A Mechanism for Discharge of Charged Excitatory Neurotransmitter

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ABSTRACT Excitatory neurotransmitter is charged, so that emptying of a transmitter-containing vesicle (discharge) would seem to require considerable energy. Even if the energy problem is surmounted and discharge thereby made possible, there is still a problem of making the discharge fast enough (considerably less than 1 ms). Proposed here is a mechanism wherein discharge of charged transmitter is accompanied by the influx of cocharged ions or coefflux of counter-charged particles (ion interchange). It is shown theoretically that ion interchange obviates the necessity for a separate energy source and can provide the observed rapid vesicle discharge.

INTRODUCTION

It is via regulated exocytosis of neurotransmitter-containing synaptic vesicles that nerve cells communicate with each other or with muscle cells. In nerve terminals the time that elapses from the stimulus by an action potential until the beginning of release (minimum delay) is about 0.5 ms at room temperature (see review by Parnas and Parnas, 1994). During this time several events take place, the last of which is the passage of the transmitter through a fusion pore that links the vesicle to the cell exterior. Khanin et al. (1994) provided considerable theoretical evidence that diffusion cannot account for discharge from synaptic vesicles in fast synapses, notably because diffusive discharge is too slow compared to the minimum delay. In addition, Alvarez de Toledo et al. (1993) showed that for large granules in slowly releasing systems, diffusive discharge is not consistent with the observed relationship between the time of the discharge and the radius of the granules.

How much faster than the minimum delay is the duration of discharge? This question was answered by Khanin et al. (1994), who showed that, regardless of the mechanism of discharge, to generate a sufficiently high (mM range) concentration of transmitter in the vicinity of the postsynaptic receptors (Matthews-Bellinger and Salpeter, 1978; Land et al., 1980), the duration of discharge must be about 100 μs in fast synapses.

The results of Khanin et al. (1994) concerning the inadequacy of diffusion as a discharge mechanism were obtained under the assumption that transmitter bears no electric charge. However, excitatory neurotransmitters are charged— acetylcholine (ACh^+) positively and glutamate negatively. Vesicles containing ACh^+ also contain negatively charged adenosine triphosphate (ATP^{3-}). Thus, in the case of ACh^+ the secretant could be neutral if it were discharged in the form of a complex with a negatively charged intravesicular molecule such as ATP^{3-}. Redman and Silinsky (1994) indeed suggested that there is corelease of ACh^+ and ATP^{3-}. However, a neutral $3ACh^+$-ATP^{3-} complex would diffuse even more slowly than the ACh-sized neutral particle that was studied by Khanin et al. (1994). The fact that synaptic discharge cannot take place via pure diffusion of neutral transmitter thus casts considerable doubt on the existence of a neutral complex. In what follows we therefore consider the discharge of charged neurotransmitters.

Here we study the possibility that the discharge of excitatory transmitters in fast synapses can be driven by a mechanism wherein charged transmitter molecules exchange places with like-charged coions. We will use the term *ion interchange* to describe this mechanism. We also study a closely related scenario in which there is a coefflux of transmitter and counter-charged particles. We sometimes loosely use "ion interchange" to describe both alternatives.

Considerable evidence that movement of other charged particles accompanies discharge of (charged) secretants from granules was obtained by Uvnas and Aborg (1984a,b) and Uvnas et al. (1985) in their experiments with chromaffin and mast cells. They proposed that such charge movements are the basis of an ion exchange mechanism for discharge from both large granules and small vesicles (Uvnas, 1973; Uvnas and Aborg, 1984b), but they did not embody their ideas in a formal theory.

We will formulate and analyze a mathematical model to investigate semiquantitatively whether the ion interchange mechanism can provide sufficiently rapid discharge. Our goal is to obtain an order of magnitude estimate for the duration of discharge and to study the effect of the key parameters on this duration. We will concentrate on the frog neuromuscular junction and acetylcholine-containing vesicles, for relevant information is most complete in that system. If information is not available for the frog, when permissible we employ data from other systems.

Analysis of our model shows that if the discharge of charged secretants through a fusion pore were unaccompanied by any other movements of charge, the potential dif-

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ference across the pore (the transpore potential) would change in such a way as to rapidly block discharge. By contrast, if efflux of charged transmitter is accompanied by influx of cocharged ions (and/or efflux of counter-charged particles) then the transpore potential transiently takes on an intermediate value so as to accelerate both the outward transmitter movement and the inward movement of coion. Rapid vesicle discharge is thereby obtained.

BIOLOGICAL BACKGROUND

We will now provide the biological background necessary to quantitate the various assumptions that we will make in the course of developing our model.

Contents of the vesicle

Vesicles from the frog neuromuscular junction have an internal radius of 18.5 nm (Heuser and Reese, 1973) and contain a concentration of ~300 mM acetylcholine (Kelly and Hooper, 1982). Synaptic vesicles also contain considerable amounts of ATP, 20–50% of the ACh concentration, and much less GTP (Wagner et al., 1978). There are small amounts of metal ions (Schmidt et al., 1980). Perhaps there are some proteins such as proteoglycan (Kelly and Hooper, 1982), but the total amount of internal protein does not seem significant and there is no dense core (Parsons et al., 1993). Charged gels might be present (Nanavati and Fernandez, 1993), but they are not taken into consideration here.

State of the vesicle neurotransmitter

Using NMR proton analysis of isolated cholinergic vesicles from *Torpedo marmorata*, Stadler and Fuldner (1980) showed that the vesicle content is in an essentially fluid state (see also Parsons et al., 1993). If so, the high contents of the vesicle could lead to high osmolarity. However, it can easily be calculated that if ACh and ATP, at the concentrations indicated above, are the main contents of the vesicle that are in a free state, the total osmolarity in the vesicle would be about 0.8 osmol, which is the same as the osmolarity in the surrounding medium (Kelly and Hooper, 1982). We may conclude that osmolarity considerations do not hamper the possibility of the vesicular transmitter, being mostly in an unbound fluid state. We assume that the *Torpedo* findings also hold for frog nerve terminals.

Fusion pore size and conductance

It is commonly assumed that the mechanisms of fusion and fusion pore formation are similar in different preparations (Monck and Fernandez, 1992; Zimmerberg et al., 1993). Therefore, in the absence of detailed information concerning pore conductance in synaptic vesicles, we employ results from measurements in granules of other secreting cells. In mast cells Spruce et al. (1990) found an initial pore conductance of approximately 300 pS. After its first rapid opening the pore expands, so that its conductance increases at an average rate of 200 pS/ms for a few milliseconds. Subsequently the pore can flicker for a while, and then completely and irreversibly open, or close again.

Curran et al. (1993) measured the same value for early pore conductance; Nanavati and Fernandez (1993) obtained a higher value, 500 pS. Values of 300 pS or lower were also obtained in mouse and guinea pig eosinophils (Lindau et al., 1995; Hartmann and Lindau, 1995). Especially important are measurements in smaller granules from human neutrophils, whose radius of 100 nm is considerably less than the 350-nm radius of mast cells. In neutrophils a mean value of 150 pS was obtained (Lollike et al., 1995). Given these observations, in our basic model we will consider the pore to open instantaneously, yielding a conductance of 300 pS. We neglect pore dilation, because the latter is far too slow to have any significant effect on a discharge that lasts for about 0.1 ms. For the dimensions of the pore we employ values of Spruce et al. (1990), who estimated the pore length to be 10–15 nm (two membrane thicknesses) and the pore radius to be about 1 nm. The difficulty of making any simple theoretical generalizations about the conductances is proved by the fact that channels (and therefore, it is reasonable to assume, narrow pores) can be highly selective, conducting very differently ions such as sodium and potassum, whose free diffusivities are virtually identical.

Selectivity of the fusion pore

Secretants of all charges and sizes, from small synaptic vesicles to large granules, are all discharged through fusion pores. If the mechanism of pore formation is the same in different systems, then it is reasonable to assume that this pore is nonselective. We took the value 300 pS measured by Spruce et al. (1990) as a basic estimate for the nonspecific conductance of the pore.

We do not explicitly take into account the fact that the radius of the pore is only twice as large as the characteristic radius of charged transmitter molecules, even though wall hindrance has a significant effect on diffusive discharge, typically slowing diffusion by a factor of 4 (Khanin et al., 1994). The reason is that directed movement of charged particles in a channel is much less sensitive to the size of the solute molecules than diffusion. According to Deen (1977), if the pore radius is twice the radius of transmitter molecules, for example, then a transmitter molecule moves in the pore with a velocity that is 1.3 times less than its velocity in an unbounded fluid in an electric field of the same magnitude.

Initial transpore potential

Angel and Michaelson (1981) reported that in *Torpedo* the electrical potential of cholinergic synaptic vesicles is negative inside (−80 mV) with respect to the cell interior. The potential across the cell membrane of −70 mV gives an

initial transpore potential difference of $\Phi - \Phi^{(e)} = -150$ mV (see Fig. 1 for notation).

For the possibly related case of large granules in mast cells, Breckenridge and Almers (1987) found highly variable positive vesicle potential (inside) with respect to the cell interior, from 10 mV to 160 mV (i.e., a transpore potential difference ranging from −60 mV to 90 mV). In view of the uncertainty in the measurements of potential, we will examine a range of initial transpore potentials. Our calculations will show that the initial value of the transpore potential is not important for the time course of discharge. Within about a microsecond, the potential difference across the fusion pore reaches a value that is independent of the initial potential difference.

