Kinetic diversity in the fusion of exocytotic vesicles

**Yasunori Ninomiya, Takuya Kishimoto,
Toshiko Yamazawa¹, Hiroshi Ikeda,
Yasushi Miyashita and Haruo Kasai²**

Departments of Physiology and ¹Pharmacology, Faculty of Medicine,
University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

²Corresponding author

The speed at which secretory vesicles fuse with the plasma membrane is a key parameter for neuronal and endocrine functions. We determined the precise time courses for fusion of small clear and large dense-core vesicles in PC12 and chromaffin cells by simultaneously measuring both plasma membrane areas and release of vesicular contents. We found that instantaneous increases in cytosolic Ca^{2+} concentration evoked vesicle fusion, but with time constants that varied over four orders of magnitude among different types of vesicles and cells. This indicates that the molecular machinery for the final Ca^{2+} -dependent fusion steps of exocytosis is highly variable and is as critical as Ca^{2+} signalling processes in determining the speed and amount of secretion of neurotransmitters and hormones. Our results suggest a new possibility that the molecules responsible for the final fusion reaction that leads to vesicle fusion are key determinants for neuronal plasticity and hormonal disorders.

Keywords: caged compounds/calcium/capacitance measurement/chromaffin cells/PC12 cells

Introduction

Secretion of neurotransmitters and hormones involves exocytotic fusion of secretory vesicles with the plasma membrane in a Ca^{2+} -dependent manner (Schweizer *et al.*, 1995). Two classes of secretory vesicles have been identified, small clear vesicles (SVs) and large dense-core vesicles (LVs) (De Camilli and Jahn, 1990; Kelly, 1993), the contents, origins and functions of which are different. SVs contain classical neurotransmitters in synapses, whereas LVs contain neuropeptides. In endocrine cells, classical hormones are contained selectively in LVs, while other chemicals may be stored in SVs (Thomas-Reetz and De Camilli, 1994). The exocytoses of the two types of vesicles are evoked by different patterns of stimulation, and play roles in distinct neuronal functions (Jan and Jan, 1982; Andersson *et al.*, 1982; Matteoli *et al.*, 1988; De Camilli and Jahn, 1990; Verhage *et al.*, 1991). Therefore, clarifying the mechanisms of SV and LV exocytosis seems crucial to the understanding of neuronal and endocrine functions. Moreover, comparison of the properties of the two exocytotic pathways will aid elucidation of the

molecular basis of Ca^{2+} -dependent exocytosis (O'Connor *et al.*, 1994; Bajjalieh and Scheller, 1995).

Two important issues remain to be addressed. First, although most studies of secretion have relied on the hypothesis of exocytosis as it was proposed originally (Katz 1969), the hypothesis was not proved until recently when the secretion of monoamines contained in individual LVs was shown to correlate closely with the increase in membrane area detected using capacitance measurement (Chow *et al.*, 1992; Alvarez de Toledo *et al.*, 1993; von Rueden and Neher, 1993; Chow *et al.*, 1996). However, no such study has been performed for SVs. Second, the speed of secretion of neurotransmitters and hormones should depend on the kinetics of both the Ca^{2+} signalling and fusion of secretory vesicles after Ca^{2+} binding. Although the dynamics of Ca^{2+} signalling in neurones and endocrine cells have been a matter of intensive investigations (Smith and Augustine, 1989; Kasai and Augustine, 1990; Chow *et al.*, 1994; Petersen *et al.*, 1994; Schweizer *et al.*, 1995), the kinetics of vesicle fusion after Ca^{2+} binding have not been quantified in those experiments where the types of secretory vesicles have been identified definitively.

Clonal PC12 cells derived from adrenal chromaffin cells possess both LVs containing monoamines (MAs) and SVs containing acetylcholine (ACh) (Greene and Rein, 1977; Rebois *et al.*, 1980; Bauerfeind *et al.*, 1993; Weihe *et al.*, 1996). Our previous capacitance measurements suggested that SVs and LVs in PC12 cells exhibit greatly differing time constants of fusion following rapid and homogeneous elevation of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) produced by photolysis of caged- Ca^{2+} compounds (Kasai *et al.*, 1996). In the present study, we confirmed this hypothesis by direct measurement of ACh secretion using skeletal muscle cells as biosensors and by amperometric measurement of MA secretion using carbon fibres. We also examined MA secretion of the adrenal chromaffin cells using carbon fibres, and found that the fusion time constants of LVs differed greatly between PC12 and chromaffin cells. Thus, the fusion time constants have been found to exhibit enormous diversity among distinct vesicles and cells. Furthermore, our data provide new evidence regarding exocytotic secretion mediated by SVs and LVs.

