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The Possible Role of Fixed Membrane Surface Charges in Acetylcholine Release at the Frog Neuromuscular Junction

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Summary. Bass and Moore [*Proc. Nat. Acad. Sci.* **55**:1214 (1966)] proposed that the vesicles containing acetylcholine undergo Brownian motion in the nerve terminals. Acetylcholine is released whenever a vesicle touches the inner face of the axolemma of the nerve terminal. The frequency at which contact is made is limited by an energy barrier that must be overcome before the vesicle can touch the axolemma. The energy barrier has two components. (1) An electrostatic repulsion between positive, fixed charges on the vesicles and a relatively positive potential at the face of the axolemma that is generated by the resting potential. (2) A layer of water molecules held to the vesicle by the surface charge. This model is inconsistent with experimental data. A modification of the model is presented. Both the vesicle and the inner face of the axolemma are assumed to have fixed, negative surface charges that are responsible for the energy barrier. By a series of simplifications, the model leads to two predictions. (1) A plot of the ln (miniature end plate potentials/sec) as a function of the concentration of ions in the axoplasm)$^{-0.5}$ should give a straight line. (2) A plot of ln(end plate potential amplitudes) as a function of (extracellular Ca^{++} concentration)$^{-0.5}$ should give a straight line. These predictions are shown to agree reasonably well with experimental data.

At the neuromuscular junction the transmitter, acetylcholine (ACh) is almost certainly stored in membrane-bounded vesicles in the motor nerve terminal. When an action potential invades the terminal several hundred vesicles release their contents into the extracellular fluid. Probably each vesicle fuses with the inner face of the axolemma and a channel is formed between the interior of the vesicle and the extracellular space. The liberated ACh interacts with the muscle end plate to elicit a depolarization, the end plate potential (e.p.p.) (Katz, 1969). The release of quanta of ACh from the nerve is a markedly nonlinear function of $[Ca^{++}]_o$; plots of log (e.p.p. amplitude) as a function of log ($[Ca^{++}]_o$) in Ringer's solution of normal osmolarity give straight lines with slopes ranging between 3 and 4 (Jenkinson,

1957; Dodge & Rahamimoff, 1967). The usual interpretation is that several Ca^{++}'s act cooperatively to trigger quantal ACh release.

Even when the nerve is not being stimulated there is an occasional spontaneous release of quantum of ACh, which generates a miniature end plate potential (min.e.p.p.). The rate of spontaneous release is markedly increased by raising the osmolarity of the Ringer's by adding NaCl or sucrose: a 50% increase in osmolarity may produce a 45-fold rise in min.e.p.p. frequency (Fatt & Katz, 1952).

Bass and Moore (1966) proposed a model to account for both the effects of changes in osmolarity and of the nerve action potential on quantal ACh release. In this paper we will summarize their model, show how it is inconsistent with certain facts, and will then introduce a variation on their model that will be tested by comparing its predictions with empirical data.

Materials and Methods

Grass frogs, *Rana pipiens*, were obtained from a commercial supplier and kept in tanks at room temperature until used.

The sciatic nerve-sartorius muscle preparation was dissected from a pithed frog. The muscle was pinned to encapsulating resin on the bottom of an acrylic plastic chamber and was stretched to about 120% of its slack length. The nerve was led into a second chamber where it rested across a pair of platinum stimulating electrodes and was covered with a mixture of vaseline and mineral oil to prevent drying. The end plate was located by following out a branch of the nerve as far as possible under the dissecting microscope, and then inserting a microelectrode several times at points along the fiber to find the point with the fastest rise-time of the e.p.p. or the min.e.p.p.

Glass microelectrodes were filled with 3 M KCl; D-C resistances ranged from 5 to 20 MΩ. An Ag-AgCl pellet in the bath served as the second electrode. The microelectrode led to a W-P Instruments electrometer amplifier.

The basic Ringer's solution contained 100 mM NaCl, 2.0 mM KCl, 2.5 mM $CaCl_2$, 3.0 mM $MgCl_2$, and 8.0 mM tris maleate buffer, pH = 7.4 (Danforth & Helmrich, 1964). When recording min.e.p.p.'s, 1.0 μg/ml neostigmine methyl sulfate (Sigma) was added to increase amplitudes. In some experiments the e.p.p. amplitude was depressed by using 1.0 to 3.0 μg/ml D-tubocurarine chloride (Schwartz-Mann) or 7 to 15 mM $MgCl_2$. Solutions were made hypertonic by adding sucrose (usually Schwartz-Mann ultra-pure). The osmolarity was determined by the freezing point depression, measured with a Fiske osmometer.

When recording e.p.p.'s the nerve was stimulated once every 2 sec with a 0.2-msec pulse that was 33 times supra-maximal. Two hundred or more e.p.p.'s were photographed for each observation. The electrode was kept at the same end plate site throughout the experiment. Fibers with membrane potential of more than −80 mV were chosen and the experiment was discontinued if the decrease in membrane potential exceeded 10% of the initial value except when studying only min.e.p.p. frequency.

The bath contained about 5 ml of solution. Solutions were changed by running in 50 ml of fresh solution, removing the excess with continuous aspiration. The inflow rate was 11 ml/min. Recording was usually begun 3 min after the completion of solution change.