FORMULATION OF THE MODEL

To check the proposed idea for the ion interchange discharge of excitatory neurotransmitter, we will now formulate a mathematical model. The essence of the model is shown in Fig. 1. An ACh-containing spherical vesicle of radius r is connected by an initially narrow nonselective transmembrane pore to the synaptic cleft. At rest the counterion ATP^{3-} balances the positive charge of ACh^+ in the vesicle. As was mentioned in the section on biological background, fusion pores have a narrow radius (in the period of interest) that is comparable to the radii of ion channels. Thus we can base our analysis on conventional approaches to describe ionic flows through channels where the flows are driven by electrochemical gradients. Accordingly, there exists a transpore potential difference, $\Phi - \Phi^{(e)}$, and a transpore conductance, g. In our basic model there is an outward current of transmitter ACh^+ and an inward current of the major extracellular coion Na^+. (Other ions will be considered shortly.) The changing values of the vesicle potential Φ are related to these currents via the capacitance of the vesicle membrane.

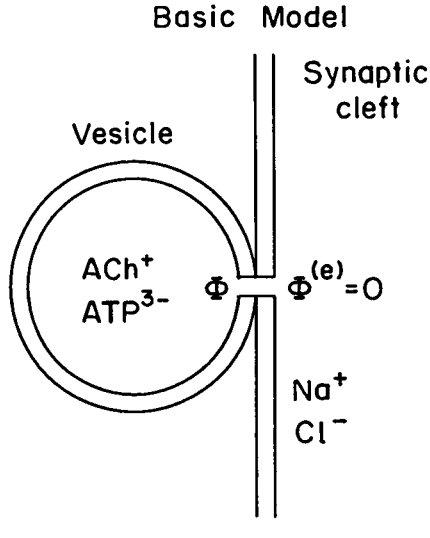

FIGURE 1 Schematic view of the synaptic vesicle connected to the plasma membrane by a narrow fusion pore. The vesicle contains charged neurotransmitter (for definiteness taken to be ACh^+) and complementary charges (ATP^{3-}). Significant concentrations of coion (Na^+) and counterion (Cl^-) are present extracellularly. The transpore potential is $\Phi - \Phi^{(e)} = \Phi$.

We denote the outward current of species j through the pore by I_j. I_j depends on the concentration of all permeable species and on the transpore potential difference, $\Phi - \Phi^{(e)}$. We have taken the potential values at the pore ends to be equal to those in the cell exterior ($\Phi^{(e)}$) and vesicle interior (Φ), respectively. (Note that because outward current is taken to be positive, the transpore potential is indeed given by $\Phi - \Phi^{(e)}$ and not by $\Phi^{(e)} - \Phi$.) The potential of the cell exterior, $\Phi^{(e)}$, can be approximated as a constant, which without loss of generality is taken to be zero. Thus the changes of the transpore potential are due to the changes of the intravesicle potential, Φ, only.

As was justified in our study of diffusive discharge (Khanin et al., 1994), we assume that equilibration of species concentrations in the vesicle occurs rapidly compared to the overall time scale of the process (100 μs). Thus we will consider average concentrations of species in the vesicle. We will assume that all nonneutralized charge is at the surface of the vesicle, an assumption that should be adequate for the small vesicles being considered here. We thus regard the vesicle as a spherical capacitor (with capacitance κ); hence we can express the charge, Q, of the vesicle as $Q = \kappa\Phi$. The vesicle charge changes, owing to fluxes of all permeable species into and out of the vesicle:

$$\frac{dQ}{dt} = -I_T, \qquad I_T \equiv \sum_j I_j. \tag{1a}$$

Here I_T is the total current of all species moving through the fusion pore. Thus, because $Q = \kappa\Phi$,

$$\kappa\frac{d\Phi}{dt} = -\sum_j I_j. \tag{1b}$$

The capacitance, κ, of a vesicle of radius r is calculated by multiplying the vesicle surface area by the capacitance per unit area of biological membrane, β: $\kappa = 4\pi\beta r^2$.

What current-voltage relationship should be assumed for the fusion pore? As with channels, the passage of particles through these pores is a highly complex process that will be influenced by "dielectric forces, local dipoles, and local charges that would make peaks and valleys in the potential profile" (Hille, 1991, p. 347). There is no universal agreement concerning an accurate model for such situations, and even if there were, such a model would be highly complex and would require unavailable information on the structure of the pore and the mechanism of flow through it. In any case, for a proof-of-principle paper such as the present one, the right course is to utilize a simple model that captures the essential qualitative features of the phenomenon in question. To obtain a sensible first-approximation model, we adopt the essence of channel theory, and assume that each

ion moves independently through the pore; interference effects are neglected.

There is nothing qualitatively new in our independence assumption when like-signed ions are considered; in perfectly selective channels identical ions move in opposite directions, whereas in unselective pores this motion is executed by different ions having the same charge. The independence assumption is riskier for the situation when oppositely signed ions move through the pore. However, the only simple alternative hypothesis in this case seems to be the assumption that the oppositely charged particles merge to make a neutral entity—which brings us back to the too-slow diffusion of neutral particles that we mentioned in the Introduction.

To describe each presumably independent ionic current through the pore, we will adopt the conventional Ohm's law, which gives an excellent first approximation for currents through many channels, albeit not for all of them (Hille, 1991). In its standard form Ohm's law has been the bedrock of neurophysiological calculations for decades. We use this formalism in a somewhat more general situation than standard calculations, for more than a single type of ion has significant flux through the pore. We neglect interactions within the pore between the different ionic types. For our purposes, perhaps the best way to view Ohm's law is as a phenomenological linear approximation to the initial portion of the actual current-voltage relation, a curve that increases with increasing voltage starting with zero current at the reversal potential. It seems well justified to use the conventional Nernst formula for the reversal potential, because this equilibrium quantity has been found to be quite insensitive to departures from an idealized view of channel flow (Hille, 1991, p. 347).

According to Ohm's law, the current I_j of charged species j through the fusion pore is

$$I_j = g_j(\Phi - E_j), \qquad E_j = \frac{RT}{z_j F}\ln\left(\frac{C_j^{(e)}}{C_j}\right). \qquad (2a, b)$$

Here E_j is the Nernst equilibrium potential for species j; $C_j^{(e)}$ and C_j are the concentrations outside and inside the vesicle, respectively ($j = 0$ for transmitter); g_j is the conductance of the pore to species j; z_j is the charge of species j; F is the Faraday constant; R is the universal gas constant; and T is the absolute temperature. Because the transpore potential depends on the concentrations of all the species that permeate through the nonselective fusion pore (Eq. 1b), the currents of all species are interdependent. We will show in the Appendix that except for tiny granules and vesicles much smaller than synaptic vesicles, it can be deduced from our model that after a very fast transient the transpore potential is given by the classic Goldman equation.

The average concentration of species C_j in the vesicle volume, V, changes because of the current of this species, I_j:

$$\frac{dC_j}{dt} = -\frac{I_j}{z_j F V}. \qquad (3)$$

This completes the equations of our model.

The extracellular concentrations of ions, $C_j^{(e)}$, $j = 1, 2, \ldots$, are regarded as fixed at their initial values, because their influx through the pore will not appreciably affect their high concentrations in the relatively large extracellular volume. By contrast, transmitter initially has a very low extracellular concentration, which will be strongly modified by transmitter efflux. To approximate the concentration of transmitter at the exterior pore mouth, $C_0^{(e)} \equiv \mathrm{ACh}^{(e)}$ (recall that we focus on acetylcholine), we used the solution for a continuous point source emitting at a fixed rate q for a time θ (Carslaw and Jaeger, 1962), where $q\theta$ equals the number of transmitter molecules in the vesicle. Taking the frog as an example and hence using the estimates $q\theta = 10{,}000$ and $\theta = 100$–200 μs, we calculated how the concentration at the pore mouth, $\mathrm{ACh}^{(e)}$, changes during the time course of discharge. (The source was located on one of two infinite impermeable parallel planes a distance 500 Å apart; the volume between the planes represents the synaptic cleft.) We found that $\mathrm{ACh}^{(e)}$ shoots up from its initial very low value of $\sim 10^{-3}$ mM to very high values (tens of mM) in a few microseconds and then rapidly (tens of microseconds) decays to a value that is lower than the postsynaptic concentration of transmitter at the end of discharge ($\sim$1 mM; see, for example, Land et al., 1980). We thus fixed the concentration at the exterior pore mouth at a representative value of 2 mM, which is consistent with our somewhat more accurate but considerably less informative numerical calculations with a varying concentration at the pore mouth (not shown). Note that the decision on which fixed concentration to select is not crucial for our estimates. As we will show in the Appendix, the duration of discharge varies only logarithmically with the fixed concentrations at the pore mouth. Also see Fig. 3 *B* (discussed below).

To account, at least roughly, for the decreasing rate of arrival of new molecules at the pore as the concentration in the vesicle decreases, a saturating conductance was postulated (Hille, 1991):

$$g_j = g_K \frac{C_j}{K + C_j}. \qquad (4)$$

Here K is the half-saturation constant, which is taken to be half the initial transmitter concentration, ACh(0). The maximum possible conductance g_K is taken to be 450 pS, so that $g_j = 300$ pS (Spruce et al., 1990) at the highest expected particle concentration $C_j = \mathrm{ACh}(0)$, and g_j decreases from this value when C_j decreases. In the absence of detailed measurements, we took all of the saturating conductances to have the same value g_K. The essence of our results would be unaffected if the saturating conductance for ACh^+ were rather smaller than that for Na^+. We now attempt to build understanding of the model (Eqs. 1–4) by treating sucessively less oversimplified cases and/or various alternative hypotheses.

