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Spontaneous Calcium Transients in Autonomic Boutons and Varicosities

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Spontaneous multiquantal events are recorded at many different boutons and varicosities for which there is evidence that the receptor patch at these individual synapses is saturated by the transmitter unit. In order to reconcile these observations, a model is considered in which calcium release from a ryanodine channel within a nerve terminal can reach adjacent active zones in single synapses in sufficient concentration to occasionally trigger exocytosis from adjacent zones synchronously, giving rise to multiquantal spontaneous events. It is shown that the spatial and temporal distribution of calcium concentration at the active zone after a spontaneous opening of a ryanodine channel can predict the amplitude and time course of observed calcium-activated potassium channel currents. Similar calcium transients are sufficient to give rise to multiquantal events. Such events suggest a multiunit hypothesis for secretion.

Introduction

Difficulties have arisen from the earliest attempts to use quantal analysis to study synaptic transmission at peripheral synapses other than the neuromuscular junction (for a review, see Bennett, 1995). First, the amplitude histograms of spontaneous excitatory post-synaptic potentials (sEPSPs) are not distributed as Gaussian distributions, but often possess multimodes so that they are distributed as a Poisson mixture of Gaussians (see, for example, Martin & Pilar, 1964; Bornstein, 1981; Warren *et al*., 1995). Thus a unit step in the evoked synaptic potential may not simply be equated with the sEPSP, as in classical quantal analysis (del Castillo & Katz, 1954). Second, in the original quantal analysis the size of the quantum as given by the distribution of spontaneous potentials was taken to reflect the size of a unit of transmitter release (Kuffler & Yoshikami, 1975), whereas there is now evidence that at peripheral synapses the size of the small sEPSP is due to saturation of the receptor patch beneath the boutons by the unit of transmitter that is spontaneously released (Rang, 1981; Bennett *et al*., 1997a). These problems with quantal analysis as applied to peripheral synapses have recently been exacerbated by studies at central synapses that also indicate that the amplitudes of sEPSPs as well as of spontaneous inhibitory synaptic potentials (sIPSPs) are distributed as Poisson mixtures of Gaussians (Edwards *et al*., 1990; Ropert *et al*., 1990; Korn *et al*., 1993; Paulsen & Heggelund, 1994). Furthermore, as at peripheral synapses,

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present evidence favours the idea that the unit of transmitter released at these central boutons in general saturates the post-synaptic receptors (Clements, 1996).

Recently, an hypothesis has been put forward to account for the apparently conflicting observations at peripheral and central synapses. This hypothesis is that the unit of transmitter release is distributed multimodally but that the smallest mode in the distribution is due to a unit of transmitter release that saturates the post-synaptic receptor patch beneath the bouton. It is conjectured that spontaneous calcium transients (SCATs) may occur in a bouton sufficiently close to the pre-synaptic membrane to excite exocytosis of transmitter from vesicles attached to two or more adjacent active zones in the bouton, each with their own receptor patch (Bennett *et al*., 1998). Multimodal histograms of sEPSPs can then be generated, even though the unit of transmitter release from the individual vesicles is sufficient to saturate the receptor patches beneath the boutons. The question of whether action potentials can initiate such calcium transients within a bouton, so that evoked release receives a contribution from both vesicular release triggered by the local calcium entry through voltage-sensitive calcium channels in the active zone as well as to vesicular release triggered by local intraterminal calcium transients, remains for further consideration.

In the present work, an investigation is made into the plausibility of the SCAT hypothesis for the generation of multimodal histograms of sEPSPs at peripheral nerve terminals. Such transients have recently been observed in single sympathetic boutons in the rat superior cervical ganglion (Lin *et al*., 1997) as well as in single sympathetic varicosities in smooth muscle (Brain & Bennett, 1997); each of these release structures is about one to two micrometres in diameter. It is likely that these calcium transients are due to calcium sparks that arise from the release of calcium from localized groups of ryanodine (Ry) channels on the endoplasmic reticulum opposing the membrane, called here the ryanodine release unit (Berridge, 1997). Evidence for this comes from the observation that SCATs still occur after blocking voltage-dependent calcium channels. It seems very likely that these calcium transients originate from Ry channels that are in close apposition to the nerve terminal membrane, as they give rise to transient hyperpolarizations of the membrane due to the opening of calcium-activated potassium channels of the BK variety that are located there (Fletcher & Chiapinelli, 1992). These channels are located in the active zones of nerve terminals (Robitaille *et al*., 1993) and have been used to assay the concentrations of calcium reached within the zones following the arrival of a nerve impulse (Issa & Hudspeth, 1994; Roberts, 1994). The present work analyses the spontaneous hyperpolarizing transients in the nerve terminal in order to determine the characteristics of the calcium sparks that give rise to them. There is evidence that exocytosis of the contents of a synaptic vesicle at many terminals occurs from a ''secretosome'', consisting of a complex of a vesicle, vesicle-associated proteins that include the calcium-sensor protein for release, and a voltage-dependent calcium channel (O'Connor *et al*., 1993; Bennett *et al*., 1997b). The calcium sparks have also been used in the present work to ascertain whether they can trigger exocytosis from the secretosomes in adjacent active zones that occur in some boutons (Sargent & Pang, 1988, 1989), and so generate multimodal distributions of sEPSPs.

Methods

CALCIUM CONCENTRATION AT THE PLASMALEMMA DUE TO THE OPENING OF A SINGLE RYANODINE CHANNEL

An intracellular calcium store is contained within a nerve terminal, and the spontaneous opening of a single Ry channel associated with the store allows calcium to diffuse from the channel to the plasmalemma. This arrangement is modeled as buffered diffusion in a semi-infinite three dimensional space, where diffusion is restricted to the region $z \geqslant 0$. The Ry channel is located at the origin in an otherwise impermeable barrier at the plane $z = 0$, and the concentration of calcium in the plane $z = d$, corresponding to the plasmalemma, is calculated.

Let $c(\mathbf{r}, t)$ denote the excess calcium concentration at point $\mathbf{r}$, where $\mathbf{r} = (x, y, z)$ is a general point in three-dimensional space, at time t after

the opening of the Ry channel. Then (Fogelson & Zucker, 1985; Parnas *et al*., 1989)

$$\frac{\partial c}{\partial t} = \frac{D}{1+B}\nabla^2 c, \tag{1}$$

where D is the diffusion constant for aqueous solution, and B describes the buffering of calcium. This approximation assumes the presence of a fixed uniform unsaturable buffer and that the binding of calcium to it is instantaneous. It is a limiting form of the rapid buffering approximation that has been extensively discussed in a recent series of papers (Wagner & Keizer, 1994; Smith *et al*., 1996; Smith, 1996; see also Roberts, 1994). The assumption of instantaneous binding is reasonable, since the equilibration time is only a fraction of a ms. [Using the parameter values of Klingauf & Neher (1997) in eqn (20) of Wagner & Keizer (1994) gives characteristic times of 20–40 μs.] The neglect of a mobile buffer is more serious, as this can affect the calcium concentration profile particularly at short times and distances. However, the inclusion of these effects would greatly complicate the present calculations and is not warranted in the light of other approximations made and the uncertain values of many of the parameters. A further approximation is to treat both the plasmalemma and the reticulum as plane membranes, impermeable to calcium. In particular, this neglects the obstructive effect the vesicles could have on calcium diffusion and this could be significant, given that vesicles can have diameters up to 70 nm.

If the presence of the plasmalemma is temporarily neglected, then eqn (1) is to be solved subject to the boundary conditions

$$c \to 0 \text{ as } |x|,|y|, \text{ and } z \to \infty \tag{2}$$

and

$$D\frac{\partial c}{\partial z} = -F(x,y)g(t) \text{ on } z = 0, \tag{3}$$

and the initial condition

$$c(\mathbf{r}, 0) = 0. \tag{4}$$

The functions $F(x, y)$ and $g(t)$ describe the spatial location and temporal opening, respectively, of the Ry channel in the surface $z = 0$; in this work, calcium influx is assumed to occur at a point, so $F(x, y)$ is a two-dimensional Dirac delta function.