Theory, Results, and Discussion

The Model of Bass and Moore

Suppose that the vesicles move in the axoplasm with Brownian motion, so their thermal energy is distributed according to the Boltzmann expression. To release its quantum of ACh the vesicle must first contact the inner face of the axolemma. Before contact can be made there is an energy barrier B that must be overcome. Only those vesicles with sufficient thermal energy to surmount the barrier can reach the axolemma and release their contents. The vesicle making contact with the axolemma is assumed to be the rate-limiting step in release. Therefore the rate of quantal release v is given by

$$v = v_0 \cdot \exp(-B/kT) \tag{1}$$

where v_0 is the total frequency at which vesicles approach the axolemma, k is Boltzmann's constant, and T is the absolute temperature (we prefer to think of v_0 as the total frequency at which vesicles that are oriented so they can release their contents approach a release site on the inner face of the axolemma). Then

$$\frac{\partial(\ln v)}{\partial B} = -1/kT = -U. \tag{2}$$

Bass and Moore (1966) suggest that there may be two major components to the energy barrier: electrokinetic B_e and hydration B_h. The total energy barrier is the sum, $B = B_e + B_h$. The two barriers are generated in their model as follows.

1. *Electrokinetic.* They suppose that a substantial fraction of the voltage drop, measured as the membrane potential, occurs in the adjacent solutions, rather than in the substance of the membrane itself. The fraction of the total resting membrane potential that will exist as a voltage drop in the axoplasm just beneath the membrane a is given by

$$a = \frac{\varepsilon_m}{\varepsilon \kappa \delta} \tag{3}$$

where ε_m is the dielectric constant of the membrane of thickness δ, ε is the dielectric constant of the axoplasm, and κ is the Debye reciprocal length parameter (m^{-1})

$$\kappa = \frac{8\pi c e^2 z^2}{\varepsilon kT} \tag{4}$$

where e is the charge on the electron (C), and c is the concentration of ions (moles/liter) of valence z.

For an appreciable fraction of the total resting potential to consist of a voltage drop in the axoplasm the dielectric constant of the membrane must be greater than that of the axoplasm adjacent to the membrane [*see* Eq. (3)]. Bass and Moore (1966) assume that ε_m is somewhat less than the dielectric constant of water (80) and that ε for the axoplasm just adjacent to the membrane is about 3. On the basis of these assumptions there is a substantial excess of negative charges in the axoplasm just beneath the axolemma, so the inner face of the axolemma is electrically positive compared to the bulk of the axoplasm. The vesicles are assumed to have a positive fixed surface charge, owing to an excess of cationic groups on their surface. There will be a repulsion between the two positively charged surfaces. The fixed surface charge on the vesicle will produce an electrical double layer in the surrounding axoplasm, by attracting anions and repelling cations. Therefore, the electrical potential will decrease relatively gradually as one moves from the surface of the vesicle into the axoplasm. This means that a repulsive force should be generated as soon as a vesicle approaches roughly within 30 to 60 Å of the inner face of the axolemma, and the repulsion will increase as the distance between the vesicle and the membrane is closed (Fig. 1*A*).

There is no doubt that repulsion between membranes with surface potentials of like sign would occur, but there are difficulties with the way in which Bass and Moore (1966) suppose that the potential on the inner face of the axolemma is generated, and in their assumption about the sign of the surface charge on the vesicles. The values they choose for the dielectric constants of the membrane and for the adjoining axoplasm seem unlikely. There is no reason to assume that the dielectric constant of a membrane made largely of lipids and proteins can come anywhere close to that of water. They argue that the dielectric constant of water in the layer adjacent to the membrane will be greatly reduced, because the water molecules will be held in a relatively fixed position by interaction with the membrane. The same argument has been used to suggest that the dielectric constant of the water surrounding an ion in solution will be greatly reduced, because the water molecules are held in a fixed position in the intense electric field set up by the ion. However, detailed calculations suggest that the dielectric constant 2 Å away from an ion is only reduced by about 17%, so in practice it is best to assume that the dielectric constant is that of ordinary water (Schellman, 1957). The possibility that an appreciable fraction of the potential drop will occur in the solutions adjacent to a membrane has been con-