ANALYSIS OF BASIC MODEL

Time course of discharge without ion interchange

To demonstrate the necessity of ion interchange we first consider an oversimplified model that takes into account only the transpore potential and the accompanying flux of (positively) charged neurotransmitter through the pore (See Fig. 2 *A*, *inset*). We employ the notation ACh for the concentration of transmitter. This gives the following versions of Eqs. 1–3:

$$\frac{d\mathrm{ACh}}{dt} = -\frac{I_{\mathrm{ACh}}}{FV}, \qquad \kappa\frac{d\Phi}{dt} = -I_{\mathrm{ACh}}, \tag{5a, b}$$

$$I_{\mathrm{ACh}} = g_{\mathrm{ACh}}\left[\Phi - \frac{RT}{F}\ln\left(\frac{\mathrm{ACh}^{(e)}}{\mathrm{ACh}}\right)\right]. \tag{5c}$$

Fig. 2 shows the results of simulating the system in Eq. 5. Transmitter concentration in the vesicle is normalized to its initial value, i.e., ACh(0) = 1. We consider two cases of initial transpore potential, −150 mV and 150 mV, which are typical of the expected range of initial transpore potential (see Biological Background). Fig. 2 *A* shows that only a small fraction of transmitter (0.5% and 2%, respectively) will be discharged. The underlying explanation for this behavior can be inferred from Fig. 2 *B*. There it is shown that in considerably less than 1 μs, because of the efflux of only a relatively small number of charged transmitter molecules, the potential across the pore reaches the transmitter equilibrium potential, E_{ACh}. Thereafter, the discharge is blocked; no more transmitter can flow out of the vesicle.

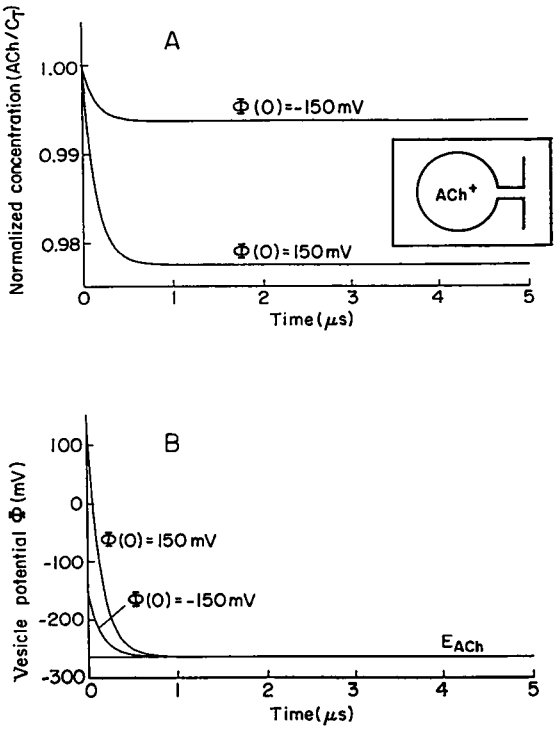

FIGURE 2 Time-course calculations at two initial vesicle potentials, taking into account the charge of the transmitter (ACh^+), but without coion. Simulations of Eqs. 5. (*A*) Normalized concentration of transmitter. (*B*) Transpore potential, Φ, and equilibrium transmitter potential, E_{ACh} (same for both initial potentials). Parameters: $g_{\max} = 300$ pS, $RT/F = 25.8$ mV (at room temperature), $V = 2.6 \times 10^{-17}$ cm^3, $\beta = 1$ μF/cm^2, $\mathrm{ACh}^{(0)} = 300$ mM, $\mathrm{ACh}^{(e)} = 0.01$ mM. Here and below the insets indicate the scope of the model under consideration.

Fig. 2 thus demonstrates that if charged transmitter is the only particle that moves through the narrow pore, then discharge is completely blocked almost immediately. Something is required to overcome this block. We thus move to the next step in our model, a demonstration that the influx of an extracellular positive ion can compensate for the efflux of a positively charged transmitter (ACh^+) and thereby can alleviate the blockage in the discharge.

Ion interchange

To account for ion interchange we augment the model in Eq. 5 by considering the influx of sodium, whose concentration is denoted by Na. (Other ions will be considered later.) We now have two equations for the average concentrations of two charged species in the vesicle:

$$\frac{d\mathrm{ACh}}{dt} = -\frac{I_{\mathrm{ACh}}}{FV}, \qquad \frac{d\mathrm{Na}}{dt} = -\frac{I_{\mathrm{Na}}}{FV}, \tag{6a, b}$$

$$I_{\mathrm{ACh}} = g_{\mathrm{ACh}}\left[\Phi - \frac{RT}{F}\ln\left(\frac{\mathrm{ACh}^{(e)}}{\mathrm{ACh}}\right)\right], \tag{6c}$$

$$I_{\mathrm{Na}} = g_{\mathrm{Na}}\left[\Phi - \frac{RT}{F}\ln\left(\frac{\mathrm{Na}^{(e)}}{\mathrm{Na}}\right)\right]. \tag{6d}$$

The equation for the potential takes the form

$$\kappa\frac{d\Phi}{dt} = -(I_{\mathrm{ACh}} + I_{\mathrm{Na}}). \tag{6e}$$

There are no accurate data on the intravesicular concentrations of sodium and other ions. It is only known that these concentrations are much smaller than the concentration of ACh (Schmidt et al., 1980; Kelly and Hooper, 1982). Therefore we will assume that $\mathrm{Na}^{(0)}$ (and later $\mathrm{Cl}^{(0)}$ and $\mathrm{K}^{(0)}$) are 1 mM, compared with $\mathrm{ACh}^{(0)} = 300$ mM. Here the superscript zero denotes intravesicular concentrations at time $t = 0$ when the pore opens.

Fig. 3 *A* (*solid lines*) shows the concentrations of transmitter and sodium in the vesicle obtained by numerical solutions of Eq. 6 for the initial transpore potential $\Phi^{(0)} = -150$ mV. (The concentrations have been normalized by dividing them by the initial concentration $C_T = \mathrm{ACh}^{(0)} + \mathrm{Na}^{(0)}$ of positive charges. Because of flux neutrality (see Appendix), in the course of time ACh will decrease and Na will increase, but their sum will not exceed C_T.) It is seen that the vesicle will be emptied in about 70 μs. Here, as in Khanin et al. (1994), we define the duration of discharge t_d as the time at which transmitter concentration decreases by

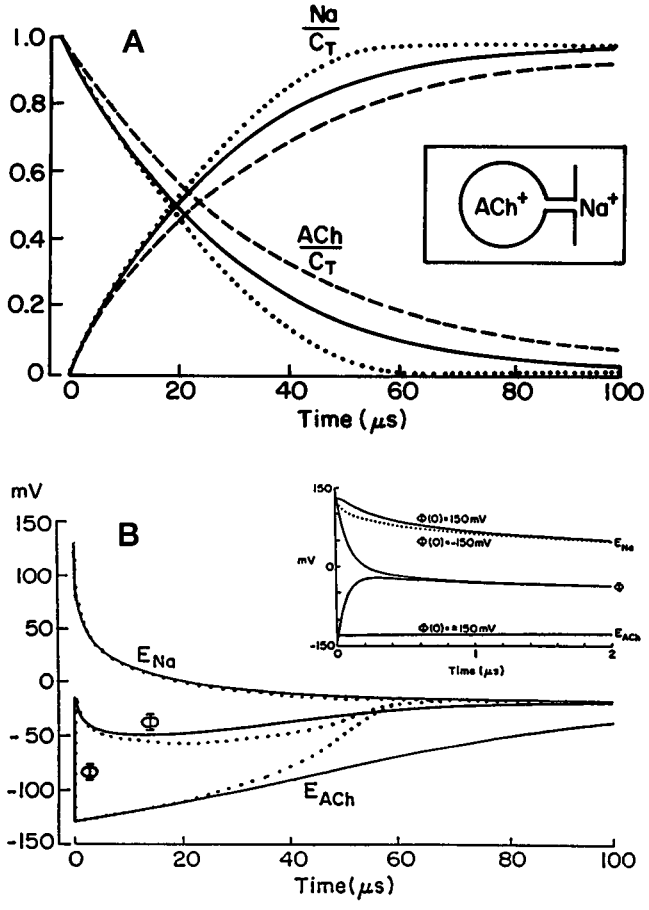

FIGURE 3 Time-course calculations using ion interchange model for discharge, Eq. 6. $\Phi(0) = -150$ mV. *Solid lines*: $Na^{(0)} = 1$ mM, $Na^{(e)} = 150$ mM. Other parameters as in Fig. 2. (*A*) Transmitter and sodium concentrations in the vesicle normalized by initial total concentration C_T. $ACh^{(e)} = 2$ mM. *Dashed lines*: $ACh^{(e)} = 5$ mM. Here and in *B*, dotted lines give results when instead of using the saturating formula (Eq. 4), we take the conductance to be constant (300 pS). (*B*) Transpore potential Φ and equilibrium potentials E_{ACh}, E_{Na}. *Inset*: The first 2 μs, for two initial vesicle potentials.

95%, because it is observed that the vesicle is essentially empty at the conclusion of discharge.