The release of calcium ions by the Ry channel is modelled as a current flowing from a point source. If the magnitude $\bar{g}$ of this current is constant for the duration of the channel open time then

$$g(t) = \begin{cases} A\bar{g} & \text{if } 0 \leqslant t \leqslant t_c, \\ 0 & \text{otherwise,} \end{cases} \tag{5}$$

where A is a scaling factor (see below). With the given boundary and initial conditions, eqn (1) is conveniently solved by Green's function techniques. The Green's function gives the concentration at the plasmalemma due to a unit instantaneous point source located at $\mathbf{r}_0 = (x_0, y_0, z_0)$. By the method of images (Carslaw & Jaeger, 1959) it is

$$G(\mathbf{r}, \mathbf{r}_0; t) = \frac{1}{(4\pi\mathscr{D}t)^{3/2}} \times \left\{ \exp\left[-\frac{(x-x_0)^2 + (y-y_0)^2 + (z-z_0)^2}{4\mathscr{D}t} \right] + \exp\left[-\frac{(x-x_0)^2 + (y-y_0)^2 + (z+z_0)^2}{4\mathscr{D}t} \right] \right\} \tag{6}$$

where the effective diffusion constant in the presence of buffering is given by $\mathscr{D} = D/(1+B)$. Convolution of the Green's function with the source current, described by $g(t)$, gives the calcium concentration due to a point source at $\mathbf{r} = 0$ as (cf. Bennett *et al*., 1995)

$$c(r, t) = \frac{1}{1+B}\int_0^t G(\mathbf{r}, \mathbf{0}, t-\tau)g(\tau)\,\mathrm{d}\tau = \frac{1}{1+B}\frac{2}{(4\pi\mathscr{D})^{3/2}}\int_0^t \frac{1}{(t-\tau)^{3/2}} \times \exp[-r^2/4\mathscr{D}(t-\tau)]g(\tau)\,\mathrm{d}\tau, \tag{7}$$

where $r = |\mathbf{r}|$. For $g(t)$ given by eqn (4) the integral in eqn (7) can be done to give (Bennett *et al*., 1995)

$$c(r, t) = \begin{cases} \dfrac{A\bar{g}}{2D\pi r}\left[1 - P\left(\dfrac{1}{2}, \dfrac{r^2}{4\mathscr{D}t}\right)\right], & \text{if } 0 \leqslant t \leqslant t_c, \\ \dfrac{A\bar{g}}{2D\pi r}\left[P\left(\dfrac{1}{2}, \dfrac{r^2}{4\mathscr{D}(t - t_c)}\right) - P\left(\dfrac{1}{2}, \dfrac{r^2}{4\mathscr{D}t}\right)\right], & \text{if } t > t_c, \end{cases} \tag{8}$$

where

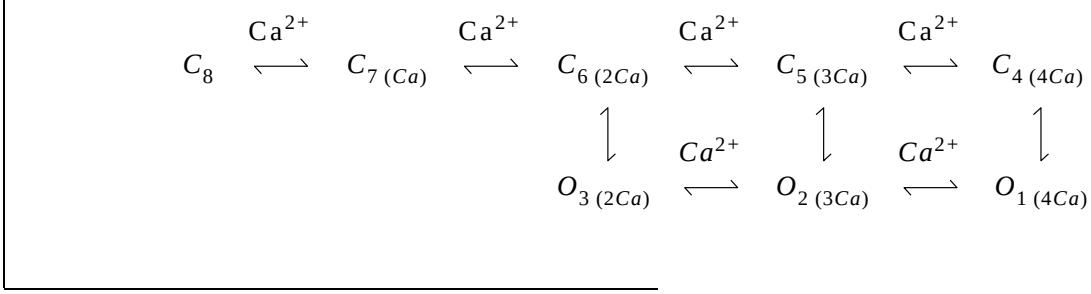
$$P(a, x) = \frac{1}{\Gamma(a)} \int_0^x \mathrm{e}^{-t} t^{a-1}\, \mathrm{d}t \tag{9}$$

is the incomplete gamma-function. If $\bar{g}$ is measured in pA then taking $A = 5.20$ gives $c(r, t)$ in μM.

The presence of the plasmalemma as the plane $z = d$ means that the calcium current flowing through the Ry channel from the intracellular store now enters a half-space between the endoplasmic reticulum and the plasmalemma. It is intuitively reasonable that the presence of two impermeable barriers will result in the doubling of the calcium concentration at the plasmalemma over that found in the single-barrier case considered above; that is, eqn (8) for $c(r, t)$ multiplied by a factor of 2 now gives the concentration of calcium at the plasmalemma. The proof rests on the method of images and is given in the Appendix. This "double-barrier" model is used in all the calculations reported in this paper.

STOCHASTIC BINDING OF CALCIUM TO A BK CHANNEL

The calcium that diffuses from a single Ry channel may bind to calcium binding sites associated with BK channels located at the plasmalemma. An increase in the open probability (P_{open}) of a BK channel with an increased intracellular calcium concentration ($[Ca^{2+}]_i$) is a characteristic of BK channels, and results from complex changes in channel kinetics. The simplest gating mechanism that describes the major features of the calcium-dependent kinetics of BK channels is given by McManus & Magleby (1991) as



This gating scheme describes the Ca^{2+}-dependence of the probability of a BK channel being in each of eight states, where C_n and O_n represent closed and open states, respectively. It exhibits three open states (O_n) and five shut (C_n) states, and incorporates four Ca^{2+} binding sites. The upper horizontal line gives the transitions between the five closed states, each transition being caused by the binding or unbinding of one calcium ion with the total number of bound ions in each state shown in brackets. The lower horizontal line shows the corresponding transitions for the closed states. The vertical arrows show the conformational changes between closed and open states; these transitions occur without the binding or unbinding of calcium ions. In general, the lifetimes of the shut states decrease

from C_8 to C_5, with C_4 then increasing. The lifetimes of the open states increase from O_3 to O_1 ($[Ca^{2+}]_i > 10$–$20\ \mu M$). The scheme accounts for the calcium-dependent shifts in both P_{open} and the channel open and shut dwell-time distributions for changes in $[Ca^{2+}]_i$. However, it does not take into account the possibility that the calcium concentration at the binding sites may not be the same as in the bulk solution (Moczydlowski & Latorre, 1983), or the voltage sensitivity of the gating of the channel (which has been considered in other models, e.g. Methfessel and Boheim, 1982; and Moczydlowski & Latorre, 1983). We employed this scheme to model the probability of a BK channel being in one of k states at time t after the opening of a Ry channel ($P_k(t)$).