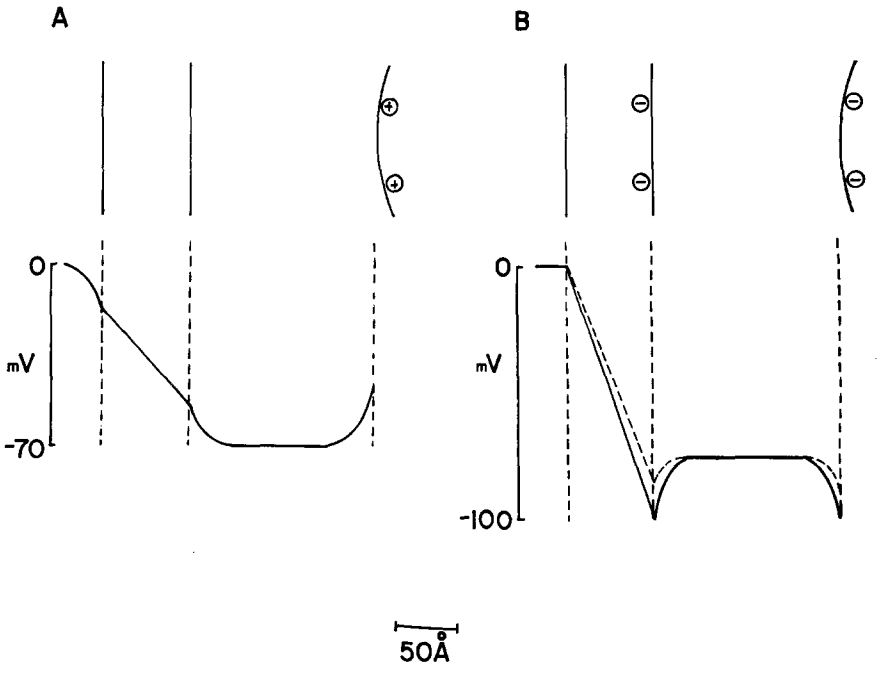

Fig. 1. (*A*) *Upper:* Diagram of the model for the regulation of the quantal release of ACh proposed by Bass and Moore (1966). The cell membrane (left) has no net fixed surface charge. The vesicle membrane (right) has a positive fixed surface charge. These charges hold water molecules in a layer surrounding the vesicle; some of the water must be stripped away before the vesicle can touch the membrane. *Lower:* Diagram of the electrical potential as a function of distance in the upper diagram. An appreciable portion of the resting potential appears as a voltage drop in the solutions adjacent to the membrane (*see text*). Therefore, the inner face of the axolemma is relatively positive compared to the axoplasm. There will be an electrostatic repulsion between the vesicle and the axolemma. (*B*) *Upper:* Diagram of the modified model. The inner face of the axolemma and the vesicle have fixed, negative surface charges (the fixed negative charges that exist on the outer face of the axolemma have been ignored to simplify the diagram). Both membranes have shells of water molecules that must be removed before the membranes can make contact. When an action potential invades the terminal, Ca^{++} enters and binds to specific anionic fixed charges at or near release sites on the axolemma, reducing the surface potential. *Lower:* Diagram of the electrical potential profile in the resting nerve terminal. The inner face of the axolemma and the vesicle are negative because of the fixed charges. The potentials fall away exponentially as a function of distance from the membranes. The dotted lines show the effect of doubling the ion concentration of the axoplasm. The magnitude of the surface potentials is reduced, because of screening of the fixed charges

sidered by Walz, Bamberg and Lauger (1969); they conclude that with physiological concentrations of ion only a small fraction of the potential drop would be in the solution.

Although there is a report that vesicles within the nerve terminal through which a steady current is being passed move as though they had a positive surface charge (Landau & Kwanbunbumpen, 1969), in these experiments the mitochondria also behave similarly. But isolated mitochondria are known to have a negative surface charge (Vos, Kuriyama & Roberts, 1968), as is the case with other biological membranes that have been studied. Even if one accepts a positive charge on the vesicles, there is a well-known experimental observation that rules out the idea that the inner face of the axolemma is relatively positive owing to the resting membrane potential. In a Ca^{++}-free Ringer's solution an action potential can invade the nerve terminal without accelerating quantal release (Katz & Miledi, 1965). The action potential would certainly cause a reversal of sign in a charged layer just beneath the axolemma that is generated by the membrane potential, so their theory clearly predicts a nonexistant acceleration of release. It is evident that an electrical barrier generated by the membrane potential does not play any substantial role in governing quantal release.

2. *Hydration.* Because of the fixed surface charge on the vesicles, Bass and Moore (1966) suggest that there will be a layer of water molecules with a taffy-like consistency held close to their surface, because the water molecule behaves as an electrical dipole. At least part of this water shell must be stripped away before the vesicle can touch the inner face of the axolemma. Changes in osmotic pressure might alter this hydration barrier by varying the cohesion of the water molecules. The difficulty with this part of the model is that increases in osmotic pressure produced by adding the permeant solute, glycerol, to the Ringer's solution produce only slight, transitory increases in min.e.p.p. frequency (Furshpan, 1956; Hubbard, Jones & Landau, 1968*b*). Apparently the effects of hyperosmolar solutions depend upon the presence of impermeant solutes that shrink the nerve terminals and thereby increase the concentration of ions within the cells. In sum, the electrokinetic barrier proposed by Bass and Moore does not affect ACh release, and their model for the action of changes in osmolarity does not agree with the data.

A Second Model Based on Resting Membrane Potential

Remler (1973) has also considered the possibility that the resting potential generates a layer of excess negative charges along the inner face of the axolemma that might be important in regulating the approach of vesicles. Using assumptions about dielectric constants similar to those of Bass and Moore (1966), but a different approach to the calculation, he estimates the

surface potential at the inner face of the axolemma as about 0.12 V. Since this is many times greater than the total resting potential one immediately suspects that something is wrong with the assumptions. He then assumes that the vesicles have a negative fixed surface charge, but somehow concludes that there will be a repulsion between the negatively charged vesicle and the surface of the inner face of the axolemma, which in his model will be positive compared to the axoplasm (In spite of this problem, his paper contains some interesting calculations on how a steady intracellular current might affect the distribution of vesicles in the terminal. This subject will not be discussed further here).

A Fixed Surface Charge Model

Most, if not all, biological membranes have a net negative surface charge, because of the anionic groups on the phospholipids (*see*, for example, Camejo, Villegas, Barnola & Villegas, 1969). Synaptic vesicles have been shown by electrophoresis to have a negative surface charge (Vos *et al.*, 1968). Electrophysiological experiments are consistent with a surface potential on the inner face of the squid axon of about -17 mV (Chandler, Hodgkin & Meves, 1965). If vesicles and axolemma have surface potentials of the same sign there will be an electrical repulsion between the two structures (Fig. 1 *B*) (Blioch, Glagoleva, Liberman & Nenashev, 1968; Kita & Van der Kloot, 1971). Increases in the ion concentration in the axoplasm, produced by hypertonic extracellular solutions, will screen the charges on the membranes, reducing the surface potentials, thereby decreasing the repulsion and making it easier for the two membranes to contact one another. If Ca^{++} enters the terminal following an action potential, it might bind to the anionic sites on the inner surface of the axolemma and momentarily reduce the surface potential. We shall now derive some predictions based on this model.

