hill, a new variant of adult hemoglobin, was found in a Caucasian and one of his three daughters. The abnormal hemoglobin had only half of the expected number of heme groups. Five amino acid residues appeared to be missing from the β-globin chains. These residues occur in linear sequence in normal β-chains in a region involved in heme-globin binding. A deletion of five amino acids in the β-chains of hemoglobin Gun Hill is postulated. The most likely mechanism for the origin of such a hemoglobin variant would appear to be unequal crossing-over during meiosis.*

The human hemoglobins (Hb) are conjugated proteins which contain four globin chains, each with an attached heme group. There is no evidence to suggest that any of the described abnormal human hemoglobins deviate from this gross structure. With the exception of Hb H (β_4) and Hb Barts (γ_4) the hemoglobins contain two unlike pairs of globin chains. The normal prototypes are Hb A $\alpha_2 \beta_2$, Hb A_2 $\alpha_2 \delta_2$, Hb F $\alpha_2 \gamma_2$.

We now report a newly found hemoglobin, designated hemoglobin Gun Hill (Hb GH), which lacks the normal complement of heme groups. There appears to have been a deletion of five amino acids in a region of each β-globin chain which functions in heme-globin binding.

The propositus was a 41-year-old man of remote German and English ancestry who had had mild jaundice from early adolescence. He had splenomegaly and signs of a compensated hemolytic state. An 8-year-old daughter also had compensated hemolysis, but lacked splenomegaly. Both the father and daughter were heterozygous for the abnormal hemoglobin. Two other daughters did not have the abnormal hemoglobin and showed no signs of hematologic disease.

Starch-gel electrophoresis (*1*) revealed two abnormal components in addition to hemoglobin A in the hemolyzates of the affected individuals (Fig. 1). The major abnormal band migrated in the position of Hb A_2; a minor abnormal component occupied a slightly more anodal position. The two abnormal hemoglobins comprised about one-third of the total hemoglobin, an estimate based upon the heme absorption of the components eluted after electrophoretic separation on starch granules (*2*), corrected for the abnormal ratio of heme to globin in Hb GH.

Our report concerns studies of the major abnormal component, which had been separated from Hb A_2, and the minor component by carboxymethylcellulose chromatography (*1*), followed by electrophoresis on starch granules.

Half of the globin chains in Hb GH lack heme groups. The concentrations of the cyanmethemoglobin derivatives of Hb GH and Hb A were determined at 540 mμ [molar extinction (ε) being 4.37×10^4] and confirmed by measurement of the pyridine hemochromogen (*3*). The protein concentrations of these solutions were calculated from values of protein nitrogen obtained by the Kjeldahl method (*4*). In Hb A, the two methods for the estimation of protein concentration gave comparable results; the value based upon the Kjeldahl nitrogen determination averaged 103 percent (range 92 to 110 percent) of the concentration obtained by the spectrophotometric method. In Hb GH, the Kjeldahl method yielded values twice those obtained when concentration was estimated from the optical absorption of the heme groups, with an average of 205 percent (range 198 to 220 percent).

The absorption spectrum of Hb GH reflected the abnormal ratio of heme to globin. A greater optical absorption in the ultraviolet region relative to the absorption at the visible wavelengths was observed in Hb GH when compared to Hb A. The shapes of both the oxyhemoglobin and the cyanmethemoglobin absorption spectra of Hb GH in the region 350 to 700 mμ were identical to those of Hb A.

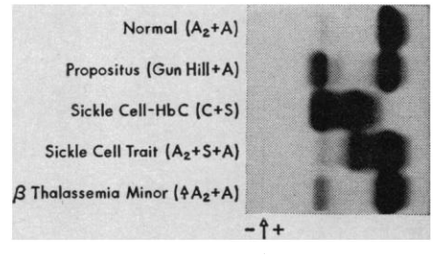

Fig. 1. Starch-gel electrophoresis in tris, boric acid, EDTA buffer at *p*H 8.6, stained with benzidine. Two abnormal components are evident in the hemolyzate of the propositus: a major component in the position of Hb A_2, and a minor component with slightly more anodal migration. Hemoglobin A_2, present in the major abnormal band, could be separated from the Hb GH component by carboxymethylcellulose chromatography.