Let k_{ij} be the rate constant for the transition from state i to state j. Then the rate of attachment of a calcium ion to each binding site is $k_{ij}[Ca^{2+}]_i$ for each relevant transition; unbinding and conformational changes have rates k_{ij}. Following standard techniques (Feller, 1950), we derive a set of coupled differential equations that describe the probability of a BK channel being in any particular state:

$$\frac{dP_1}{dt} = k_{41}P_4 - k_{14}P_1 + k_{21}cP_2 - 4k_{12}P_1,$$

$$\frac{dP_2}{dt} = k_{52}P_5 - k_{25}P_2 + 2k_{32}cP_3 - 3k_{23}P_2 + 4k_{12}P_1 - k_{21}cP_2,$$

$$\frac{dP_3}{dt} = k_{63}P_6 - k_{36}P_3 + 3k_{23}P_2 - 2k_{32}cP_3,$$

$$\frac{dP_4}{dt} = k_{54}cP_5 - 4k_{45}P_4 + k_{14}P_1 - k_{41}P_4,$$

$$\frac{dP_5}{dt} = 2k_{65}cP_6 - 3k_{56}P_5 + 4k_{45}P_4 - k_{54}cP_5 + k_{25}P_2 - k_{52}P_5,$$

$$\frac{dP_6}{dt} = 3k_{76}cP_7 - 2k_{67}P_6 + 3k_{56}P_5 - 2k_{65}cP_6 + k_{36}P_3 - k_{63}P_6,$$

$$\frac{dP_7}{dt} = 4k_{87}cP_8 - k_{78}P_7 + 2k_{67}P_6 - 3k_{76}cP_7,$$

$$\frac{dP_8}{dt} = k_{78}P_7 - 4k_{87}cP_8, \qquad (10)$$

where $c \equiv c(r, t)$ is the calcium concentration as given by eqn (8); the integers multiplying various terms are statistical factors which allow for the fact that a transition can be caused by a calcium ion binding to, or unbinding from, several different sites. Using the fact that $\Sigma_{i=1}^{8} P_i = 1$, this set of eight equations can be reduced to seven. McManus & Magleby (1991) give kinetic data for five BK channels. In our model, we employ their forward and backward rate constants for channel 1 (McManus & Magleby, 1991: Table 1).

Solution of eqn (11) (we use a fourth-order Runge-Kutta integration method with a constant step size, Press *et al*., 1992), now gives the probability of a BK channel being in each of its eight states at time t after the opening of a Ry

TABLE 1

Values of parameters used in the numerical simulations involving calcium diffusion, BK channel kinetics, and exocytotic kinetics

Quantity	Value	Reference
Calcium diffusion coefficient	$0.6\ \mu m^2\ ms^{-1}$	Fogelson & Zucker, 1985
Bound to free calcium ratio	100	Augustine *et al*., 1987
Ryanodine channel current	4–16 pA	Smith *et al*., 1988
BK channel current	7 pA	Dryer *et al*., 1991
BK channel separation	20 nm	Roberts, 1994; Robitaille *et al*., 1993
BK channel Ca^{2+} binding kinetics	Channel 1	McManus & Magleby, 1991
Exocytotic Ca^{2+} attachment rate (k^a)	$15 \times 10^6\ M^{-1}\ s^{-1}$	Bennett *et al*., 1997b
Exocytotic Ca^{2+} detachment rate (k^d)	$750\ s^{-1}$	Bennett *et al*., 1997b
Exocytotic rate of conformational change (β)	$2000\ s^{-1}$	Bennett *et al*., 1997b

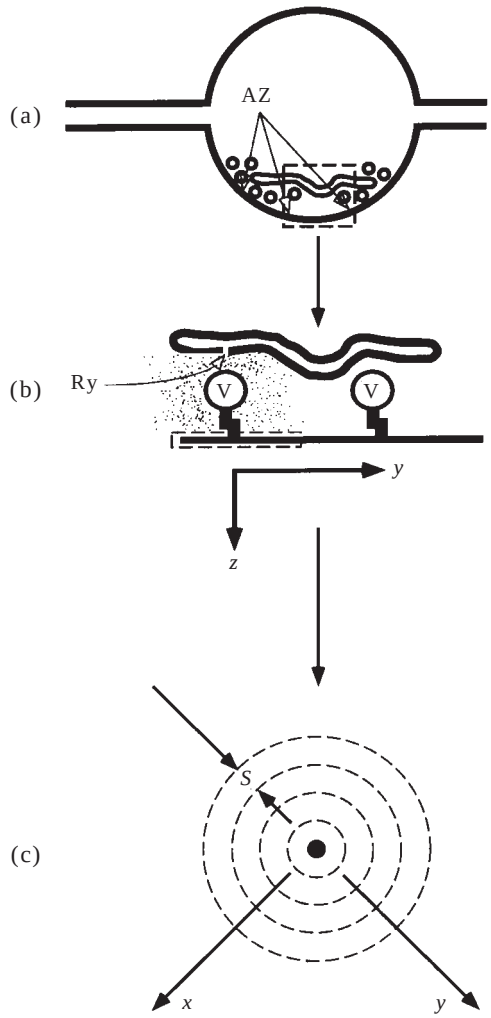


FIG. 1. Diagrams of the calcium-transient model: (a) a single bouton or varicosity possesses one or more active zones (AZ–indicated by arrows), around which are clustered docked vesicles attached to the active zone; (b) higher power view of the boxed area in (a). This shows single vesicles each docked in a secretosome (consisting of a vesicle, vesicle-associated proteins and a voltage-sensitive calcium channel) at two adjacent active zones that are y nm apart. Endoplasmic reticulum is positioned above these zones at a distance z nm from the secretosome; (c) higher power view of the boxed area in (b). This shows in plan view the distribution of calcium-activated potassium channels (of the BK type) in a concentric circular array around a central channel that is positioned next to a secretosome (●). The distance between these concentric circles of channels is S nm.

channel. The total open probability of a BK channel at time t is then given by summing the probability of the channel being in its three open states

$$P_{open}(t) = P_1(t) + P_2(t) + P_3(t) \qquad (11)$$

SPATIAL ARRANGEMENT OF BK CHANNELS AT THE PLASMALEMMA

BK channels are assumed to be located at the plasmalemma in a plane distance $z = d$ from the Ry channel [Fig. 1(b)]. They exhibit a concentric arrangement about a reference channel K_0 located in the plasmalemma at the position $r = (0, 0, d)$ [Fig. 1(c)], such that the reference channel is directly opposite the Ry channel. The radial separation of each concentric ring of BK channels from the reference channel is then given by

$$Y_n = nS, \quad n = 1, 2, 3, \ldots, N, \qquad (12)$$

where S is the distance between each ring of channels and N is the total number of rings [Fig. 1(c)]. The average number of channels at each radial separation Y_n is then assumed to be

$$S_n = 2\pi n, \quad n = 1, 2, 3, \ldots, N. \qquad (13)$$

According to this scheme there are approximately 20 BK channels present when $N = 2$ and the reference channel is counted. In the model, this is the number of BK channels assumed to be located at the plasmalemma under a Ry channel (see Fletcher & Chiapinelli, 1992).

CALCULATION OF THE TOTAL BK CHANNEL CURRENT

The average current $I_n(t)$ generated by the opening of a representative BK channel (located at the position $r = (0, Y_n, d)$) is given as a proportion of the maximum current (I_m) scaled by the total open probability ($P_{open}(t)$) calculated for the channel:

$$I_n(t) = P_{open}(t) I_m. \qquad (14)$$

Summation then gives the average current $I_T(t)$ generated by the population of BK channels activated at the plasmalemma:

$$I_T(t) = \sum_{n=1}^{N} I_n(t) S_n + I_0(t), \qquad (15)$$

where I_0 is the average current generated by the reference channel.