Predicted Effects of Changes in Axoplasmic Ion Concentration

The repulsive force B_e between two spheres with constant fixed surface charges can be estimated by an equation derived by Wiese and Healy (1970):

$$B_e=\frac{\varepsilon a_1 a_2\{\psi_1(0)^2+\psi_2(0)^2\}}{4(a_1+a_2)}\left[\frac{2\psi_1(0)\cdot\psi_2(0)}{\psi_1(0)^2+\psi_2(0)^2}\right.$$
$$\left.\cdot\ln\left\{\frac{1+\exp(-\kappa l)}{1-\exp(-\kappa l)}\right\}-\ln\{1-\exp(-2\kappa l)\}\right] \tag{5}$$

where ε is the dielectric constant (F/m), a_1 and a_2 are the radii (m) of spheres with surface potentials at infinite distance from one another of $\psi_1(0)$ and

$\psi_2(0)$ (V), l is the shortest distance between the surfaces of the spheres (m), and κ is the Debye reciprocal length parameter (m^{-1}). To approximate the case of a vesicle within the nerve terminal, we take the axolemma as a sphere of very large radius, so $a_1 \gg a_2$, and for simplicity assume that $\psi_1(0) = \psi_2(0)$, then

$$B_e = \frac{\varepsilon a_v \psi(0)^2}{2}\left[\ln\left\{\frac{1+\exp(-\kappa l)}{1-\exp(-\kappa l)}\right\} - \ln\{1-\exp(-2\kappa l)\}\right] \tag{6}$$

where a_v is the radius of a vesicle, about 250 Å, and

$$\frac{\partial B_e}{\partial\{\psi(0)^2\}} \simeq \frac{\varepsilon a_v}{2}\left[\ln\left\{\frac{1+\exp(-\kappa l)}{1-\exp(-\kappa l)}\right\} - \ln\{1-\exp(-2\kappa l)\}\right] = V. \tag{7}$$

To develop some rough quantitative predictions, we shall make two additional approximations. We assume that the radius of the vesicle a_v is not altered by changes in osmolarity. We also assume that changes in the concentration of ions in the axoplasm exert their major effect on the magnitude of the surface potential; the effects of variations in the Debye reciprocal length [Eq. (4)] will be ignored.

In a symmetrical electrolyte solution the surface potential on a planar surface is related to the surface charge by the Gouy expression

$$\sinh\left(\frac{zF\psi(0)}{2RT}\right) = \frac{136\sigma}{c^{0.5}} \tag{8}$$

where c is the concentration of electrolyte in the bulk solution (moles/liter), R is the gas constant (joules · °K^{-1} · moles^{-1}), T is the absolute temperature (°K), σ is the surface charge (charges/Å^2), and the factor 136 has units of (moles/liter)$^{0.5}$ · Å^2 · charges^{-1}. When $\sinh(x) < 1$, $\sinh(x) \simeq x$. Then

$$\frac{\partial\{\psi(0)\}}{\partial(1/c^{0.5})} \simeq \frac{2RT \cdot 136\sigma}{zF} = W \cdot \sigma. \tag{9}$$

If there are two membranes, each with the same surface charge density

$$\frac{\partial\{\psi(0)^2\}}{\partial(1/c)} \simeq W^2 \cdot \sigma^2. \tag{10}$$

Consequently, if an electrical repulsion between the fixed surface charges in the membranes play a major role in determining the rate of quantal release,

so $B \simeq B_e$

$$\frac{\partial(\ln v)}{\partial(1/c)} \simeq \frac{\partial(\ln v)}{\partial B_e} \cdot \frac{\partial B_e}{\partial\{\psi(0)^2\}} \cdot \frac{\partial\{\psi(0)^2\}}{\partial(1/c)} \simeq -U \cdot V \cdot W^2 \sigma^2. \tag{11}$$

This means that a plot of ln(min.e.p.p./sec) as a function of $(1/c)$ should show that the data points fall on a straight line. We have assumed that intracellular concentrations c are equal to half of the osmolarity of the extracellular solution, which was determined by the depression of the freezing point. When our data was plotted on these coordinates the points clearly did not fit the predicted line. This might mean that theory as we have developed it contains too many simplifying assumptions [like the linearizations and geometrical approximations involved in Eq. (7)] or that electrostatic repulsion between the membranes does not play a rate-determining role in ACh release.

Fixed charges on a membrane will hold water molecules in an oriented layer adjacent to the membrane. Some of these water molecules must be stripped away before the membranes could touch one another. Remler (1973) suggested that the magnitude of this hydration barrier B_h can be approximated by

$$B_h \simeq \frac{m \cdot \psi(0) \cdot n_w}{d} \tag{12}$$

where d is the distance a water molecule must be moved to take it out of the first layer next to the membrane into the second layer, a distance of roughly 5 Å, n_w is the number of water molecules that must be shifted, and m is the dipole moment of the water molecule $(C \cdot M)$. Then

$$\frac{\partial B_h}{\partial\{\psi(0)\}} \simeq \frac{m n_w}{d} = X \tag{13}$$

and therefore if $B \simeq B_h$

$$\frac{\partial(\ln v)}{\partial(1/c^{0.5})} \simeq \frac{\partial(\ln v)}{\partial B_h} \cdot \frac{\partial B_h}{\partial\{\psi(0)\}} \cdot \frac{\partial\{\psi(0)\}}{\partial(1/c^{0.5})} \simeq -U \cdot X \cdot W \cdot \sigma. \tag{14}$$