Results

Quantal ACh secretion

We performed capacitance measurements in undifferentiated PC12 cells, and stimulated the cells by photolysis of a caged- Ca^{2+} compound, dimethoxynitrophenamine tetrasodium salt (DM-nitrophen), to produce rapid and homogeneous increases in $[\text{Ca}^{2+}]_i$ (Ca^{2+} jumps). ACh secretion from PC12 cells was detected using an adjacent

Fig. 1. Quantal ACh release from PC12 cells. (A) A phase-contrast image of a PC12 cell (right) and skeletal myoball (left) pair during whole-cell recording. (B) Quantal secretion of ACh from a PC12 cell induced by a Ca^{2+} jump detected in a skeletal myoball upon photolysis of a caged- Ca^{2+} compound, DM-nitrophen, present in the PC12 cells. Changes in capacitance trace (C) and $[\text{Ca}^{2+}]_i$ (D) in the same PC12 cell. Magnified traces of evoked EPC-like currents (E) and spontaneous ones (F). Arrowheads indicate the artifact of flash irradiation. Simulated currents (dashed lines, Materials and methods) were superimposed on the EPC-like currents. The distance from the release site and muscle surface is represented by d . (G) Distribution of times-to-peak (t_p) of the evoked EPC-like currents, and estimated distance of release site from muscle (d) (see Materials and methods).

skeletal myoball as a biosensor (Figure 1A). Ca^{2+} jumps in the PC12 cells (Figure 1D) induced increases in capacitance of the PC12 cells (Figure 1C) and transient inward currents in the myoballs (Figure 1B). Large inward currents were detected in 38 out of 181 myoballs in contact with PC12 cells, and 35 of the 38 showed clearly resolved transient currents resembling end plate currents (EPC) of neuromuscular synapses (Figure 1B and E). The mean number of Ca^{2+} -evoked EPC-like currents was 1.37 in 35 myoballs. These EPC-like currents were concluded to be mediated by nicotinic ACh receptors, because they were blocked by curare (0.2 mM, $n = 7$, data not shown). In addition, spontaneous currents with amplitudes and time courses similar to those of the evoked currents were observed (Figure 1F) only when the recorded myoballs were in close proximity to PC12 cells. Thus, the currents recorded in myoballs reflect Ca^{2+} -evoked and spontaneous secretion of ACh from PC12 cells.

The time-to-peak of the EPC-like currents was quite variable, ranging from 0.5 to 10 ms (2.21 ± 2.1 ms, mean $\pm$ SD, $n = 59$) (Figure 1G). This may have been due to a variation in distance between the sites of ACh secretion and the myoball ACh receptors; because the PC12 cells were plated onto cultured myoballs shortly before recording (Materials and methods), ACh secretion

may not have been restricted to the region of contact between the two cells. Consistent with this explanation, the time courses of evoked and spontaneous currents could be fitted by a simple mathematical model based on diffusion of ACh from an instantaneous point source that is placed at various distances from the myoballs (Figure 1E and F, dashed lines, Materials and methods). The resulting estimated distances, which ranged from 1 to 11 μm (Figure 1G), were always shorter than the diameter of PC12 cells (20 μm). Because most of the release sites were within 4 μm of myoballs, it appears that a myoball can detect ACh secretion from $\sim 10\%$ of the surface area of a PC12 cell. The charge carried by EPC-like currents ranged from 450 fC to 36 pC; assuming that a charge of 6 pC is produced by activation of a receptor by two molecules of ACh (Sakmann, 1992), each current results from the secretion of at least 150–12 000 ACh molecules. These data indicate that each EPC-like event is caused by the instantaneous secretion of a large amount of ACh from a surface site on PC12 cells.