STOCHASTIC BINDING OF CALCIUM TO THE EXOCYTOTIC VESICLE-ASSOCIATED PROTEIN

Following Bennett *et al*. (1997b) we assume that four sites on a particular vesicle-associated protein must each bind a calcium ion before a conformational change and exocytosis of a vesicle is triggered. The simplest case occurs when all sites have the same affinity, in which case the kinetic scheme is

$$S_0 \underset{v_1}{\overset{4\mu_1}{\rightleftarrows}} S_1 \underset{2v_2}{\overset{3\mu_2}{\rightleftarrows}} S_2 \underset{3v_3}{\overset{2\mu_3}{\rightleftarrows}} S_3 \underset{4v_4}{\overset{\mu_4}{\rightleftarrows}} S_4 \overset{\beta}{\longrightarrow} S_{ex},$$

where S_i, $i = 1, \ldots, 4$, denotes the state with i calcium binding sites occupied, S_{ex} denotes the state with all four sites occupied after the conformational change, $\mu_i(t)$ and $v_i(t)$ are the respective rates of attachment and detachment of a calcium ion at the i-th step and $\beta(t)$ is the rate for the final exocytotic step. (See Bennett *et al*., 1997b, for a discussion of more complex schemes.) It is then easy to write a set of coupled differential equations for the probabilities $P_i(t)$ that the system is in state i at time t:

$$\begin{aligned}
\frac{dP_0}{dt} &= -4\mu_1 P_0 + v_1 P_1, \\
\frac{dP_1}{dt} &= 4\mu_1 P_0 - (3\mu_2 + v_1)P_1 + 2v_2 P_2, \\
\frac{dP_2}{dt} &= 3\mu_2 P_1 - (2\mu_3 + 2v_2)P_2 + 3v_3 P_3, \\
\frac{dP_3}{dt} &= 2\mu_3 P_2 - (3v_3 + \mu_4)P_3 + 4v_4 P_4, \\
\frac{dP_4}{dt} &= \mu_4 P_3 - (4v_4 + \beta)P_4, \\
\frac{dP_{ex}}{dt} &= \beta P_4, \qquad (16)
\end{aligned}$$

with initial conditions

$$P_0(0) = 1, \quad P_k(0) = 0, \quad k = 1, \ldots, 4, \quad P_{ex}(0) = 0. \quad (17)$$

The rate of exocytosis is then dP_{ex}/dt and the probability of exocytosis occurring is the asymptotic value of dP_{ex}/dt for large t. The attachment rates μ_i are taken to be proportional to the calcium concentration at the position of the vesicle-associated protein, and the detachment rates v_i are taken to be constants, independent of time:

$$\mu_i(t) = k_i^a c(r, t), \quad v_i(t) = k_i^d, \quad i = 1, \ldots, 4, \qquad (18)$$

and the rate of the conformational change β is also taken to be a time-independent constant. A further simplification is to assume no cooperativity in binding or unbinding, so that $k_i^a = k^a$ and $k_i^d = k^d$, $i = 1, \ldots, 4$.

DETERMINATION OF PARAMETER VALUES REPLICATING OBSERVED SPONTANEOUS OUTWARD CURRENTS IN THE CALCIFORM TERMINAL

The numerical values of the parameters used in the calculations involving the opening of the Ry channel and the diffusion of calcium from this channel to bind with the BK channels located at the plasmalemma are listed in Table 1. Dryer *et al*. (1991) report a mean current amplitude of 10.18 pA for a single BK channel in an inside-out patch held at -35 mV. We employed the conservative value of 7 pA for our simulated BK channel current assuming a holding potential of -60 mV. Table 1 also gives the source of the rate constants for the kinetic scheme describing the attachment and detachment of calcium ions to binding sites associated with the BK channel [see eqn (11)] as well as the rate constants for the attachment and detachment of calcium ions to the exocytotic vesicle-associated protein and the rate constant for the conformational change of the exocytotic vesicle-associated protein leading to exocytosis of the vesicle [see eqn (16)].

We characterised the mean amplitude, time to peak, time constant of decay (given as the time taken for the membrane potential to fall to $1/e$ of its peak value), and half width of spontaneous hyperpolarizations of the membrane potential (given in Fig. 1 of Fletcher & Chiapinelli, 1992). An equivalent circuit incorporating a BK conductance was then employed to generate a representative membrane hyperpolarization. The current transform of the representative membrane transient was then obtained from this equivalent circuit. Subsequently, the SCAT model was employed to explore the effect on the temporal and spatial characteristics of these

transients by altering the geometry of the Ry and BK channels, while Ry channel open time and separation from the plasmalemma were varied as were the numbers of BK channels and their density.

Results

THE SPONTANEOUS CALCIUM TRANSIENT (SCAT) MODEL

In this model, synaptic boutons or varicosities possess vesicles that are located in secretosomes that are docked at active zones, and are frequently in close apposition to a Ry release unit in an overlying endoplasmic reticulum [Fig. 1(a)]. Some synaptic boutons and varicosities possess more than one active zone, with the distance between the zones being of the order of several hundred nanometres [in the y direction, see Fig. 1(b)]. The distance between a particular secretosome of interest and its overlying Ry release site is of the order of a hundred nanometres or less [in the z direction, see Fig. 1(b)]. Surrounding the synaptic vesicle in the secretosome are concentric rings of calcium-activated potassium channels of the BK variety [arranged so that they are S nm apart, see Fig. 1(c)]. The properties of this model will be investigated below, first to see if it correctly describes the spontaneous hyperpolarizing transients in the terminals, and second whether it provides an explanation for the appearance of multiunit spontaneous potentials in neurons and muscles.

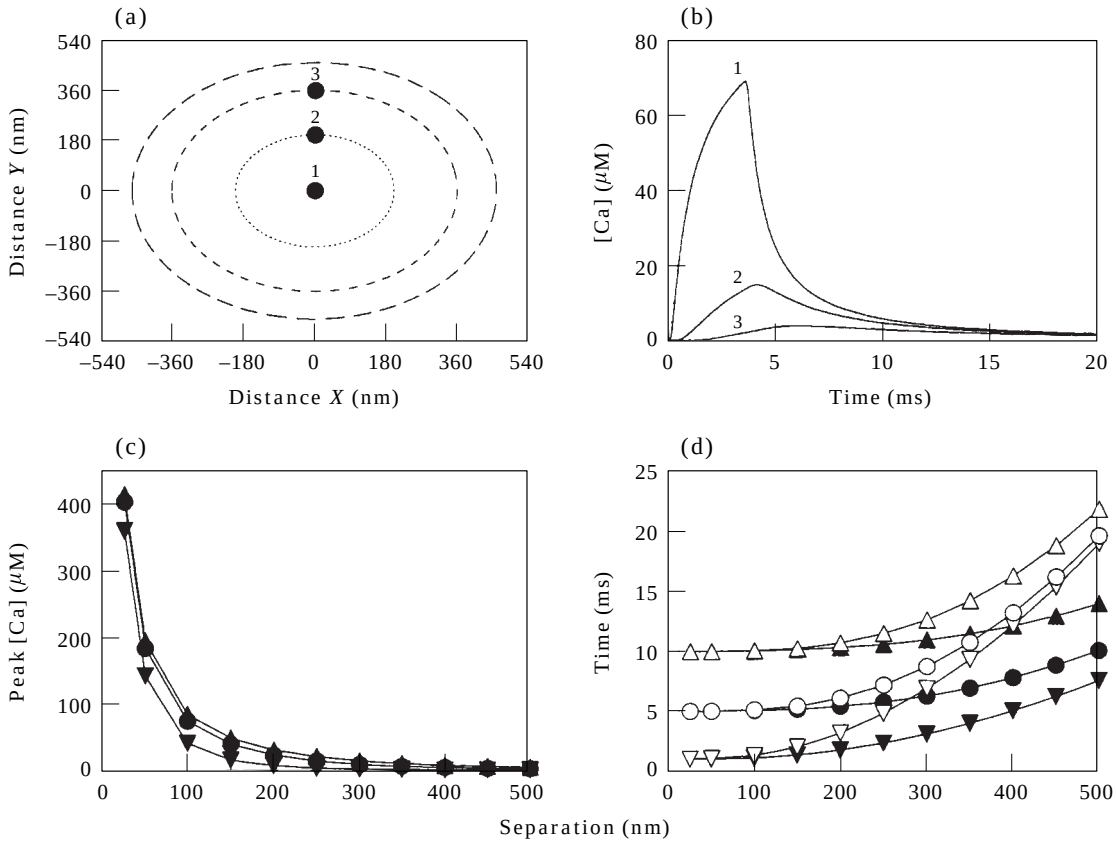


FIG. 2. Theoretical changes in calcium transients in a bouton or varicosity following release of calcium from a ryanodine release unit: (a) shows spatial contour maps of the extent of the calcium concentration equivalent to one-half its peak value at 5 ms (. . .), 10 ms (– – –) and 15 ms (— —) after the opening of a ryanodine channel (open time 3.5 ms, current 4 pA), positioned 100 nm above the secretosome [see Fig. 1(b)]; (b) shows the time course of calcium concentration after the opening of the Ry channel, at positions marked 1, 2 and 3 in (a); (c) gives the peak value of the calcium transient when the ryanodine channel is located at distances of $z = 25$–500 nm (abscissa) above the secretosome for open times of the ryanodine channel of 1, (▼) 5 (●), and 10 (▲) ms [see Fig. 1(b)], (d) gives the time to peak (filled symbols) and the duration of its half-width (open symbols) of calcium transients when the ryanodine channel is separated by distances of 25–500 nm (abscissa) above the secretosome [see Fig. 1(b)] for the different open times given by the legend in (c).