The essence of the ion interchange mechanism for discharge is revealed by the present simulations. We have seen that if transmitter is the only charged species that participates in the discharge, after the pore opens the vesicle potential rapidly approaches the transmitter equilibrium potential E_{ACh}. When sodium is taken into account, its equilibrium potential is also relevant. The inset in Fig. 3 *B* illustrates, and analytic calculations in the Appendix confirm, that after the pore opens, irrespective of its initial value, the vesicle potential rapidly takes up a value between the two competing local equilibrium potentials. It can be shown, in fact, that in the present case after the initial fast transient this value is the average of the values of the two equilibrium potentials weighted by the varying conductances of Eq. 4 (Goldman equation; see Appendix). The dotted curves in Fig. 3 illustrate a simplified case wherein conductances are taken to be constant and equal. We see here that after the fast transient Φ becomes a simple average of E_{Na} and E_{ACh}.

The dashed curves in Fig. 3 are for the choice $ACh^{(e)} = 5$ mM, as opposed to $ACh^{(e)} = 2$ mM for the solid curves. The similarity of the results is one indication that the choice of $ACh^{(e)}$ is not crucial.

We note by examining $E_{Na} - \hat{\Phi}$, as seen in Fig. 3 *B*, that the driving force for ACh is still quite high at $t = 70$ μs, when only 5% of the ACh remains, a situation that we define as the termination of release. But at this time the concentration of ACh is 15 mM (5% of the initial concentration of 300 mM) compared to the extracellular concentration $ACh^{(e)} = 2$ mM. Thus considerable further discharge will take place. The situation is different for sodium. At $t = 70$ μs the Na^+ concentration in the vesicle is 285 mM, much higher than the extracellular concentration $Na^{(e)} = 150$ mM. In spite of the relatively low driving force for Na^+, there is a balance between Na^+ influx and ACh^+ efflux. The reason is that according to the concentration-dependent conductance formula (Eq. 4), the Na^+ conductance is much greater than the ACh^+ conductance. As expected, when constant conductance is assumed, the Na^+ and ACh^+ driving forces are equal (Fig. 3 *B*, *inset*).

As expected from the analogous result in Fig. 2, Fig. 3 *B* (*inset*) shows that regardless of its initial value the potential very rapidly adjusts to a quasiequilibrium state. Because its value has almost no effect, in what follows we will confine ourselves to a resting vesicle potential of -150 mM, the best available estimate.

The time to reach the equilibrium potential in Fig. 2, tenths of a microsecond, is the same magnitude as the time to reach the quasiequilbrium average potential in Fig. 3 *B* (*inset*). This very short time is not an artifact of our particular model. It is a classical result of electrophysics that local equilibrium is attained in a domain of characteristic length r in a time of order of magnitude given by the so-called Debye time, t_{De} (Jackson, 1974; Rubinstein, 1990). In the present instance,

$$t_{De} = \frac{\epsilon r^2}{D}. \tag{7}$$

Here ϵ is a small, dimensionless parameter (see Eq. A1), and D is the diffusion coefficient of transmitter. From the theory of diffusion it follows that local equilibrium is reached in the time it takes to diffuse the distance $\sqrt{\epsilon}r$. When $r = 20$ nm, the radius of synaptic vesicles, the Debye time indeed has a value of magnitude ~0.1 μs.

We conclude from our analysis of the basic model that the essential requirements for discharge have been satisfied: blockage was relieved and fast discharge was obtained.

REFINEMENT OF THE BASIC MODEL

Having examined the essential aspects of our ion interchange model, we now consider a number of refinements that a priori could have a significant effect on the results.

Effect of chloride and other ions

The extracellular concentration of chloride is about that of sodium: $Cl^{(e)} = 130$ mM (Plonsey and Barr, 1988). It seems that the negative ion chloride might significantly slow down the discharge of transmitter by annulling the effects of sodium inflow. The relevant mathematical model requires adding to Eq. 6 an equation for the vesicular chloride concentration Cl:

$$\frac{dCl}{dt} = \frac{I_{Cl}}{FV}, \qquad I_{Cl} = g_{Cl}\left[\Phi + \frac{RT}{F}\ln\left(\frac{Cl^{(e)}}{Cl}\right)\right]. \quad (8a, b)$$

Equation 6e becomes

$$\kappa\frac{d\Phi}{dt} = -(I_{ACh} + I_{Na} + I_{Cl}). \quad (8c)$$

We find upon analyzing this model that Cl^- has no significant effect on the time course of discharge (Fig. 4 *A*). The main reason is that the negative vesicular potential hinders the entrance of extracellular Cl^-, in contrast to the potential's attractive effect on extracellular Na^+. Indeed, Fig. 4 *B* shows that Φ and E_{Na} effectively add to produce a relatively large driving force for sodium, in contrast to chloride, where the driving force is small, owing to cancellation in $\Phi - E_{Cl}$. As a consequence there is no significant flux of chloride ions.

In the frog, other positively charged ions (K^+, Ca^{2+}, Mg^{2+}) have a small effect (which accelerates discharge) because the extracellular concentration of these ions is low (not shown). In squid, for example, where Ca^{2+} concentration is quite high, calcium can join with Na^+ in accelerating the discharge of transmitter, particularly as divalent ions "count double."

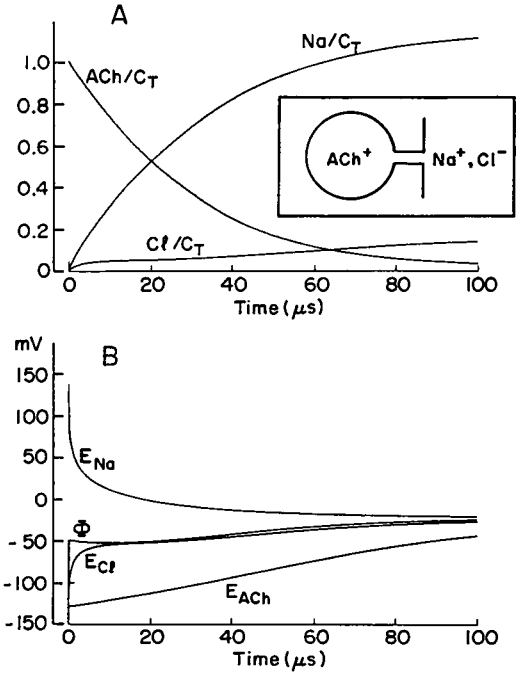

FIGURE 4 Same as Fig. 3, except that chloride, Cl^-, is included. Here $Cl^{(0)} = 1$ mM, $Cl^{(e)} = 120$ mM. Note: $E_{Cl}(0) = -123$ mV.

Noninstantaneous pore opening

As we mentioned, measurements on pore opening show only that this occurs in less than 100 μs, but this time is of the magnitude of the discharge time. One might think that taking into account the noninstantaneous opening of the pore would have an effect on the time course of the discharge. Small ions of sodium can start to flow through the small pore precursor before the pore becomes permeable to large transmitter molecules. However, this effect is negligible: only when the pore is permeable to transmitter does ion interchange start, after which discharge occurs as with instantaneous pore opening (calculations not shown). We note that because there are no significant particle movements until the pore is large enough to permit passage of of transmitter, such an opening must take only a few microseconds, given that the minimum delay is as brief as 0.2 ms (Llinas et al., 1982) and that high postsynaptic concentrations require discharge to be completed in about 100 μs.

Concurrent discharge of transmitter and counterion

Another variant of the model deals with the possibility that mobile counter-charges stored in the vesicle (e.g., ATP for ACh-containing vesicles; see Redman and Silinsky, 1994) move with the transmitter through the pore. In the simulations shown in Fig. 5, ACh and ATP move separately, each along its own electrochemical gradient. Note that the normalized time course of discharge of the two is the same (Fig. 5 *B*, *inset*). This is because the higher concentration of ACh (ACh(0) = 300 mM, ATP(0) = 100 mM) is compensated by the higher charge of ATP ($z_{ACh} = 1$, $z_{ATP} = 3$).

We find that with the model of Fig. 5 the time course of neurotransmitter discharge is the same as in our basic model (compare Fig. 5 to Fig. 3). Indeed, it is to be expected that there is little difference between compensating for the efflux of positively charged transmitter with the coinflux of positive ions or with the coefflux of negative ions.

Vesicle channels

Synaptic vesicles from *Torpedo marmorata* were found to contain large-conductance (80–100 pS) potassium-preferring channel(s) (Rahamimoff et al., 1988). It has been suggested that the presence of channels in synaptic vesicles may be related to the secretion process in a number of distinct ways (e.g., Stanley and Ehrenstein, 1985). Here we study the possibility that the concentrations of different species in the vesicle might be affected not only by fluxes through the fusion pore, but also through the potassium channels.