Ca^{2+} jumps that were sufficiently large ($>30 \mu\text{M}$) to trigger the maximal fast exocytosis of PC12 cells (Kasai *et al.*, 1996) rapidly evoked transient EPC-like currents (Figure 2A). Most currents appeared within 100 ms following a Ca^{2+} jump (Figure 2B), with a peak rate of

Fig. 2. Latency of the EPC-like currents and capacitance changes in PC12 cells immediately following Ca^{2+} jumps. (A) Ten examples of EPC-like currents from different myoballs adjacent to PC12 cells to which Ca^{2+} jumps $>30 \mu\text{M}$ were applied. (B) Latency distribution of 68 evoked EPC-like currents. The latency was defined as the time between the onset of the flash artifact and that of the EPC-like current. According to the simulation (Materials and methods), the latency was prolonged by <3 ms due to the diffusional delay of ACh from the PC12 cells to the myoball in most cells. (C) Magnified histogram of (B). The decay in the appearance of EPC-like currents is fitted with a single exponential function with a time constant of 24 ms. (D) Averaged capacitance traces obtained from a total of eight experiments including four traces shown in (A), where capacitance measurements fulfill the criteria described in the Materials and methods. (E) A time derivative of the capacitance trace. The capacitance trace was binned with a width of 5 ms before differentiation. The dashed line shows a double exponential fit of the data as indicated. Open arrows indicate the peak in the time derivative of the capacitance trace.

secretion occurring after 20 ms (Figure 2C, arrow). The decay in the rate of ACh secretion was exponential, with a time constant of 24 ms (Figure 2C). Figure 2D shows the averaged changes in membrane capacitance among the cells where similarly large Ca^{2+} jumps were applied. Membrane capacitance exhibited time-dependent increases following Ca^{2+} jumps, very similar to ACh secretion; the averaged capacitance trace showed a sigmoidal onset (Figure 2D, arrow), with a time derivative that peaked at 25 ms (in Figure 2E, arrow). The rate of increase in membrane capacitance decayed with two exponentials following a Ca^{2+} jump, with the most rapid component, 29 ± 7 ms (mean $\pm$ SD, $n = 9$, Figure 2E), being very similar to the time constant for ACh secretion (Figure 2C). The slight difference in the two time constants may reflect the fact that the SVs of PC12 cells were heterogeneous and SVs containing larger amounts of ACh might fuse at a faster rate than the rest. The second component of the capacitance increase, which had a time constant of 250 ms (Figure 2E), might be interpreted similarly. The observation that the kinetics of ACh secretion (Figure 2B) correlate well with the fast component of increase in membrane area (Figure 2C and E) suggests

Fig. 3. Latency of quantal secretion of monoamines (MAs) and capacitance changes in PC12 cells. (A) Little quantal MA secretion (arrow) within 200 ms after Ca^{2+} jumps. (B) Three examples of amperometric currents recorded over a period of 30 s after Ca^{2+} jumps. Each spike represents individual quantal secretion. (C) Time course of $[\text{Ca}^{2+}]_i$ changes, averaged over eight cells, including those shown in (B). (D) Latency distribution of quantal MA secretion obtained from 32 cells. (E) Averaged capacitance changes recorded from eight cells including those shown in (B). (F) A time derivative of the averaged capacitance trace. Filled arrows denote the peak of the time derivative of capacitance trace.

the involvement of exocytosis of SVs in ACh secretion (Katz, 1969).

Quantal monoamine secretion

We next used a carbon fibre electrode (Beam *et al.*, 1986) to measure MA secretion from PC12 cells (Figure 3). Unlike ACh secretion, quantal MA secretion seldom occurred within 100 ms following Ca^{2+} jumps (Figure 3A, arrow). Instead, MA secretion was more tonic and took place over tens of seconds (Figure 3B and C). This slow time course of MA secretion corresponded fairly well with a slow component (Kasai *et al.*, 1996) of capacitance increase (Figure 3E and F, arrows), except that the capacitance tended to decay earlier due to the simultaneous occurrence of endocytosis (Kasai *et al.*, 1996; Smith and Betz, 1996). It appeared that MA secretion roughly paralleled changes in $[\text{Ca}^{2+}]_i$ (Figure 3C), consistent with the known Ca^{2+} dependence of MA secretion (Ahnert-Hilger *et al.*, 1987). The time constant of MA secretion was estimated to be >10 s, because the MA secretion rate remained constant for up to 10 s in some cases (Figure 3B), where $[\text{Ca}^{2+}]_i$ remained at a level higher than $20 \mu\text{M}$ for >10 s (data not shown). This estimate is fully consistent with others based on measurements of the slow component of capacitance increase (Kasai *et al.*, 1996). This supports the hypothesis that secretion of MAs involves exocytosis of LVs, the time constant of fusion of which is >400 times larger than that of SVs. Similar observations were made for two other clones of PC12 cells supplied by different laboratories.