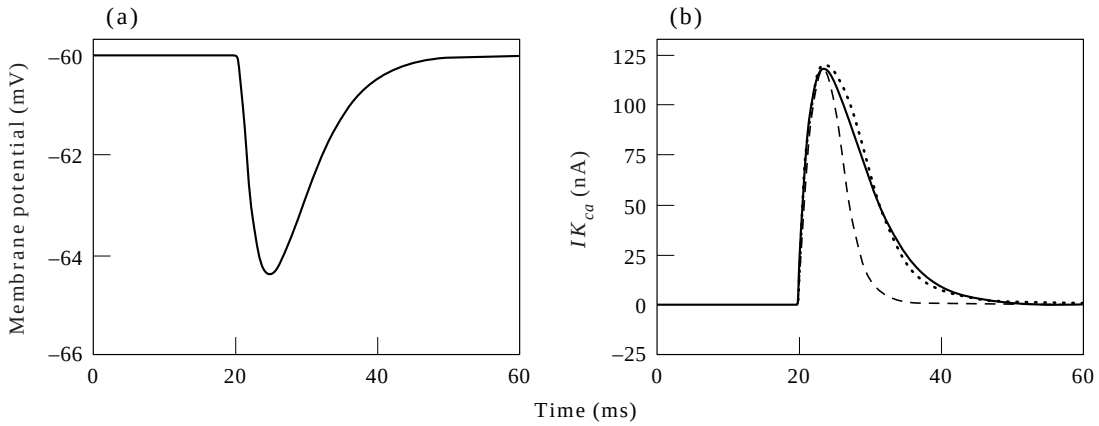


FIG. 3. Comparison between the observed hyperpolarizing transients and the theoretical predictions of the calcium-transient model: (a) mean voltage transient of the events reported by Fletcher & Chiapinelli (1992) is generated with the equivalent circuit described in the text; (b) the current through the BK channel conductance (IK_{Ca}) that generates the voltage shown in (a) is given by (—). The calcium-transient model predicts a similar IK_{Ca} for ryanodine channel currents of 16 pA located 100 nm from the plasmalemma (. . .). For ryanodine channel currents of 4 pA located 25 nm from the plasmalemma the model predicts an IK_{Ca} given by the (– – –).

SCATS IN NERVE TERMINALS

Equation (8) (with a multiplicative factor of 2 to allow for the double-barrier effect) has been used to calculate the calcium concentration at the plasmalemma due to the opening of a Ry release unit situated a distance of 100 nm above the pre-synaptic membrane. The release unit opens for 3.5 ms and carries a current of 4 pA. Figure 2(a) shows three concentration contours on the plasmalemma for times 5, 10 and 15 ms, respectively, after the Ry release unit opens; each contour gives the spatial location at which the calcium concentration is one-half its value at the centre at that time. The temporal profiles of the calcium concentration at the positions marked 1, 2 and 3 in Fig. 2(a) are shown in Fig. 2(b). At position 1, directly opposite the Ry release unit, the concentration increases steeply to a peak of 69 μM just after the release terminates at 3.5 ms and then declines rapidly. For positions 2 and 3 the peak concentrations are much lower and are attained later. Figure 2(c) shows how the peak calcium concentration at the plasmalemma depends on the open time of the Ry release unit and on its distance from the plasmalemma. There is little concentration change for open times beyond 5 ms. The concentration decreases rapidly with increasing separation of the Ry release unit and the plasmalemma, but is still of the order of 10 μM at a separation of 300 nm. The sequestering processes, other than the buffer, work on a longer time frame than the diffusion times for calcium, so that it is legitimate to consider the duration of the calcium pulse that appears at different distances from the opening Ry release unit. Figure 2(d) shows the way in which the time to peak and the duration at half-height of the calcium transient increase with increasing separation of the Ry release unit and the plasmalemma.

SPONTANEOUS HYPERPOLARIZING TRANSIENTS IN NERVE TERMINALS

Intracellular recordings with high impedance electrodes from the calyx of avian ciliary ganglia reveal a hyperpolarizing transient with a mean amplitude of 4.4 mV, a mean rise time of 4.8 ms, a mean decay time constant (time to $1/e$ of the peak amplitude) of 8.1 ms, and a mean width at half-height of 9.4 ms (Fletcher & Chiapinelli, 1992). It is very likely that these spontaneous hyperpolarizing transients have their origins in the activation of BK channels by the release of calcium from the endoplasmic reticulum, as they are increased greatly in frequency by caffeine and blocked by charybdotoxin. These transients can be generated by a model of the calyx consisting of a resistance of 39 MΩ in parallel with a capacitance of 26 pF, with a resting potential of -60 mV and a driving force for the conductance introduced by the BK

channels of −90 mV. The BK channel conductance in this circuit is modeled as an alpha function with a time to peak of 3.7 ms, and a maximum conductance of 190 pS. The amplitude of a representative hyperpolarization is matched by scaling this conductance to give the conductance predicted by 24 BK channels [Fig. 3(a)].

Calcium-activated BK channels are found in arrays within the active zones of nerve terminals. The SCAT model has therefore been used to see if the characteristics of the hyperpolarizing transients can be predicted on the basis that they arise from the SCATs within the active zone as a consequence of the release of calcium from a Ry release unit. The spatial distribution of BK channels is not known for boutons and varicosities (see Issa & Hudspeth, 1994, for hair cells, and Robitaille *et al*., 1993, for neuromuscular junction). For simplicity, the BK channels are assumed to be organized in concentric rings around a secretosome within the active zone, with different densities of the channels determined by the distance (S nm) between the rings, and different numbers of channels given by the number of rings present [Fig. 1(c)]. Calcium transients at the rings due to the opening of a Ry release unit at different distances from the rings, given by the coordinate z in Fig. 1(b), have been determined and used to generate the resulting IK_{Ca} currents by solving eqns (8)–(15). Figure 3(b) shows two representative examples