Consequently, plots of ln(min.e.p.p./sec) against $(1/c^{0.5})$ should give straight lines. Fig. 2 shows some of our data (Kita & Van der Kloot, 1971) plotted on these coordinates. The fit of the points to the predicted line is good. Therefore, the data fit well to the predictions of a model based on the idea that changes in osmolarity of the Ringer's solution alter the frequency of spontaneous releases by changing the ion concentration in the nerve termi-

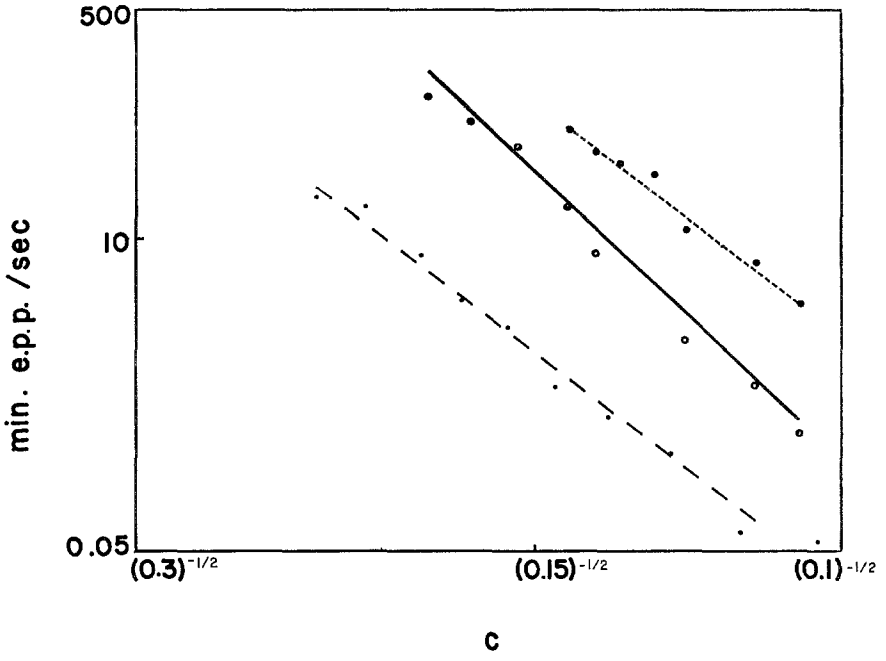

Fig. 2. Three examples, each from an experiment on a single end plate in the frog sartorius muscle, of the effects of changing the tonicity of the Ringer's solution on min.e.p.p. frequency. The reasons for the choice of the coordinates are given in the text. --- slope $= -6.6$, $r = 0.989$. —— slope $= -8.5$, $r = 0.995$. — — — slope $= -6.8$, $r = 0.992$

nals. The ion concentration determines the surface potentials generated by fixed charges. The surface charges are responsible for the major barrier for quantal release, perhaps by holding water molecules close to the membrane of the vesicles and close to the release sites on the axolemma. The next step is to see what further predictions one can make from the surface charge model and to see whether or not the data fit the predictions.

Predictions about the Effect of Ca^{++} Entry on Release Rates

When the nerve is depolarized in the presence of normal Ca^{++} (2.5 mM) Ringer's fluid there is a great increase in the rate of quantal release. Suppose that the action potential allows Ca^{++} to enter the nerve terminal, and that over the range of concentrations usually studied (roughly 0.1 to 3.0 mM) the amount that enters is directly proportional to $[Ca^{++}]_0$. The Ca^{++} that enters the terminal can bind to anionic groups (A^-) near release sites on the inner face of the axolemma

$$Ca^{++} + 2A^- \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Ca\,A_2 \qquad (15)$$

so

$$[A^-] \simeq \left(\frac{k_2[\mathrm{Ca}A_2]}{k_1[\mathrm{Ca}^{++}]}\right)^{0.5}$$

and as long as $[\mathrm{A}^-] \gg [\mathrm{Ca}A_2]$

$$\frac{\partial([A^-])}{\partial([\mathrm{Ca}^{++}]^{-0.5})} \simeq \left(\frac{k_2[\mathrm{Ca}A_2]}{k_1}\right)^{0.5} = Y \tag{16}$$

since σ is proportional to $[A^-]$

$$\frac{\partial\{\psi(0)\}}{\partial\sigma} \simeq \frac{2RT \cdot 136}{zF \cdot c^{0.5}} = \frac{W}{c^{0.5}}. \tag{17}$$

Since we assume the $[\mathrm{Ca}^{++}]_{in}$ is directly proportional to $[\mathrm{Ca}^{++}]_0$

$$\frac{\partial(\ln v)}{\partial([\mathrm{Ca}^{++}]_0^{-0.5})} \simeq \frac{-U \cdot X \cdot Y \cdot W}{c^{0.5}}. \tag{18}$$

Plots of ln(e.p.p. amplitude) or of ln(quantal output) as a function of $([\mathrm{Ca}^{++}]_0^{-0.5})$ are shown in Fig. 3. The data, taken from three different sources, give a satisfactory fit to the prediction.