To determine whether LVs fuse at the same time constant in other cells, we also measured the rate constant of MA secretion in adrenal chromaffin cells. In these cells, Ca^{2+} jumps ($>20 \mu\text{M}$) triggered large amperometric

Fig. 4. Time courses of quantal secretion of monoamines from an adrenal chromaffin cell. (A) Quantal MA secretion induced by a Ca^{2+} jump. (B) Capacitance change of the chromaffin cell during the Ca^{2+} jump. (C) Time derivative of the capacitance change shown in (B). The trace was low-pass filtered with a cut-off frequency of 100 ms. (D) Change in $[\text{Ca}^{2+}]_i$ during the Ca^{2+} jump. Filled arrows indicate the peak of the secretion of MAs, and open arrows the peak of an additional fast component of secretion and capacitance changes.

currents composed of many quantal secretory events (Figure 4A). The time course of these amperometric currents was correlated with a slow component of the capacitance increase (Figure 4A–C, filled arrows). The time constant for quantal MA secretion ranged between 0.2 and 2 s ($n = 9$), with a mean of 1.04 s (± 0.45 s SD), at $[\text{Ca}^{2+}]_i > 20 \mu\text{M}$. In addition to the slow component, the capacitance traces exhibited a very rapid component (Figure 4B and C, open arrows) (Augustine and Neher, 1992; Heinemann *et al.*, 1994) that was not reflected proportionately in the amperometric measurements (Figure 4A, open arrows). The rapid component sometimes preceded the amperometric current by more than 0.5 s (Oberhauser *et al.*, 1996) when increases in $[\text{Ca}^{2+}]_i$ were less than 10 μM (data not shown). It is likely that the fast component represents exocytotic events involving vesicles other than typical LVs, an obvious candidate being SVs (Thomas-Reetz and De Camilli, 1994).

Discussion

In the present study, the time constants of exocytotic secretion involving SVs and LVs in PC12 cells and LVs in chromaffin cells were investigated using two independent methods, one for detecting quantal secretion of vesicular contents and the other for measuring increases in plasma membrane areas. For all three examples, the time constant estimated using one of the methods was similar to that estimated using the other, although the time constants were highly variable among distinct vesicles and cells. The implications of these findings will be discussed below.

Vesicular hypothesis of secretion

The major features of ACh secretion from PC12 cells were found to be similar to those of ACh secretion from the presynaptic terminals of neuromuscular junctions (Katz, 1969). First, the time courses of the EPC-like

currents recorded from muscle cells were well fitted by an instantaneous point source of ACh at a surface site on PC12 cells (Figure 1E and F). Second, such quantal secretion also occurred spontaneously. We then tested the validity of the vesicular hypothesis for SV exocytosis by comparing the time course of ACh secretion and capacitance increase when secretion was triggered synchronously throughout a cell by Ca^{2+} jumps. The close temporal correspondence between quantal secretion and increase in membrane area in a millisecond time resolution indicates that secretion of ACh coincides with formation of electrical connections between SVs and the extracellular space, consistent with the vesicular hypothesis. A similar correlation between MA secretion and increase in membrane area of PC12 and chromaffin cells for LVs represents new experimental evidence for exocytosis of LVs. Our data also justify the use of capacitance measurement in studying exocytosis.

One surprising observation was that the number of EPC-like currents produced by a Ca^{2+} jump was far smaller than the number of SV fusion events estimated from the capacitance measurements (Figure 2). A capacitance increase of 200 fF represents exocytosis of 3000 SVs per cell, assuming that one SV has a diameter of 50 nm and a specific capacitance of 1 $\mu\text{F}/\text{cm}^2$. In contrast, a Ca^{2+} jump produced only 0.31 EPC-like currents in all cells examined ($n = 178$); even assuming that these currents were released from 10% of the surface area of the PC12 cell (see above), only 3.1 currents were produced per PC12 cell. This suggests that the majority of fusing SVs contain too little ACh to be detected by the myoballs. Thus, it is important to clarify why PC12 cells contain an abundance of SVs although they secrete very little ACh. One possibility is that the exocytotic vesicles perform functions other than secretion. This possibility is also consistent with the finding that non-secretory Chinese hamster ovary cells (CHO cells) exhibited a high degree of Ca^{2+} -dependent exocytotic activities (Morimoto *et al.*, 1995; Ninomiya *et al.*, 1996). The Ca^{2+} -dependent exocytotic pathways in the clonal cell lines might play housekeeping roles, such as roles in cell membrane repair (Bi *et al.*, 1995).