To quantitate the effect of the ionic channels on the

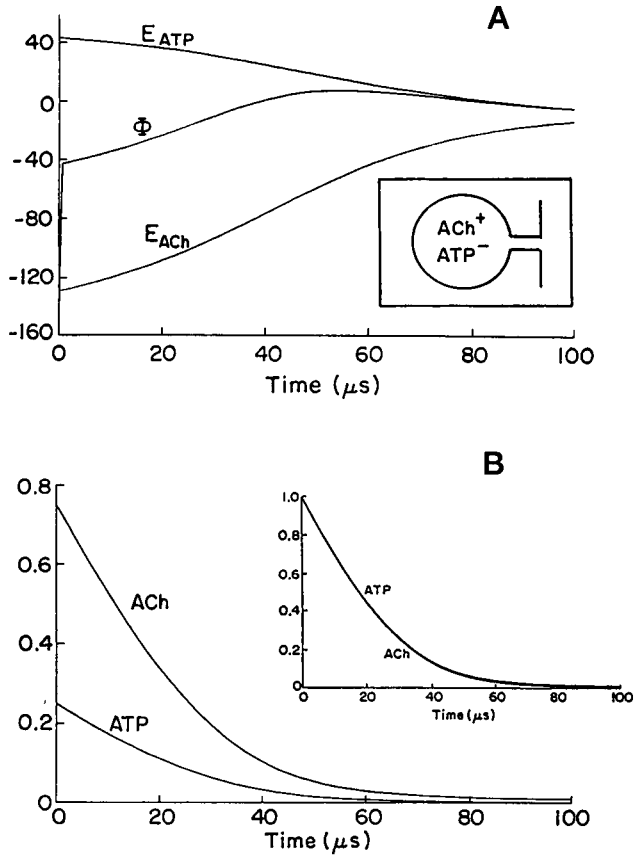

FIGURE 5 Concurrent discharge of transmitter ACh^+ and a counter-charged ion ATP^{3-}. (*A*) Potentials. (*B*) Concentrations, normalized by initial ACh^+ concentration. *Inset*: Both concentrations normalized by their initial values, showing parallel discharge of the two charged particles. Here ATP(0) = 100 mM (one-third of initial transmitter concentration in the vesicle), $ATP^{(e)}$ = 1 mM, $ACh^{(e)}$ = 2 mM, as in Fig. 3. Other parameters as in Fig. 2. The appropriate version of the basic equations (Eqs. 2 and 3) were used and are given below:

$$\frac{dACh}{dt} = -\frac{I_{ACh}}{FV}, \qquad \frac{dATP}{dt} = \frac{I_{ATP}}{3FV}, \qquad I_{ATP} = g_{ATP}\left[\Phi + \frac{RT}{3F}\ln\frac{ATP^{(e)}}{ATP}\right],$$

$$\kappa\frac{d\Phi}{dt} = -(I_{ACh} + I_{ATP}).$$

vesicle membrane, we consider the following variation of the basic model (Eqs. 6–7). The outward current I_j^{ch} of charged species j through the channels is described by Ohm's law, in parallel with Eqs. 2a,b:

$$I_j^{ch} = g(\Phi - \Phi^{(int)} - E_j^*), \qquad E_j^* = \frac{RT}{F}\ln\frac{C^{(int)}}{C_j}. \quad (9a, b)$$

Here $\Phi - \Phi^{(int)}$ is the transvesicle potential difference and E_j^* is the corresponding equilibrium potential for species j. The intracellular potential Φ is taken to be constant. Because the extracellular potential $\Phi^{(e)}$ is zero, Φ is the same as the potential $\Phi - \Phi^{(e)}$ across the plasma membrane (which we take to have the typical value −70 mV).

The concentration of species in the vesicle and changes in the vesicle potential depend on both the currents through the pore and those through the channels:

$$\frac{dC_j}{dt} = -\frac{I_j + I_j^{ch}}{z_j FV}, \qquad \kappa\frac{d\Phi}{dt} = -\left(\sum_j I_j + \sum_j I_j^{ch}\right). \quad (10a, b)$$

The potassium channels are likely to be closed under resting conditions. Otherwise, the vesicular potassium would be in equilibrium with the intracellular potassium, yielding a high intravesicular concentration of potassium that is contrary to the experimental findings (Schmidt et al., 1980). The negative transvesicle potential at rest ($\Phi(0) - \Phi^{(int)} = -80$ mV; Angel and Michaelson, 1981) indeed ensures that the channels are closed at rest.

Rahamimoff et al. (1988) found that the vesicle channels open upon depolarization of the vesicle membrane. This depolarization, the transvesicle potential difference $\Phi - \Phi^{(int)}$, occurs owing to the opening of the fusion pore and the consequent change in Φ due to the onset of charged fluxes through the pore (see, for example, Fig. 3).

The channels open instantaneously when the transvesicle potential reaches a threshold depolarization, which we take to be 20 mV (Edry-Schiller et al., 1991). From Fig. 3 *B* we see that from its initial value of −150 mV Φ almost instantly reaches a value of −50 mV and then rises further. Because $\Phi^{(int)} = -70$ mV, the transvesicle potential $\Phi - \Phi^{(int)}$ will rapidly increase from −50 mV to +20 mV, a depolarization of 100 mV, and the channels will open. We assume that the channels remain open during the discharge process, which is justified by the observation of Rahamimoff et al. (1988) that the channels remain open from milliseconds to even seconds.

In a simulation to examine the possible effects of vesicle channels, we considered ACh^+, Na^+, and K^+, with only K^+ passing through the vesicle channels and only ACh^+ and Na^+ passing through the pore. We thus considered Eq. 6, modifying Eq. 6e by the addition of the potassium channel current I_K^{ch} to the other currents. I_K^{ch} is given by Eq. 9 if $\Phi - \Phi^{(int)} > -60$ mV; otherwise, $I_K^{ch} = 0$. There is an equation analogous to Eqs. 6a,b for the vesicular potassium concentration K. Fig. 6 shows that taking into account a total potassium channel conductance of 300 pS, equal to the pore conductance, has an insignificant facilitating effect on the time course of neurotransmitter discharge. Essentially the same result is obtained for five channels, each of 100 pS conductance (not shown). Given the size of the vesicle, it is doubtful that more than five potassium channels are present. The relatively small effect of the potassium channels is due to the fact that the influx of potassium acts to slow the influx of sodium when sodium is the only other positive ion that flows in. If chloride channels were present in vesicles with negatively charged transmitter, we would similarly expect only a small effect in accelerating discharge.

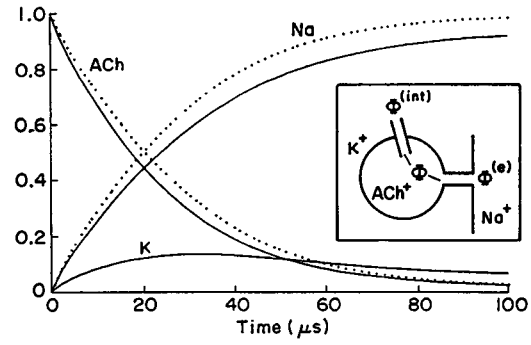

FIGURE 6 Effect of potassium channels on the time course of discharge. Solid lines represent normalized concentrations of transmitter, sodium, and potassium when potassium channel is included. *Dotted lines*: Normalized concentrations of transmitter and sodium in the absence of potassium channels, as in Fig. 2. Threshold depolarization for channel opening is 20 mV; total channel conductance is 300 pS.

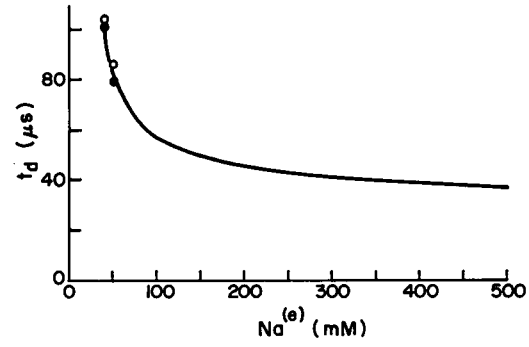

FIGURE 7 Discharge time t_d as a function of $Na^{(e)}$, the extravesicular sodium concentration, according to numerical simulations of Eq. 6 (*solid line*). For $Na^{(e)}$ = 40, 50 mM, *empty and solid circles* give calculations, respectively, according to the approximate formula (Eq. A13) and to the further approximation (Eq. 11). The three calculations of t_d give indistinguishable results for $Na^{(e)} \geq 100$. In all calculations, conductance is constant, as in Fig. 3 (*dotted lines*).

SENSITIVITY OF CONCLUSIONS

A simple analytical formula is the best way to demonstrate parameter dependence. Based on the smallness of the parameter ϵ of Eq. 7, we have developed an approximate formula for the duration t_d of ion interchange discharge (see Appendix). Only transmitter ACh^+ and its coion Na^+ are taken into account because, as we have seen, the interchange of ACh^+ and Na^+ gives the essence of the matter. We make the additional assumption that the initial transmitter concentration is large compared to the initial sodium concentration in the vesicle. Expression A13 results are a good approximation of the numerical results (see Fig. 7). To make the behavior of the integral in Eq. A13 transparent, the integral is approximated by a simple function of c. Dimensional variables are employed. We obtain

$$t_d = \frac{N_T}{g}\frac{F^2}{RT}\left[\frac{c + 4.79}{8.37c - 17.4}\right], \qquad N_T = \mathrm{ACh}(0)V,$$

$$c = \ln\frac{\mathrm{Na}^{(e)}}{\mathrm{ACh}^{(e)}}. \tag{11}$$

We chose the coefficients in the function of c to give a good approximation in the range $3 < c < 5$, which corresponds to the large range $40 < \mathrm{Na}^{(e)} < 500$ for $\mathrm{Tr}^{(e)} = 2$ mM. See Fig. 7.