As shown in Eq. (18), the relation between release rate and $[\mathrm{Ca}^{++}]_0$ is inversely proportional to the square root of the concentration of ions in the axoplasm. An increase in the osmolarity of the Ringer's solution should decrease the slope of the line produced by plotting ln(e.p.p. amplitude) as a function of $([\mathrm{Ca}^{++}]_0^{-0.5})$. In normal Ringer's, the slope of the line (λ_r) is about 4 (Fig. 3). According to the theory, the slope in hypertonic Ringer's, λ_h,

$$\lambda_h = \frac{\lambda_r \cdot c_r^{0.5}}{c_h^{0.5}} \tag{19}$$

where c_r is the estimated intracellular concentration in normal Ringer's and c_h is the estimated intracellular concentration in hypertonic Ringer's. In Ringer's solution +180 mM sucrose the slope was about 2.9; the predicted slope is also 2.9. For the two examples in Ringer's +220 mM sucrose the slopes were 2.4 and 2.1; the predicted slope is 2.7. The effects of the hypertonic solutions may be somewhat greater than predicted by the straightforward theory. This is easy to understand since the model assumes that there are only univalent ions in the exoplasm. If there are also divalent ions,

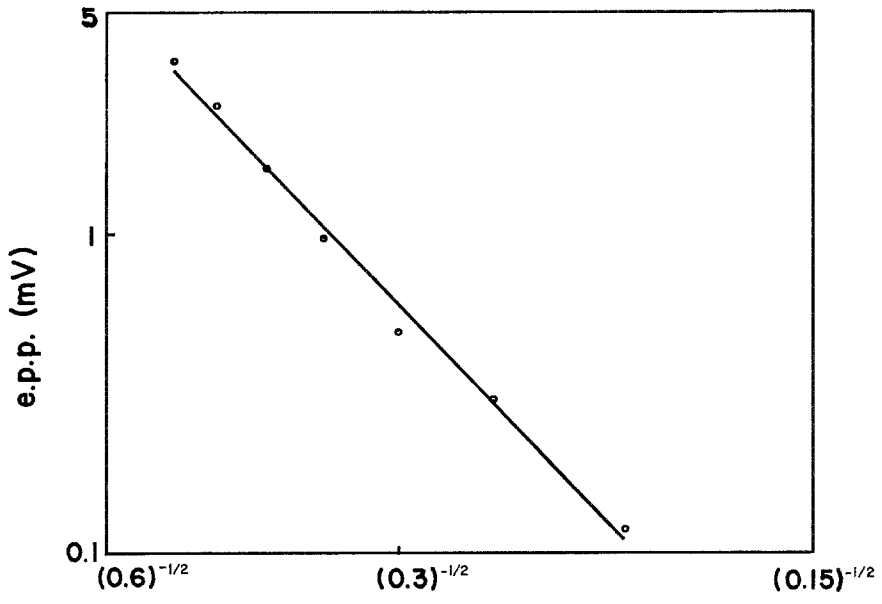

Fig. 3 *A*

Fig. 3. Three examples of the effects of changing $[Ca^{++}]_0$ on quantal release from stimulated nerve terminals. (*A*) At the frog neuromuscular junction, data from Dodge and Rahamimoff (1967). The Ringer's solution contained 1.0 mM $MgCl_2$. Slope $= -4.1$, $r = 0.996$. (*B*) At the rat neuromuscular junction, data from Hubbard, Jones and Landau (1968*a*, Fig. 2). The calculated quantal output m represents the mean value for from 6 to 14 junctions. Tubocurarine chloride was added to prevent the e.p.p. from reaching threshold for setting up action potentials. Slope $= -2.3$, $r = 0.990$. (*C*) From a frog neuromuscular junction in Ringer's solution containing 0.5×10^{-6} g/ml of tubocurarine chloride; slope $= -4.1$, $r = 0.983$. In four similar experiments on the effects of changing $[Ca^{++}]_0$ in hypertonic Ringer's, the mean $r = 0.993 \pm 0.006$ (SD)

such as Mg^{++}, they will have a greater screening effect and surface potentials in hypertonic Ringer's solution will be lower than predicted by the theory. In general, we feel that the fit between the theory, with all of its obvious simplifications, and the data is good.

Effect of Extracellular $[Ca^{++}]$ *on Release Rates*

There is a further prediction that can be made and tested. Ca^{++} also plays a role in determining the rate of spontaneous release. When the $[Ca^{++}]_0$ is reduced there is a marked decrease in min.e.p.p. frequency (Blioch *et al.*, 1968; Kita & Van der Kloot, 1971; Quastel, Hackett & Cooke,