Diversity in fusion time constants

The time constants that we obtained in this study mainly represent the time constants of fusion of vesicles after Ca^{2+} binding to Ca^{2+} sensors for exocytosis, because large Ca^{2+} jumps were applied, so that Ca^{2+} binding occurred rapidly. In fact, the time constant of quantal secretion was not smaller with greater increases in $[\text{Ca}^{2+}]_i$ in PC12 and chromaffin cells (data not shown). Further, since the exocytosis was independent of cytosolic ATP under our experimental conditions (Ahnert-Hilger and Gratzl, 1987; Neher and Zucker, 1993; Ruden *et al.*, 1994; Parsons *et al.*, 1995; Kasai *et al.*, 1996), exocytosis only involves fusion-ready vesicles downstream of final ATP-dependent steps (Hay and Martin, 1993), a mechanism also postulated for synaptic vesicles (Sudhof, 1995). Thus, our measurements of time constants should reveal properties of the final reactions that lead to vesicle fusion. The time constant for the final reaction was predicted to be 0.3 ms for synaptic vesicles (Heidelberger *et al.*, 1994), while it was estimated as 24 ms, 1.04 s and >10 s for

SVs of PC12 cells, LVs of chromaffin cells and LVs of PC12 cells, respectively. Thus, we have demonstrated that the time constants of the final reactions vary over four orders of magnitude among distinct vesicles and cells. The origin of this diversity in time constants may simply reflect the time constant of the single final fusion step. Alternatively, the diversity represents differences in the degrees of readiness of vesicles to participate in the various sequential steps of exocytosis that occur after the Ca^{2+} binding step, and that have not yet been resolved. It will be an important future challenge to identify the molecules responsible for the diversity. Although most fusion-related proteins that are found in synapses are also expressed in PC12 cells (Kelly, 1993), more detailed comparisons of the chemical and structural features among these vesicles and cells could help to clarify the molecular determinants of the time constants of Ca^{2+} -dependent exocytosis (Sudhof, 1995).

Ca^{2+} signalling is characteristic of each secretory cell type (Smith and Augustine, 1989; Kasai and Augustine, 1990; Chow *et al.*, 1994; Petersen *et al.*, 1994; Schweizer *et al.*, 1995). In particular, presynaptic terminals possess active zones in which voltage-gated Ca^{2+} channels are localized closely with SVs and mediate the generation of very fast and large increases in $[\text{Ca}^{2+}]_i$ in the vicinity of SVs, resulting in rapid exocytosis. Our results show that such fast Ca^{2+} signalling is not sufficient for fast synaptic transmission, because secretory vesicles in other cells did not fuse rapidly even when the fusion-ready vesicles were exposed to similarly rapid and large increases in $[\text{Ca}^{2+}]_i$. In theory, the fusion time constant is an important factor determining the probability that an individual vesicle will fuse in response to a given Ca^{2+} signal. Hence, the observed diversity in fusion time constants implies that the final fusion step can be an effective regulatory site for secretion of neurotransmitters and hormones. It has been considered that changes in neurotransmitter secretion underlie synaptic plasticity (Dolphin *et al.*, 1982), and that reduction in the rate of exocytosis in endocrine cells is responsible for hormonal disorders (Wollheim and Sharp, 1981). Therefore, understanding the diversity in the fusion time constants might lead to further clarification of the molecular basis of neuronal plasticity and hormonal disorders.

Materials and methods

Preparations

PC12 cells, kindly provided by K.Inoue [47th passage from the original PC12 clone (Greene and Tischler, 1976)], were subcloned. We mainly used clone A4, grown in Dulbecco's modified Eagle's medium (DMEM)-based culture medium without nerve growth factor (Kasai *et al.*, 1996). Skeletal myotubes were prepared from embryonic mouse skeletal muscles as described (Rando and Blau, 1994) and treated with colchicine to form myoballs (Beam *et al.*, 1986). PC12 cells were plated acutely on the cultured myoballs at room temperature (22–24°C) immediately before electrophysiological experiments were conducted. The bathing solution contained (in mM) 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 Na-HEPES, 10 glucose at pH 7.4 and 320 mOsm. Whole-cell recordings were carried out from a pair of a PC12 cell and myoball in contact (Figure 1A). Cultured chromaffin cells were obtained from bovine adrenal medulla as described (Neher and Zucker, 1993). Recording solutions used for the chromaffin cells were the same as those for PC12 cells.