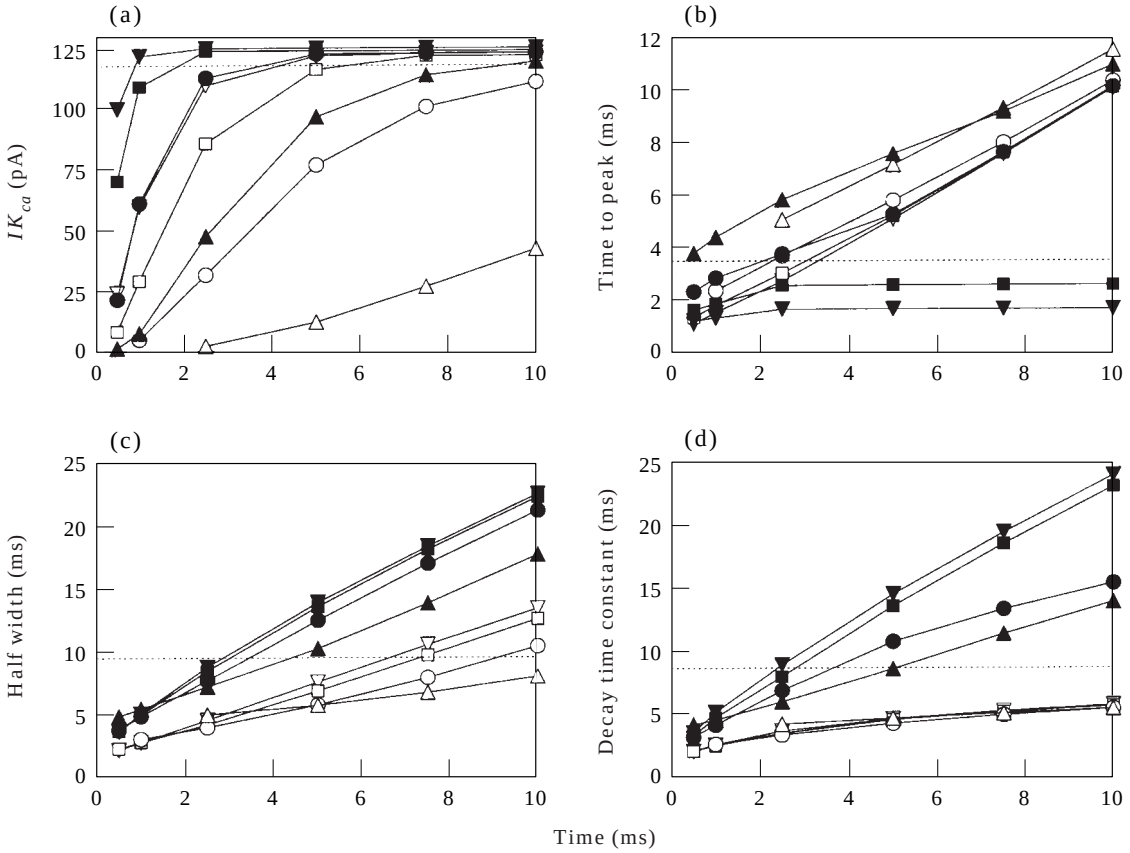


FIG. 4. The theoretical characteristics of hyperpolarizing transients in the calcium-transient model involving the diffusion of calcium from a spontaneously opening ryanodine release unit (open symbols: 4 pA, filled symbols 16 pA) opening for times of 0.5–10 ms (given in the abscissa). The release unit is separated by a distance of 25 to 200 nm from a group of 20 calcium-activated potassium channels (7 pA BK channels) situated concentrically around a secretosome and separated by ≈20 nm. In each case the values are indicated by a different symbol as given in the legends in (b) and (d); (a) amplitude of the potassium transient [(▽) 25 nm; (□) 50 nm; (○) 100 nm; (△) 200 nm; (. . .) 118 pA], (b) time to peak of the transient [(. . .) 3.5 ms]; (c) duration of the transient at half-height [(. . .) 9.5 ms]; (d) decay time constant of the transient [(. . .)] 8.6 ms]. The horizontal dashed line in each panel gives the values according to the analysis of the hyperpolarizing transients given in Fig. 3.

of hyperpolarizing transients that result from these calculations for two sets of values. These were for dimensions $z = 25$ (dashed line) and $z = 100$ nm (dotted line), $y = 20$ nm, and Ry currents of 4 (dashed line) and 16 pA (dotted line), respectively. The open time of the Ry channel was 3.5 ms, the BK current was 7 pA, and there was an average of 20 BK channels located concentrically at the plasmalemma. The 16 pA Ry channel transient shows a good match to the observed current [Fig. 3(b) solid line]. A Ry current of this magnitude can be generated by the synchronous opening of 4 Ry channels.

The sensitivity of the observed IK_{Ca} to changes in the open time of the Ry release unit and of its position with respect to BK channels is shown in Fig. 4 for channel currents of 4 pA (unfilled symbols) and 16 pA (filled symbols). In each panel of this figure the dotted horizontal line represents the observed characteristics of the IK_{Ca} defined from the observations of Fletcher & Chiapinelli (1992). The IK_{Ca} current is typically quite sensitive to changes in both the distance of the Ry channel from the plasmalemma and in the open times of the Ry channel. The IK_{Ca} current saturates when the Ry channel is located within 50 nm of the plasmalemma for Ry channel currents of 4 to 16 pA and open times greater than about 5 ms [Fig. 4(a)]. The time to peak of IK_{Ca} becomes independent of the open time of the Ry channel for Ry currents of 16 pA and distances less than 50 nm [Fig. 4(b)]. In contrast, the decay time constant of the IK_{Ca} generated by a 4 pA Ry channel current is effectively independent of the distance of the Ry channel from the plasmalemma, between 25 and 200 nm, and is little influenced by the open time of the Ry channel [Fig. 4(d)]. This is not the case for the half width of the IK_{Ca}, which is dependent on the open time of the Ry channel over all distances between 25 and 200 nm and current strengths between 4 and 16 pA [Fig. 4(c)].

SPONTANEOUS EXCITATORY POST-SYNAPTIC POTENTIALS AT NERVE TERMINALS

It has already been noted in the Introduction that amplitude–frequency histograms of sEPSPs in autonomic ganglion cells as well as spontaneous synaptic potentials in the central nervous system are often best described by a Poisson distribution rather than a Gaussian distribution. Such results indicate that there could be spontaneous multiunit events. Present analysis of the distribution and number of receptors beneath a bouton on sympathetic ganglion cells indicate that these are between 100 and 200 in number and that they are saturated to within stochastic limits following the release of a quantum of transmitter (Bennett *et al*., 1997a). This being the case, it is necessary to consider a scenario in which adjacent active zones within the same bouton or varicosity, each with their own receptor patch, simultaneously release a quantum of transmitter and so give rise to a multiunit event in the amplitude–frequency

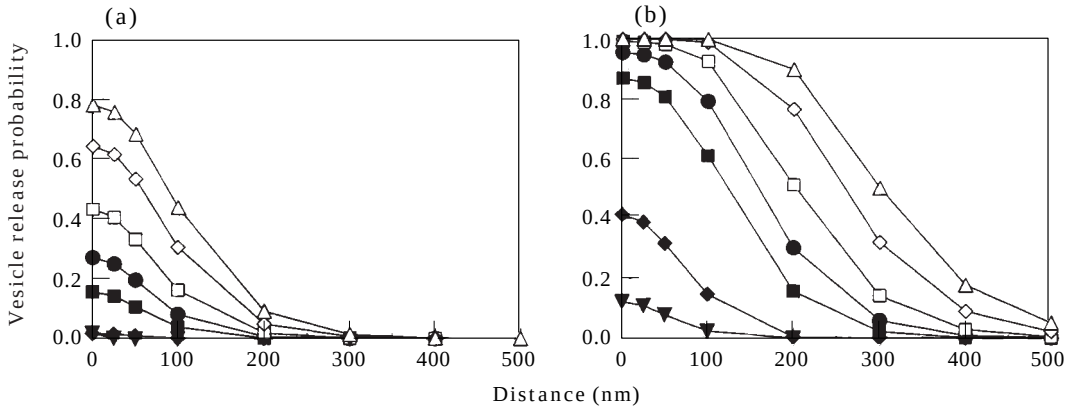


FIG. 5. Predictions of the calcium-transient model for the probability of vesicle release for two different magnitudes of ryanodine current when the ryanodine release unit is 100 nm from the plasmalemma. The probability of vesicle release is given for different radial distances of 25–500 nm (indicated on the abscissa): (a) ryanodine current of 4 pA. (▼) 0.5 ms, (◆) 1.0 ms; (■) 2.5 ms; (●) 3.5 sms; (□) 5.0 ms; (◇) 7.5 sms; (△) 10.0 ms; (b) results for a ryanodine current of 16 pA.

histogram of sEPSPs (Bennett *et al*., 1997a). The coupling of adjacent active zones in the same bouton can be achieved using the SCAT model. Similar model parameters as those used to generate the characteristics of the spontaneous hyperpolarizing currents are again utilized, only with the additional feature of placing a secretosome at the middle of the concentric array of BK channels. Solving eqns (16) to (18) for the action of the calcium transient on the calcium sensor in the secretosome then gives the probability of secretion of a quantum from the secretosome.