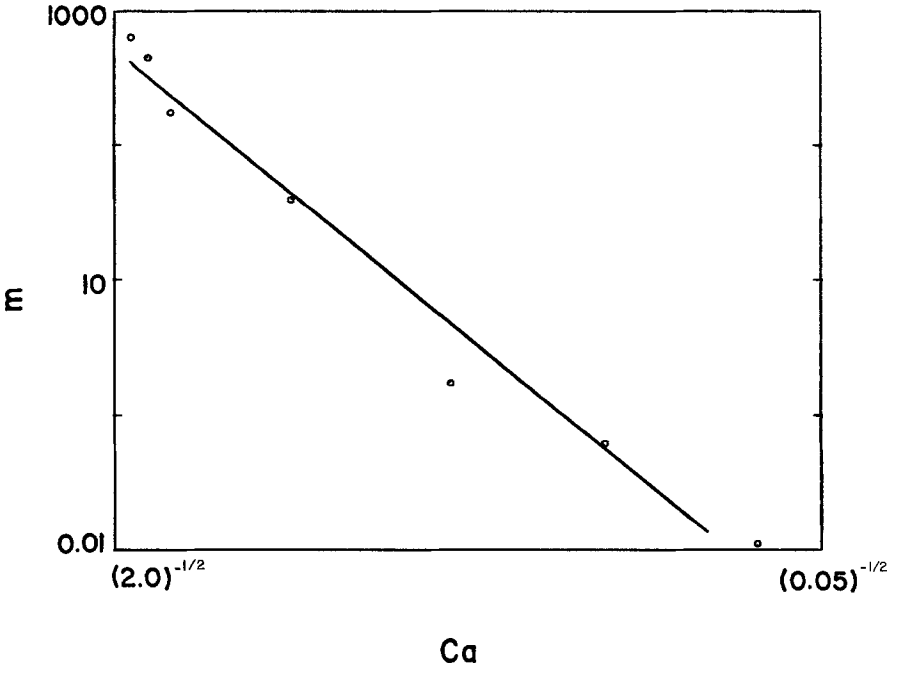

Fig. 3*B*

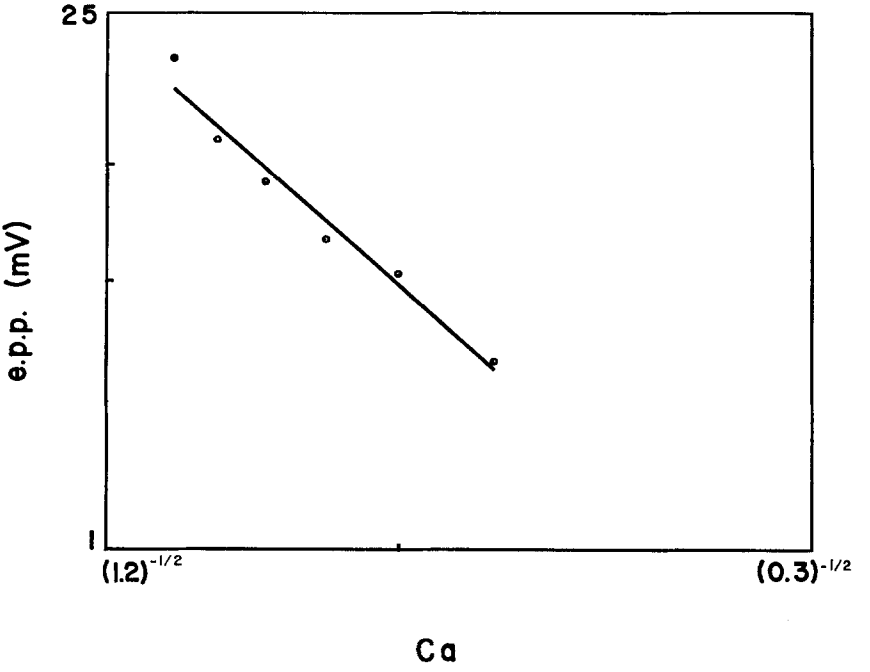

Fig. 3*C*

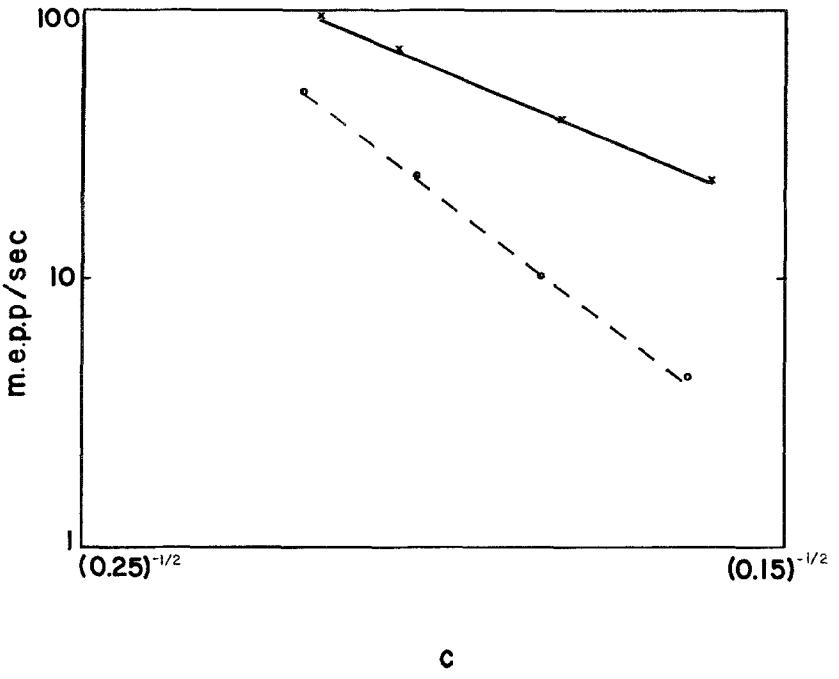

Fig. 4. Recording from a single junction in the frog sartorius muscle in Ringer's solution made without the addition of Ca^{++}, —— (slope = − 4.3, $r = 0.999$) and in the same Ringer's solution containing 1.0 mM MgEGTA, — — — (slope = − 7.7, $r = 0.999$)

1971). It seems reasonable to suppose that normally there is a slow Ca^{++} influx into the terminals. This Ca^{++} might well bind specifically to certain of the fixed anionic groups near release sites on the inner face of the membrane, lowering the surface potential and increasing the release rate. In low Ca^{++} Ringer's there should be an increase in the surface charge σ. Plots of ln(min.e.p.p./sec) as a function of $(1/c^{0.5})$ in low Ca^{++} solutions should have a more negative slope [Eq. (18)]. Examples of these experiments are shown in Fig. 4. The min.e.p.p. frequency was determined at several different osmolarities, first in Ringer's made without adding Ca^{++} and then in the same Ringer's with the addition of 1 mM MgEGTA [ethyleneglycol bis(aminoethylether)-N,N′-tetra-acetic acid] which will bring the Ca^{++} to a still lower concentration. The slope in Ca^{++}-free Ringer's is −4.3; in MgEGTA Ringer's the slope is −7.7 (Fig. 4). In a second experiment the slope changes from −8.8 in Ca^{++}-free Ringer's to −10.0 in MgEGTA Ringer's. It is also notable how well the data from Ca^{++}-deficient Ringer's fit to the predicted straight lines: in eight experiments the correlation coefficients were 0.987 ± 0.015 (mean ± SD).