Parameter sensitivity of the discharge time t_d can be read off from Eq. 11. The initial vesicle potential has no effect. This is to be expected because, as we have seen, the vesicle potential rapidly changes to an intermediate value that is independent of the initial conditions. The discharge time t_d exhibits only weak logarithmic dependence on extravesicular concentrations. Two parameters strongly affect t_d, which is proportional to the total number N_T of transmitter particles to be discharged and to the pore resistance $1/g$. Doubling the number of transmitter molecules in the vesicle, N_T, is predicted to double the discharge time. This effect could be exactly cancelled by doubling the conductance g. For the conductance of the pore, g, in synaptic vesicles we used the initial conductance of the pore in mast cells, 300 pS (Spruce et al., 1990). Synaptic vesicles are much smaller than mast cell granules; if their conductance is in the range that we have cited for neutrophils (which are considerably smaller than mast cells), 150 pS, then the calculated discharge time t_d is 140 μs, still in the range required to achieve sufficient postsynaptic levels of transmitter.

SUMMARY AND DISCUSSION

Energy considerations

We have shown that if only charged transmitters were to move through the pore, discharge would rapidly be blocked (Fig. 8, *top row*). One possible way of overcoming the block is to supply energy to the system. To estimate a lower bound for the energy required to discharge a representative quantum of 10,000 molecules (Kuffler and Yoshikami, 1975), we have utilized a standard fluid mechanics calculation of the viscous dissipation that occurs if a spherical particle of ACh moves a distance L (the pore length) in an unbounded fluid, at the required speed (Lauffer, 1989, p. 37). The estimated amount of energy for such fast discharge is 10^{-9} erg. This could, in principle, be provided from the hydrolysis of all of the ATP molecules stored in the vesicle. However, even with a hydrolysis turnover number of thousands per second, release of this energy would take much longer than the duration of discharge and would require a very substantial amount of hydrolyzing enzyme.

The presence of charge means that without the concomitant influx of like ions (or efflux of counter-charged ions), the "shooting energy" of the previous paragraph must be supplemented by an additional "capacitive energy" of 10^{-7} erg. If such energy could somehow be provided, the potential of the vesicle at the end of the discharge would reach

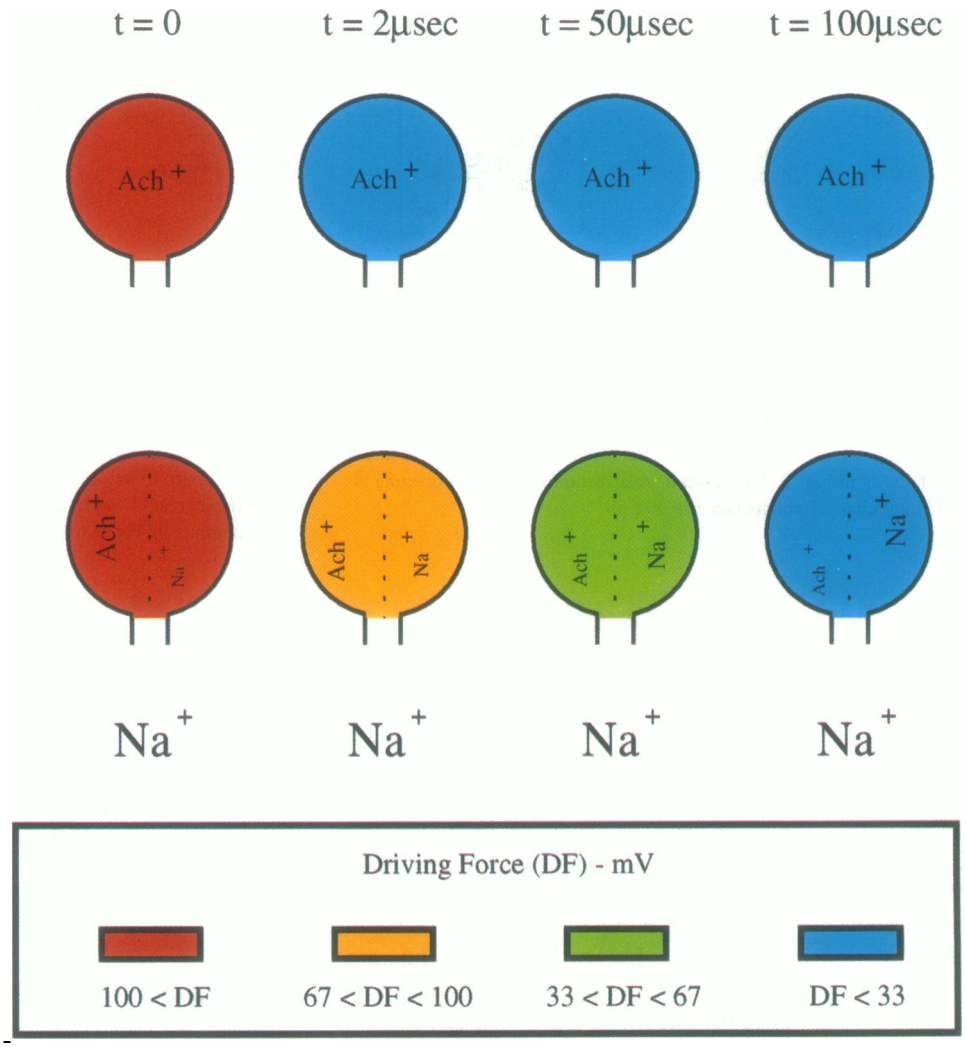

FIGURE 8 Schematic illustration of discharge from vesicles via pore. (*Top row*) Taking into account the charge of transmitter (assumed positive for definiteness), but without positively charged coion. Color indicates outward driving force (DF) for ACh^+. Discharge is blocked in ~1 μs. (*Bottom row*) The major coion Na^+ is taken into account. Color indicates driving force: outward for ACh^+ (*left*), inward for Na^+ (*right*). Size of letters indicates concentration. The fixed extracellular Na^+ concentration is indicated. Almost all of the ACh^+ is driven out in 100 μs, in exchange for the Na^+ that is driven into the vesicle.

tens of volts, far too high for a biological membrane to survive.

By contrast, ion interchange can provide a mechanism for discharge of charged neurotransmitters (Fig. 8, *bottom row*). There are no additional energy costs, because the required concentration gradients are maintained in any case. Moreover, ion interchange is fast enough to generate the observed high transmitter concentration at the postsynaptic critical area and thereby to account for the rising phase of the miniature end-plate currents. The basic model for discharge presented here is built on the concept that the charged secretants leaving the vesicle through a fusion pore are interchanged with coions that enter the vesicle through the same fusion pore (see Fig. 1). The same speed of discharge is generated if the efflux of charged transmitter is accompanied by an efflux of counter-charged particles moving along their own electrochemical potential (see Fig. 5).

Formula for discharge time

The essence of our ion interchange theory is summarized in the approximate formula (Eq. 11) for the time of discharge. Every required parameter that appears in Eq. 11 is estimated from the literature, although data concerning the critical pore conductance g had to be taken from granular release from mast cells and neutrophils. With appropriate changes of signs the model describes the discharge of negatively charged transmitter (glutamate; not shown). In this case, chloride will play the role of the principal exchanging coion. Because the extracellular concentrations of Na^+ and Cl^- are both in the 100 mM range, the theory predicts similar discharge times for both positively and negatively charged transmitters. We can conclude that our simple ion interchange model gives the right time range for excitatory neurotransmitter discharge in fast systems.

Possible experimental tests of the theory

It is important to test the ion interchange theory experimentally. One natural way is by changing the ionic concentrations in the synaptic gap and seeing whether alterations in the discharge time are in accord with our quantitative theory. However, discharge cannot be directly measured in fast synapses. A bound on the discharge time can be obtained

from the measurable minimum delay, and a relation, albeit not a strong one, has recently been established between the duration of discharge and the postsynaptic rise time (Khanin et al., 1996). Perhaps these indirect approaches can lead to an experimental check of the theory, but the situation is further complicated by the result of Eq. 11 that extracellular ionic concentrations influence the duration of discharge only logarithmically.

Indeed, Van der Kloot (1995) challenged our "proposal that ACh is released from the vesicle in exchange for Na^+" (mentioned by Khanin et al., 1994). The challenge was based on two findings: 1) Katz and Miledi (1969) recorded miniature end-plate potentials when sodium ions were completely replaced by an isotonic $CaCl_2$ solution. 2) Van der Kloot replaced all of the extracellular sodium by isotonic sucrose. Because the data were noisy, only the qualitative conclusion that "risetimes . . . are in the normal range" was drawn. It was inferred from 1) and 2) that it is "unlikely that Na^+ plays any substantial role in the release of ACh."

We have three comments. 1) Normally Na^+ is indeed the principal interchanging ion (for ACh^+) in the standard version of our theory, but any positive ion suffices. 2) Because the dependence of discharge duration on the concentration of the interchanging ions is logarithmic (see Eq. 11), it follows that decreasing the concentration of such ions by two orders of magnitude, as Van der Kloot did, should roughly double the duration of discharge. (This turns out to be a conservative estimate, because as a function of the logarithm of the concentration, t_d is a curve that rises with a typical slope that is considerably less than unity; see Appendix.) 3) The rise time is even less sensitive than the duration. For example, doubling the discharge duration from 100 to 200 μs only increases the rise time by about 20% (Khanin et al., 1996).