Figure 5 shows the probability of exocytosis from a secretosome for Ry channel currents of 4 and 16 pA [Fig. 5(a) and (b), respectively], and for different open times of the channel. It will be noted that when the Ry channel is separated from the plasmalemma by 100 nm and is open for 3.5 ms, the probability of vesicle exocytosis increases greatly as the Ry channel current is increased from 4 to 16 pA (from 0.08 to 0.79). For secretosomes situated on the plasmalemma 300 nm away, these values reduce to 0.001 and 0.06, respectively.

The probability of multiquantal release in chick ciliary ganglion cells can now be estimated and compared with the results given by Martin & Pilar (1964). From their Figure 7, it can be estimated that the relative frequency of multiquantal events to the total number of events is about 0.14. The conditions under which this proportion of multiquantal events is predicted by the SCAT model is given in Fig. 6 for a geometry

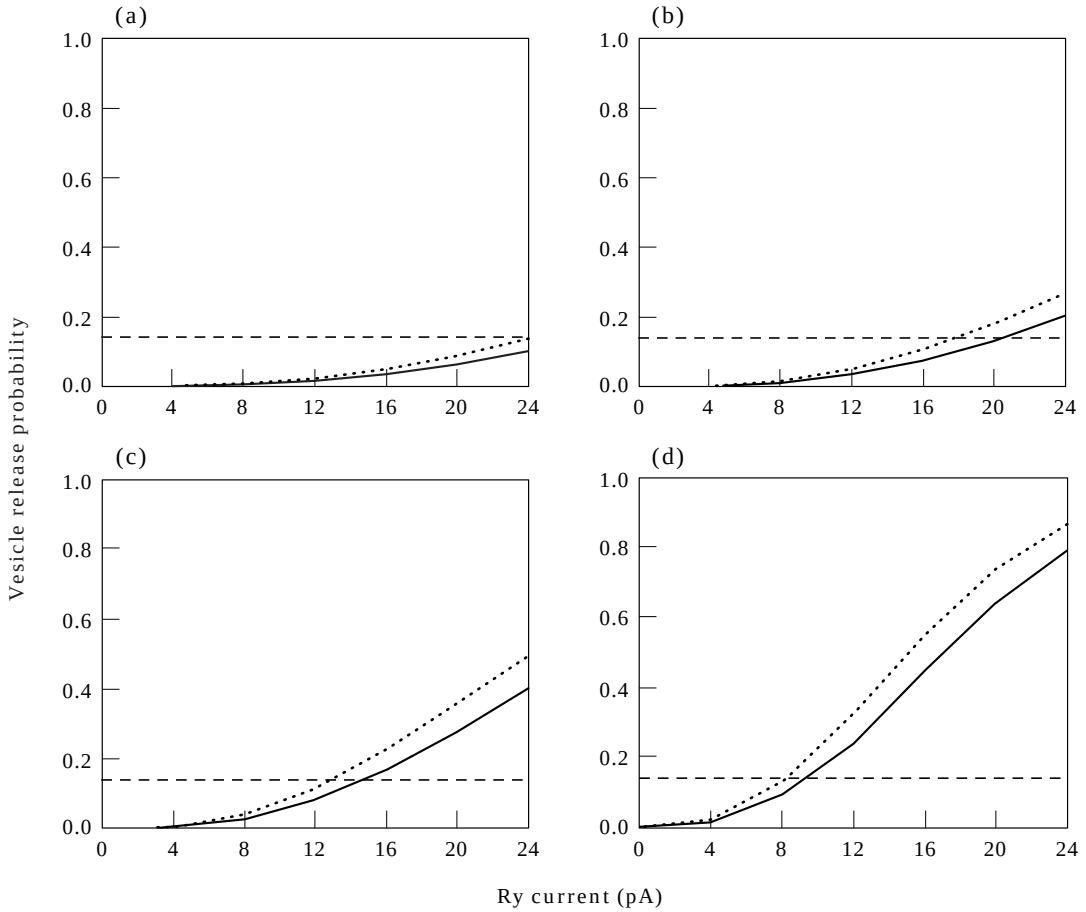


FIG. 6. Effect of the magnitude of the Ry channel current and open time on the probability of multiquantal release of neurotransmitter. The ordinate gives the probability of quantal release occurring at a site 300 nm in the *y* direction (see Fig. 1) as well as at the origin, conditional upon there being release at at least one of these sites. The abscissa is the Ry channel current. Results are given for Ry channels located at either 25 nm or 100 nm in the *z*-direction from the plasmalemma with open times of 2.5 ms (a), 3.5 ms (b), 5.0 ms (c), and 8.5 ms (d). The dashed horizontal line gives the value (0.14) for multiquantal release observed by Martin & Pilar, (1964). (. . .) 25 nm; (—) 100 nm.

in which a secretosome is located at the plasmalemma 300 nm in the y-direction from the reference position secretosome. For example, the proportion 0.14 can be reached if the Ry release unit is located 25 nm from the plasmalemma and this has a current of 24, 18, 13 and 9 pA for open times of 2.5, 3.5, 5.0 and 8.5 ms, respectively [panels (a), (b), (c) and (d), respectively]. If the Ry release unit is located 100 nm from the plasmalemma then the proportion of 0.14 can be reached by currents of more than 24, 20, 15 and 10 pA for open times of 2.5, 3.5, 5.0 and 8.5 ms, respectively [panels (a), (b), (c) and (d), respectively].

Discussion

SCATs in nerve terminals

Coordinated calcium fluctuations occur throughout the large (greater than 10 μm diameter) boutons at the neuromuscular junctions on snake muscles (Melamed *et al*., 1993). These fluctuations may consist of oscillations with a period of several seconds and involve coordinated changes in calcium concentration in adjacent boutons. SCATs have also been reported for synaptic boutons in the typical size range of about 1 to 2 μm, and occur at both mammalian autonomic synapses as well as neuromuscular synapses (Brain & Bennett, 1997; Lin *et al*., 1997). These SCATs do not oscillate and the coupling of calcium changes in adjacent boutons and varicosities is clearly due to diffusion from the bouton undergoing the transient to the adjacent boutons. These transients originate from the spontaneous release of calcium from Ry channels in the endoplasmic reticulum of the boutons and varicosities, as the transients are not affected by voltage-sensitive calcium channel blockers such as nifedipine, omega-conotoxin GIVa, or nickel; the last two agents have a profound effect on the impulse evoked calcium transients in the boutons (Lin *et al*., 1997).

The characteristics of the SCATs may be compared with that of the calcium sparks observed in muscle following the release of calcium from a small group of Ry channels. The sparks can be observed to reach a maximum in about 10 ms over a 2 μm extent in cardiac muscle and then decline so that the duration at half-height is about 20 ms (Cheng *et al*., 1993). Different estimates have been made for the number of Ry channels that open to give these sparks. There appear to be about four in the case of cardiac muscle in which the set are thought to make up an elementary unit of release in association with a membrane L-type calcium channel that might trigger the release of calcium from the set of Ry channels (Cannell *et al*., 1994); this set of Ry channels then gives rise to a calcium current of about 1.0 pA (Stern, 1992; Cannel *et al*., 1995). In the case of skeletal muscle it appears that the elementary unit of release is a single Ry channel, giving rise to a calcium current of about 0.1 pA, perhaps coupled to a single L-type calcium channel at the membrane (Tsugorka *et al*., 1995); this may under some circumstances evoke release from adjacent voltage-dependent Ry channels in the endoplasmic reticulum (Klein *et al*., 1996). Estimates of the conductance of an open Ry channel are between 70 pS (the cardiac Ry receptor; Anderson *et al*., 1989) and 177 pS (skeletal muscle reticulum; Smith *et al*., 1985), with most estimates about 100 pS (Ashley, 1989; Rousseau & Meissner, 1989; Smith *et al*., 1988).