Effects of Highly Hypertonic Solutions

In solutions at tonicities above 400 mosmoles/liter, further increases in osmolarity do not produce further increases in min.e.p.p. frequency, and may even cause a decrease. The reasons for this effect are unknown; they are not predicted by the model. The calculated correlation coefficients are all for Ringer's solutions below 400 mosmoles/liter.

Obviously, this is not the only aspect of the effects of changes in tonicity that the present simplified theory cannot account for. For example, in strongly hypertonic Ringer's there is a block in neuromuscular transmission, owing to a decrease in the number of quanta released by the stimulated nerve (Thesleff, 1959). When the concentration of ions on the axoplasm is increased there will be a slowing in the rate of the reaction

$$Ca^{++} + 2A^{-} \overset{k_1}{\rightarrow} Ca\,A_2$$

because the increased ion concentration in the solution screens the charges on the reactants from one another (Glasstone, Laider & Eyring, 1941); for the reaction shown above

$$\ln k_1 = \ln k_0 - 4.6\,c^{0.5} \tag{20}$$

where k_1 is the rate constant for the reaction in a solution with a concentration of univalent ion c, and k_0 is the rate constant in an infinitely dilute solution. Raising the concentration of ions from 0.1 to 0.2 M will reduce the rate constant to about half of its former value. This is equivalent to lowering the $[Ca^{++}]_0$ to about half of the normal level. Such a reduction in $[Ca^{++}]_0$ will not decrease quantal release from a stimulated nerve to anything approaching the decrease caused by doubling the osmolarity (in the range of concentrations in which release is depressed by osmolarity). So the decrease in the rate of reaction between entering Ca^{++} and the fixed anions could not account for all of the blocking effects of strongly hypertonic solutions on stimulated release. Perhaps the decrease in stimulated release is related to the decrease in min.e.p.p. frequency observed above 400 mosmoles/liter. Further experiments are obviously needed. Our model also fails to account for the transitory effects of changes in osmolarity found at the mammalian neuromuscular junction (Hubbard *et al.*, 1968*b*).

Effects of Temperature

Hubbard (1970) suggested that models of the type devised by Bass and Moore can be rejected because they predict a simple and straightforward

relation between temperature and min.e.p.p. frequency. In mammals, the relation between temperature and min.e.p.p. frequency is far from simple (Hubbard, Jones & Landau, 1967). As the temperature is raised the min.e.p.p. frequency increases until a peak is reached at about 16 °C. At temperatures between 18 and 20 °C there is a decline in frequency. At temperatures above 30 °C there is an increase in frequency with a rise in temperature; the increase is very marked between 34 and 38 °C. Nevertheless, we do not believe that these complexities permit a firm rejection of a physical theory. They could be accounted for by phase transitions in the structure of the membranes themselves or in the structure of water in the layers adjacent to the membrane. There is reason to believe that the water next to a membrane will undergo abrupt structural changes at temperatures between 13 and 16 °C and also between 30 and 32 °C (Drost-Hansen, 1971).

Conclusions

We have taken ideas about the possible role of membrane surface charges in regulating ACh release (Bass & Moore, 1966; Blioch *et al.*, 1968; Remler, 1973) and have derived concrete predictions about the effects of changes in osmolarity and of $[Ca^{++}]_0$ on release rates. The derivations are clearly based on broad simplifications. Nonetheless, the data appears to fit well with a model in which the vesicles undergo Brownian motion, and only those with sufficient thermal energy to overcome a barrier can reach a release site on the inner face of the axolemma. This barrier is the rate-limiting step in quantal release. The major component of the barrier is generated by fixed surface charges on the membranes. Increases in intracellular ion concentration, produced by hypertonic extracellular solutions, will screen the fixed charges, lower the surface potentials, reduce the energy barrier, and increase the spontaneous release rates. When the nerve is stimulated, Ca^{++} enters the terminal and binds to anionic groups near or at release sites on the inner face of the axolemma, thereby reducing the surface charge and accelerating quantal release. The effects of changes in tonicity on spontaneous release, and of changes in $[Ca^{++}]_0$ and osmolarity (below 400 mosmoles/liter) are consistent with this model. One of the attractions of the model is that it can be easily tested further; to give only one possibility, lowering the pH in the nerve terminal should decrease the surface charge and increase the frequency of spontaneous release.

It has been suggested that the major role of surface charge in preventing contact between vesicles and axolemma is by holding layers of water mole-

cules close to the membranes (Bass & Moore, 1966; Remler, 1973). As far as we are aware, there is too little information about the solvation energies of phospholipid membranes and the part played by membrane charges in holding the water to firmly support this conclusion. Alcohols, which are effective in lowering the solvation energies of lyophylic colloids (Shaw, 1970), increase the rate of quantal release (Gage, 1965; Okada, 1967; Quastel *et al.*, 1971). But since these agents also alter membrane permeabilities and interfere with active Ca^{++} transport (Van der Kloot, 1970), the present evidence is insufficient to permit a conclusion on this point.

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