Fig. 9 demonstrates the above points quantitatively. The time course of discharge under the two extreme experimental conditions of Van der Kloot (1995) and one of Katz and Miledi (1969) is simulated. It can be seen that as expected from Eq. 11, under the condition of Katz and Miledi (1969), in which a high concentration of Ca^{2+} is present (isotonic solution), t_d is very brief. But even under the conditions of Van der Kloot (1995), in which the total concentration of positive ions is rather low, t_d is merely increased by 50%. With these values of t_d, the expected rise times can be extracted from figure 2 of Khanin et al. (1996). We find that the rise time under the conditions of Katz and Miledi (1969) is ~150 μs, and under the conditions of Van der Kloot (1995) it is ~200 μs. Note that with the low concentration of positive ions used in the experiments of Van der Kloot (1995) and with our fixed representative (but not very accurate) choice of $ACh^{(e)} = 2$ mM, the final steady-state transmitter concentration in the vesicle is slightly higher (~7%) than the postulated concentration at the end of the discharge (5%). It should be emphasized, however, that there are no precise data on the extent to which the vesicle is emptied. Thus, there is no preference between assuming that 5% or 7% of the initial transmitter concentration remains in the vesicle at the end of discharge.

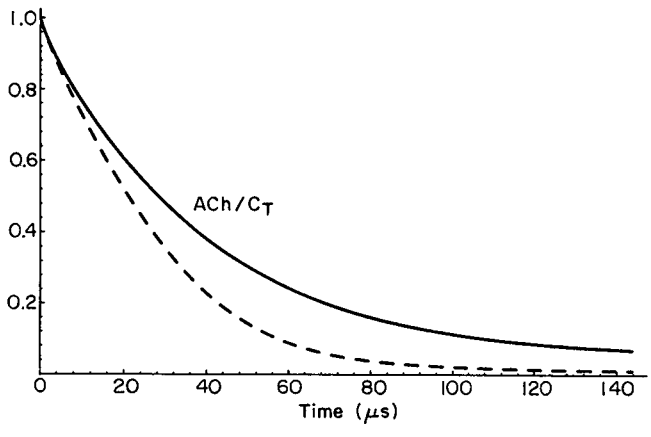

FIGURE 9 Simulation of the time course of discharge under the conditions of Van der Kloot (1995) (*solid line*) and Katz and Miledi (1969) (*dashed line*). *Solid line*: $Na^{(e)} = 0$ mM, $Ca^{(e)} = 2.5$ mM, $K^{(e)} = 2$ mM. *Dashed line*: $Na^{(e)} = 0$ mM, $Ca^{(e)} = 83$ mM, $K^{(e)} = 0$ mM, $ACh^{(e)} = 2$ mM.

In opposition to their author, we thus conclude that Van der Kloot's (1995) results are fully in accord with the version of our theory that postulates interchange of positive transmitter with positive extracellular ions. In addition, we remind the reader that another version of the theory would work in the complete absence of positive extracellular ions, because it relies on the joint discharge of positive transmitter and negative mobile charges.

Perhaps testing the ion interchange theory will be possible in slowly releasing systems in which direct measurements of the discharge process have been already performed (e.g., Wightman et al., 1991; Chow et al., 1992; Alvarez de Toledo et al., 1993) (secretants from slowly releasing systems are also charged; Kandel and Schwartz, 1985, p. 150). In contrast to synaptic vesicles, however, a large fraction of the secretants in slowly releasing granules are bound to the protein complex or are trapped in gels. Thus, secretants in large granules must be freed before they are discharged. This process probably occurs during the "foot" that always precedes the actual process of discharge (Chow et al., 1992; Alvarez de Toledo et al., 1993). The presence of the foot when the granule is already connected to the cellular membrane via the fusion pore indicates that a long time is required for the unbinding of secretory products that are eventually to be discharged. In addition, the process of unbinding of secretants is usually accompanied by matrix degranulation and swelling (Curran and Brodwick, 1991; Fernandez et al., 1991; Verdugo, 1994). Therefore, for granules the process of discharge must be linked with the processes of granule swelling and freeing of secretory products, perhaps in conjunction with fusion pore formation and expansion. It is our view that in granules, once the charged secretant is freed, its discharge proceeds by ion interchange. If it turns out that ion interchange is not the principal mechanism of discharge from granules, it must coexist with whatever governs the discharge

of charged particles. Otherwise, very high energies will be required for discharge to persist.

Ultrarapid pore opening

Interesting relevant studies have appeared since the original submission of this paper. Wahl et al. (1996) conclude from a Monte Carlo simulation of transmitter discharge from a vesicle in a hippocampal synapse that to match the time course of evoked postsynaptic currents either a much larger pore than that reported by Spruce et al. (1990) opens extremely fast (7.5–10 nm in <1 μs) or that some mechanism other than passive diffusion is involved in discharging glutamate from the vesicle. Very similar results were obtained by Stiles et al. (1996), with the help of a supercomputer, but precisely the same conclusion was reached by Khanin et al. (1994) by analytical calculations (which were not so accurate, but which are accurate enough to make the point). Indeed, if there is ultrarapid pore opening, then discharge of an uncharged particle can occur by diffusion.

In support of the hypothesis of ultrarapid opening, Stiles et al. cite a paper by Torri-Tarelli et al. (1985), who conclude from quick-freezing micrographs that about half a microsecond after synaptic delay there appears an "omega figure" (open vesicle) with a radius on the order of 10 nm. Accordingly, in their calculations, Stiles et al. (1996) assume linear expansion of the pore radius, starting with zero, and ending with a value of 10 nm at 500 μs. However, one can raise various difficulties concerning the results of the experiments and their interpretation. 1) For technical reasons, there were various nonphysiological aspects of the experiments, with unknown influences. 2) The rate of expansion used by Stiles et al. (1996) assumes that the pore opens linearly. It could well take a good deal of time for pore expansion to "get going," so that most of the rapid expansion would take place after discharge was completed. 3) The pore could be so delicate that it is ripped open by the sudden freezing, so that the observed omega figure is not physiological.

Our focus on charged neurotransmitter brings out an important point that is not considered at all by Stiles et al. (1996). The excitatory transmitters ACh^+ and glutamate are charged. As we have pointed out, it is possible that ACh^+ exists as a complex with another charged entity for which ATP^{3-} is the strongest candidate. But then the neutral particle would be much larger than ACh^+. The correspondingly smaller diffusivity would spoil the good quantitative agreement found by Stiles et al. (1996).

Suppose that the ACh^+ exited the vesicle in charged form, but that the pore opened very rapidly. Then yet another theory would be required. For a very wide pore, the influence of the walls is expected to be negligible. The essence of the matter is thus given by the classical calculation of liquid junction potentials using the Nernst-Planck-Poisson equations (Hickman, 1970). According to this approach, the fluxes of the various charged particles behave in a manner that is qualitatively and semiquantitatively similar (although for somewhat different reasons) to the behaviors shown in this work with the model based on Ohm's law. That is, an intermediate potential will arise that drives Ach^+ efflux faster than free diffusion of a chargeless particle. We have performed calculations (unpublished) that show that this is the case even if only Na^+ and Ach^+ are considered.

In summary, the consideration of charge is the principal difference between our theory and that of Stiles et al. (1996). An important secondary difference is the assumption of ultrarapid pore expansion by Stiles et al. They provide interesting speculations on molecular machinery that might bring about such an expansion. In our judgment, it is more likely that to permit rapid release, evolution chose to take advantage of the "free ride" provided by existing charged particles.

Of course, one seeks a decisive experiment to distinguish between the two theories for discharge of excitatory neurotransmitter—that diffusion is sufficient because of ultrafast pore opening or, alternatively, that owing to the presence of charge, something like interchange is necessary whether or not the pore opens rapidly. Unfortunately, at the moment such a critical experimental test seems impossible.

In the cholinergic system ATP^{3-} and ACh^+ coexist within the vesicles, and hence even if all of the external positive ions are replaced, the alternative version of the interchange (coefflux of positive and negative molecules) will still hold. For glutamate we know even less about the totality of molecules that are involved in discharge.

Incidentally, our approach leads to the speculation that use of the coefflux mechanism for discharge might be the reason that ATP^{3-} was "chosen" to reside in the synaptic vesicles together with Ach^+.

Electrically neutral inhibitory transmitters

There are cases of fast discharge involving electrically neutral neurotransmitters, notably the inhibitory transmitters GABA and glycine. In view of the fact that diffusion is not fast enough (Khanin et al., 1994), unless the synaptic pore opens much faster than granule pores (Stiles et al., 1996; Wahl et al., 1996), it is a challenge to find mechanisms for the fast discharge of neutral transmitters. Some time ago one solution was suggested (Nelson and Blaustein, 1982): perhaps transmitters bind to charged particles that "ferry" the transmitter out of the vesicle. This brings us back to ion interchange.

APPENDIX: ANALYTICAL FORMULA

We show here the consequences of certain simplifications of our basic equations. Notably, we demonstrate that the classical Goldmann equation describes the transpore potential, and we derive a useful approximation formula for the discharge time.

It turns out that the complexity of ion exchange in its most general manifestation is considerably reduced if one limits consideration to atypical vesicles (radius = tens of nanometers; initial transmitter concentration = hundreds of mM). For such a vesicle the characteristic time for significant changes in voltage, $t\phi$, is much smaller that the characteristic

time for changes in concentration, t_c. We estimate $t\phi$ by κ/g, the time it takes to discharge a vesicle of capacitance κ via a pore of conductance g. To estimate t_c, we divide the initial vesicle charge $C_T VF$ (representative mobile species concentration in the vesicle, times volume, times charge per mole) by a characteristic current through the pore. The latter is the conductance g times a characteristic voltage RT/F. If we define $\epsilon = t_\phi/t_c$, then

$$\epsilon = \frac{\kappa RT}{F^2 V C_T}. \tag{A1}$$

Note that both t_ϕ and t_c are inversely proportional to the pore conductance g. Thus ϵ is independent of g. This is fortunate, for g has not been measured for vesicles. For control parameters t_ϕ is 0.14 μs, and t_c is 100 μs, in agreement with our computer simulations. Hence $\epsilon \ll 1$.