Spontaneous miniature outward currents (SMOCs)

SMOCs have been recorded in skeletal muscle simultaneously with the appearance of SCATs, indicating that the latter probably gives rise to the former (Nelson *et al*., 1995). Furthermore, the temporal characteristics of the SMOCs in this muscle are similar to those of the SCATs, namely a rise time of about 17 ms and a duration at half-height of about 65 ms; they occupy a few microns. Ry and thapsigargin block both the SCATs and the SMOCs with the latter shown to be due to calcium-activated potassium currents (Nelson *et al*., 1995). Mathers & Barker (1981) reported that SMOCs could also be recorded in cultured mouse dorsal root ganglion cells. These SMOCs are likely to be due to the release of calcium from internal stores as they are accelerated in number before being abolished by caffeine; furthermore, calcium probably acts on calcium-sensitive potassium channels to produce

the SMOCs as they are blocked by tetraethylammonium ions (Mathers & Barker, 1984). SMOCs were subsequently reported for a number of other neurons such as those in the rabbit pelvic ganglion (Nishimura *et al*., 1988) and in bullfrog ganglia in which their characteristics have been described in detail (Satin & Adams, 1987). The current involved in SMOCs is typically less than 300 pA, has a rise time of less than 3 ms and a time constant of decay at the resting potential of about 9 ms; these authors estimated that about 10 IK_{Ca} channels are required to generate the 50 pA size SMOCs observed in this neuron. SMOCs have also been observed in the calyx of the chick ciliary ganglion (Fletcher & Chiappinelli, 1992), in which under current-clamp recording they typically have a rise time of less than 5 ms and a duration at half-height of less than 10 ms, requiring about 15–60 IK_{Ca} channels to be initiated. The size and temporal characteristics of the SMOCs in nerve terminals are then similar to those in the neuron soma. The difference in the temporal characteristics of the SMOCs in neurons and nerve terminals on the one hand and that in skeletal muscle on the other may be due to the former involving the opening of only a single Ry channel whereas the latter involves the opening of several channels.

CALCIUM-ACTIVATED POTASSIUM CHANNELS

Channels of the BK type occur in nerve terminals such as those of hair cells that have channels with a conductance of between 130 and 320 pS, open times of between 0.1 and 1.2 ms and are fully activated at -35 mV in the presence of a calcium concentration of 50 μM (Sugihara, 1994). These cells also possess SK calcium-activated potassium channels of about 30 pS which are about five times less sensitive to calcium at -35 mV than the BK channels (Art *et al*., 1995).

THE MULTIMODAL HISTOGRAMS OF SEPSPS AND THE UNIT OF TRANSMITTER RELEASE

The present work shows that using the properties of SCATs required to generate the observed SMOCs in nerve terminals is such that they may be responsible for the release of vesicles from adjacent active zones within the same bouton or varicosity. The spatial limits on this are such that the probability of multivesicular release falls to very small values if the zones are more than about 300 nm apart, in the case of a Ry channel opening within one active zone and affecting another, or about 600 nm in the case of a Ry channel opening midway between active zones. Boutons in autonomic ganglia possess an average of about three active zones, each with individual receptor clusters that are separated by distances of the order of 600 nm (Sargent & Pang, 1989; Wilson Horch & Sargent, 1996). It is suggested then that spontaneous multiquantal secretion occurs as a consequence of this spatial layout of active zones together with appropriately distributed Ry channels.

An important assumption in this model concerns the value of the affinities for calcium of the different binding sites on the calcium sensor that triggers exocytosis. We have recently provided arguments for the values chosen in the present work when considering how calcium entry through voltage-dependent calcium channels in secretosomes at the active zone during an action potential triggers exocytosis from vesicles (Bennett *et al*., 1997b). However there are no reliable estimates of the values of these affinities at this stage, even if those on the putative calcium sensor synaptotagmin are considered (Südhof, 1995). Furthermore, there is no information at present on the location of Ry channels of endoplasmic reticulum in the region of the active zones of synapses. There is then no evidence to support the present speculations that these receptors may occur in spatial configurations that allow the spread of calcium at high concentrations (in the μm range) to two or more active zones. If, however, Ry release channels can be shown to be distributed at sites between the multiple active zones found in many boutons then the present work shows that release of calcium from them may be responsible for the multimodal and skewed histograms of sEPSPs that are observed at many synapses (Bennett, 1995). This idea may not be applicable to some central synapses for which the histograms of sEPSPs are skewed with large variance, but do not show multimodal peaks. Indeed, it may be that this variance is solely due to the variance of the size of transmitter packets that are released

onto a large receptor patch, as has recently been argued (Frerking *et al*., 1997).

The present work suggests an alternative to the conventional quantal hypothesis that the unit response arises from a single packet of transmitter released onto a large and therefore unsaturated receptor patch. Rather, the unit of transmission at these synapses is possibly the saturated receptor patch. This may receive multiple quanta during either spontaneous or evoked transmission, following the opening of a single voltage-dependent calcium channel at the active zone (Bennett *et al*., 1995) or the opening of a Ry release channel. However, it still only gives rise to the same signal, namely that of the saturated receptor patch. Multiunit behaviour at those synapses for which it has been observed, may occur either spontaneously or during evoked transmission by the recruitment of quantal release from several adjacent active zones within the same bouton or varicosity through the action of the Ry release unit. This mode of action is fundamentally different from that described by the quantal hypothesis and gives rise to a multiunit hypothesis for synaptic transmission.

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APPENDIX

It is now necessary to prevent the flow of calcium ions across both the plasmalemma (the plane $z = d$) and the membrane of the endoplasmic reticulum (the plane $z = 0$), so images must be used for both. This leads to an infinite sequence of image sources, of which only the first few are of any practical importance.

Following Carslaw and Jaeger [1959, p. 374, eqn (19)], the Green's function of eqn (6) is to be replaced by

$$G(\mathbf{r}, \mathbf{r}_0; t) = \frac{1}{(4\pi\mathscr{D}t)^{3/2}} \exp\left(-\frac{(x-x_0)^2 + (y-y_0)^2}{4\mathscr{D}t}\right) \times \sum_{n=-\infty}^{\infty} \left[\exp\left(-\frac{(2nd + z_0 - z)^2}{4\mathscr{D}t}\right) + \exp\left(-\frac{(2nd - z_0 - z)^2}{4\mathscr{D}t}\right)\right]. \quad \text{(A.1)}$$

For the case of interest, $(x_0, y_0, z_0) = (0, 0, 0)$ and $z = d$, giving for the concentration at the point (x, y, z) on the plasmalemma due to an instantaneous point source at the origin,

$$G(\mathbf{r}, \mathbf{0}; t) = \frac{1}{(4\pi\mathscr{D}t)^{3/2}} \exp\left(-\frac{(x^2 + y^2)}{4\mathscr{D}t}\right) \times 2 \sum_{n=-\infty}^{\infty} \exp\left(-\frac{(2nd - d)^2}{4\mathscr{D}t}\right). \quad \text{(A.2)}$$

Here, the sum over n contains two terms of the type $\exp(-d^2/4\mathscr{D}t)$; all other terms involve $\exp(-9d^2/4\mathscr{D}t)$ or smaller and are negligible.