Capacitance measurement and flash photolysis of a caged- Ca^{2+} compound

The patch pipette for PC12 cells contained (in mM) 120 Cs glutamate, 10 CsCl, 40 Cs-HEPES and 200 μM benzothiazole coumarin (BTC,

Molecular Probes, Eugene) as a Ca^{2+} indicator, and 10 DM-nitrophen (Calbiochem, La Jolla) plus 5 CaCl_2 , as a caged- Ca^{2+} compound, at pH 7.3. The patch pipette for skeletal myoballs contained (in mM) 140 Cs glutamate, 10 CsCl, 40 Cs-HEPES and 10 Cs_2EGTA at pH 7.3. Whole-cell currents of skeletal muscle were filtered at 5 kHz and sampled at 10 kHz.

A recording chamber was placed on an inverted microscope (Olympus IX) and observed through an objective lens, UApO 40 $\times$ /340 Oil (Olympus). Capacitance measurements were carried out using a patch-clamp amplifier, AxoPatch 1D (Axon Instruments, Foster City) (Kasai *et al.*, 1996). The membrane potential of PC12 cells was maintained at -20 mV, and 1 kHz sine waves with a peak-to-peak amplitude of 100 mV were applied. Capacitances of PC12 cells were calculated from one cycle of sine waves and stored at 1 kHz (Figures 1 and 2), and from 10 cycles of sine waves and stored at 83 kHz (Figures 3 and 4). We selected our capacitance data according to the following criteria: (i) the resistance of the seal before photolysis was >1 G Ω ; (ii) the access resistance was <8 M Ω ; and (iii) the amplitude of the transient current was <100 pA (5 nS). Based on these criteria, the maximal error in the estimate of capacitance was assumed to be $<0.16\%$ of total membrane capacitance (Maruyama *et al.*, 1993).

We used a xenon flash lamp as an actinic light source for the caged- Ca^{2+} compound (High-Tech Instruments, UK) connected directly to a two-port light guide (IMT-2-RFA-caged, Olympus). The Ca^{2+} jumps were applied by flash photolysis of DM-nitrophen 1–3 min after the rupture of the patch membrane. Ca^{2+} measurements were performed as described (Kasai *et al.*, 1996) using a ratioable long-wavelength Ca^{2+} -indicator dye, BTC, and a monochromator (T.I.L.L. Photonics, Munich). Rises in $[\text{Ca}^{2+}]_i$ were estimated ratiometrically before and 0.5 s after the photolysis (Figures 1 and 2), while they were estimated at 83 Hz (Figures 3 and 4).

Amperometric detection of monoamine secretion

Oxidative currents due to MAs were recorded using the patch-clamp amplifier (CE2400, Nihon Koden, Tokyo) and carbon fibre electrode (Pro CFE, Axon Instruments, Foster City) applied with a positive potential (800 mV). Amperometric currents were filtered at 5 kHz and sampled at 1 kHz (Figure 3A), and filtered at 0.1 kHz and sampled at 83 Hz (Figures 3B and 4). Artifacts of amperometry due to flash irradiation were subtracted using the trace from the same cell at the second or third flash where no secretion was detected.

Simulation of time courses of EPC-like currents

In the simulation of EPC-like currents, we assumed the existence of an instantaneous point source of ACh at a given distance (d) from the surface of the myoball that is an infinite and absorbing plane for ACh, and that activation of ACh receptors requires binding of two ACh molecules, and hence the activation is proportional to the square of ACh flux into the muscle plane. The image plane method (Chow *et al.*, 1992) yields a solution for ACh flux into a given point of the muscle plane at a given time t , and the square of ACh flux integrated over the muscle plane is obtained as

$$J = \frac{d^2}{32(\pi D)^2 t^4} \exp\left(-\frac{d^2}{2Dt}\right)$$

where D is the ACh diffusion constant ($0.87 \mu\text{m}^2/\text{ms}$) (Almers and Tse, 1994). The EPC-like currents were then simulated by calculating a convolution of J with the time course of ACh receptor closing: $\exp(-t/2 \text{ ms})$ (Sakmann, 1992). Satisfactory fits were obtained by adjusting distances, d , and scaling the amplitudes of simulated currents. Distances of ACh point sources from the myoball (d) were calculated numerically from t_p using the above simulation, taking into account a diffusional delay between the ACh secretion and the onset of EPC-like currents.

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