The formal consequences of the smallness of ϵ will be derived shortly. Here we note the most salient conclusions. Because the time scale for voltage changes is so much shorter than that for concentration changes, after a brief transient the voltage adjusts almost instantaneously to changes in concentration. Because the voltage is in such a "quasi-steady state" ($d\Phi/dt \sim 0$) during discharge (to first approximation), there is no net flow of charge through the pore. In other words, there is flux neutrality. In particular, for each charged molecule of transmitter that leaves the vesicle, there must be a corresponding ionic movement that cancels the alteration in charge that can be attributed to this transmitter efflux.

Assuming that Φ is in a quasi-steady state, we obtain from Eq. 1b

$$\sum_j I_j = 0 \quad \text{or} \quad \sum_j g_j(\Phi - E_j) = 0.$$

From the last equation it follows that

$$\Phi = \frac{\sum_j g_j E_j}{\sum_j g_j}. \tag{A2}$$

That is, Φ is an average of the equilibrium potentials of the transmitter and the various ions, weighted by their conductivities. This is the Goldmann equation for the transpore potential (which in our case, $\Phi^{(e)} = 0$, equals the vesicle potential).

To interpret Eq. A2, let us focus on the simple case of Fig. 3, with monovalent transmitter and coion and with constant conductance g. Here Φ reduces to the ordinary average of the two relevant equilibrium potentials. It is this average potential that provides the electrochemical gradient that forces charged transmitter out and its coion in, at equal rates. In general, when $\epsilon \ll 1$, it is the weighted average of Eq. A2 that provides the intermediate potential that forces ionic movements, and these preserve flux neutrality. When ϵ is not small, which can be the case for very small vesicles, computer simulations show that discharge is still fast. The basic principle is exactly the same, but the intermediate potential that forces discharge is no longer the average of the equilibrium potentials, and ion and coion flow rates are no longer equal (not shown).

To make a semiquantitative prediction and to determine which of the parameters has a relatively strong effect on discharge time, we now turn to an approximate analytical treatment of our mathematical model, via standard singular perturbation methods (Lin and Segel, 1988).

Solution for the initial transient

Let us first introduce scaled dimensionless dependent variables:

$$x = \frac{\mathrm{ACh}}{C_T}, \qquad y = \frac{\mathrm{Na}}{\epsilon C_T}, \qquad \phi = \frac{F\Phi}{RT}, \qquad \tau = \frac{t}{t_\phi} = \frac{tg}{\kappa}. \tag{A3a, b, c}$$

When several ions of various valences and charges are present, the overall concentration scale is $C_T = \Sigma z_j C_j^{(0)}$. In the present case (where $z_x = z_y = 1$),

$$C_T = \mathrm{ACh}^{(0)} + \mathrm{Na}^{(0)} \tag{A3d}$$

is the total initial concentration of mobile species inside the vesicle. RT/F is a good scaling for equilibrium potentials, E_j, and for characteristic transpore potentials, for these arise from the currents that depend crucially on the equilibrium potentials. As a further conformation, at room temperature $RT/F \sim 25.8$ mV, and indeed the potentials have magnitude of tens of millivolts. Finally, Na is scaled with ϵC_T because the initial concentration of Na^+ in the vesicle is small.

In terms of the variables (Eq. A3), the governing system of Eq. 6 is

$$\frac{dx}{d\tau} = -\epsilon(\phi - \ln x^{(e)} + \ln x), \tag{A4a}$$

$$\frac{dy}{d\tau} = -(\phi - \ln y^{(e)} + \ln(\epsilon y)), \tag{A4b}$$

$$\epsilon\frac{d\phi}{d\tau} = \frac{dx}{d\tau} + \epsilon\frac{dy}{d\tau}, \tag{A5}$$

where ϵ has been defined by Eq. A1, and

$$x^{(e)} = \frac{\mathrm{ACh}^{(e)}}{C_T}, \qquad y^{(e)} = \frac{\mathrm{Na}^{(e)}}{C_T},$$
$$x^{(0)} = \frac{\mathrm{ACh}^{(0)}}{C_T}, \qquad y^{(0)} = \frac{\mathrm{Na}^{(0)}}{C_T}. \tag{A6}$$

Writing, for example,

$$x(\tau, \epsilon) = x_0(\tau) + \epsilon x_1(\tau) + \cdots$$

and expanding Eqs. A4 and A5 in powers of ϵ, at order of unity we obtain $dx_0/d\tau = 0$. Thus $x_0 = x^{(0)}$. Note here that to first approximation the initial charge neutrality is retained throughout the transient: ACh is constant, whereas Na is of order ϵ. Also at order unity we obtain

$$\frac{dy_0}{d\tau} = -(\phi_0 - \ln y^{(e)} + \ln(\epsilon y_0)). \tag{A7a}$$

Terms proportional to ϵ yield

$$\frac{dx_1}{d\tau} = -(\phi_0 - \ln x^{(e)} + \ln x^{(0)}), \qquad \frac{d\phi_0}{d\tau} = \frac{dx_1}{d\tau} + \frac{dy_0}{d\tau}. \tag{A7b, c}$$

The system of Eq. A7 is nonlinear and cannot be solved analytically; its numerical solution, however, is exactly the same as the numerical solution of the initial system of equations (not shown).

To obtain initial conditions for the posttransient solution, we must estimate the values of Φ and y_1 at the end of the initial transient. A simple and sufficiently accurate approach (which avoids the ln ϵ terms in more formal expansions) is to approximate $\ln(\epsilon y_0 \tau)$ by a constant: $\ln(\epsilon \hat{y}_0)$. Then the system becomes linear and can easily be solved:

$$\phi(\tau) = \left(\phi(0) - \frac{a}{2}\right)\exp(-2\tau) + \frac{a}{2}, \qquad a \equiv \ln\frac{x^{(e)}y^{(e)}}{x^{(0)}\epsilon\hat{y}_0}, \tag{A8a}$$

$$y_0(\tau) = \frac{1}{2}\left(\phi(0) - \frac{a}{2}\right)\exp(-2\tau) - \left(\frac{a}{2} + \ln y^{(e)} - \ln\epsilon\hat{y}_0\right)\tau + B,$$

$$B = y_0(0) - \frac{1}{2}\left(\phi_0(0) - \frac{a}{2}\right). \tag{A8b}$$

For large times, τ, $\phi_0 \to a/2$. Taking $\epsilon\hat{y}_0$ equal to the initial value $\epsilon\hat{y}_0(0)$ yields

$$\Phi_0 \to \frac{1}{2}[E_{ACh}(0) + E_{Na}(0)]. \quad \text{(A9)}$$

That is, independent of its initial value, to first approximation the vesicle potential rapidly approaches the average of the two equilibrium potentials.

Posttransient solution

We choose the appropriate new time scale $T = t/t_c = t\epsilon g/\kappa$, and corresponding dependent variables X, Y, and Φ. Expanding in powers of ϵ, we obtain at zeroth order

$$\frac{dX_0}{dT} = -(\Phi_0 - \ln x^{(e)} + \ln X_0), \quad \text{(A10a)}$$

$$\frac{dY_0}{dT} = -(\Phi_0 - \ln y^{(e)} + \ln Y_0), \quad \text{(A10b)}$$

$$\frac{dX_0}{dT} + \frac{dY_0}{dT} = 0. \quad \text{(A10c)}$$

Equation A10c yields, upon matching with $x + y = 1 + O(\epsilon)$,

$$X_0(T) + Y_0(T) \equiv 1, \quad \text{(A11)}$$

so that once again charge neutrality holds (to a first approximation), for all times.

Equating the sum of Eqs. A10a,b to zero, from Eq. A10c we obtain

$$\Phi_0(T) = \frac{1}{2}\left(\ln\frac{x_e}{X_0(T)} + \ln\frac{y_e}{Y_0(T)}\right). \quad \text{(A12)}$$

This formula, a specific case of the Goldmann equation, explicitly shows that after the initial transient, at all times the vesicle potential is given by the average of the transmitter and sodium potentials. Results from Eq. A12 are indistinguishable from the numerical results for Φ in Fig. 3 *B*.

Substituting Eq. A12 for the potential Φ_0 into Eq. A10a for $X_0(T)$ and using Eq. A11, we obtain an equation for $X_0(T)$. Integration yields the time T for the transmitter fraction in the vesicle to attain the value X^*:

$$T(X^*) = 2\int_{X^*}^{1} \frac{dX}{c + \ln X - \ln(1 + y^{(0)} - X)}, \quad \text{(A13)}$$

$$c = \ln\left(\frac{y^{(e)}}{x^{(e)}}\right) = \ln\left(\frac{Na^{(e)}}{ACh^{(e)}}\right).$$

Here $y^{(0)}$ is the (usually negligible) initial sodium fraction of mobile species in the vesicle. There is no discernible difference between the approximate analytic solution for $X_0(T)$, which is implicit in Eq. A13, and the numerical solution for control parameters, which is graphed as ACh/C_T in Fig. 3 *A*. Formula A13 can be approximated by a fractional-linear function of c (see Eq. 11).

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