

Neurotransmitter release evoked by nerve impulses without Ca^{2+} entry through Ca^{2+} channels in frog motor nerve endings

E. M. Silinsky, M. Watanabe, R. S. Redman, R. Qiu*, J. K. Hirsh, J. M. Hunt, C. S. Solsona, S. Alford† and R. C. MacDonald*

*Departments of Molecular Pharmacology and Biological Chemistry, *Biochemistry and †Physiology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, IL 60611, USA*

1. The requirement for extracellular Ca^{2+} in the process of evoked acetylcholine (ACh) release by nerve impulses was tested at endplates in frog skeletal muscle. Ca^{2+} -containing lipid vesicles (Ca^{2+} liposomes) were used to elevate cytoplasmic Ca^{2+} concentrations under conditions in which Ca^{2+} entry from the extracellular fluid was prevented.
2. In an extracellular solution containing no added Ca^{2+} and 1 mM Mg^{2+} (' Ca^{2+} -free' solution), Ca^{2+} liposomes promoted the synchronous release of ACh quanta, reflected electrophysiologically as endplate potentials (EPPs), in response to temporally isolated nerve impulses.
3. Motor nerve stimulation generated EPPs during superfusion with Ca^{2+} liposomes in Ca^{2+} -free solutions containing the Ca^{2+} channel blocker Co^{2+} (1 mM), and the Ca^{2+} chelator EGTA (2 mM). As a physiological control for Ca^{2+} leakage from the liposomes to the extracellular fluid, the effect of Ca^{2+} liposomes on asynchronous evoked ACh release mediated by Ba^{2+} was examined. In contrast to the effects of 0.2–0.3 mM extracellular Ca^{2+} , which generated EPPs but antagonized Ba^{2+} -mediated asynchronous ACh release, Ca^{2+} liposomes generated EPPs but did not reduce asynchronous release mediated by Ba^{2+} . The effects of Ca^{2+} liposomes were thus not due to leakage of Ca^{2+} from the liposome to the extracellular fluid.
4. Morphological studies using fluorescently labelled liposomes in conjunction with a confocal microscope demonstrate that lipid is transferred from the liposomes to nerve endings and liposomal contents are delivered to the nerve terminal cytoplasm.
5. The results suggest that when intracellular Ca^{2+} is elevated using liposomes as a vehicle, evoked ACh release can occur in the absence of Ca^{2+} entry via Ca^{2+} channels.

The evoked release of neurotransmitter substances is extremely sensitive to the concentration of extracellular Ca^{2+} (for reviews see Silinsky, 1985; Augustine, Charlton & Smith, 1987). There are two main loci of this sensitivity: (i) the Ca^{2+} channel which, in response to an action potential, translocates Ca^{2+} from the extracellular fluid to a strategic component of the secretory apparatus, and (ii) the intracellular and membrane sites, at which cytoplasmic Ca^{2+} binding reduces an energy barrier to neurotransmitter release and promotes exocytosis (Katz, 1969; Silinsky, 1981, 1985; Augustine & Eckert, 1984; Augustine, Adler & Charlton, 1991).

While the essential need for Ca^{2+} entry through Ca^{2+} channels is indisputable for neurally evoked neurotransmitter secretion, the importance of voltage-sensitive Ca^{2+} binding proteins as mediators of secretion has been a

subject of considerable controversy. For example, work in our laboratory using ion-containing lipid vesicles to deliver Ca^{2+} , Sr^{2+} and Mg^{2+} to the cytoplasm of frog motor nerve endings suggests that voltage-sensitive Ca^{2+} binding proteins in the nerve ending confer divalent cation selectivity on the process of evoked, physiologically functional acetylcholine (ACh) secretion reflected as endplate potentials (EPPs) (Kharasch, Mellow & Silinsky, 1981; Mellow, Perry & Silinsky, 1982; Silinsky, 1985). Comprehensive studies in other laboratories have independently concluded that the kinetics of the evoked ACh release process are not determined by changes in the intracellular Ca^{2+} concentrations but rather by the activation and inactivation of a voltage-sensitive Ca^{2+} binding protein (Parnas & Parnas, 1988; Hoehner, Parnas & Parnas, 1989; Dudel, 1990). Such a mechanism, whereby transient nerve terminal depolarization activates a Ca^{2+}

binding protein and produces an enormous enhancement of ACh release in a very brief interval, could provide an ideal control mechanism to assist in the efficient transmission of information from nerve to muscle.

Unfortunately, some of the conclusions concerning the requirement for voltage-sensitive Ca^{2+} binding proteins in invertebrates (e.g. Hochner *et al.* 1989) may be based upon experiments made under conditions in which small amounts of residual Ca^{2+} can enter through voltage-sensitive Ca^{2+} channels (Mulkey & Zucker, 1991; Zucker, Delaney, Mulkey & Tank, 1991). Indeed, studies on invertebrate nerve endings have led to the conclusions that action potentials are unable to evoke transmitter release in the absence of Ca^{2+} influx and that the kinetics of neurotransmitter release are likely to be determined by the Ca^{2+} concentration profile (Mulkey & Zucker, 1991; Zucker *et al.* 1991).

We thus decided to test the possibility that voltage-sensitive Ca^{2+} binding proteins are capable of mediating evoked ACh release from vertebrate nerve terminals. To this end, we used Ca^{2+} -containing lipid vesicles (Ca^{2+} liposomes) as vehicles to deliver Ca^{2+} to frog motor nerve endings under conditions in which Ca^{2+} entry cannot occur. Such liposomes have been found previously to interact with the membrane of the secretory cells and to increase the local intracellular Ca^{2+} concentration at strategic regions of secretion (Theoharides & Douglas, 1978; Rahamimoff, Meiri, Erulkar & Barenholz, 1978; Gutman, Lichtenberg, Cohen & Boonyaviroj, 1979; Kharasch *et al.* 1981; Mellow *et al.* 1982). The results contained herein provide electrophysiological and microscopic evidence in support of these earlier liposome results and demonstrate that neurally evoked ACh release in the frog can occur in the absence of Ca^{2+} entry through Ca^{2+} channels.

METHODS

Electrophysiological preparation

The electrophysiological correlates of ACh release were recorded from endplates in frog skeletal muscle using conventional intracellular recording methods (del Castillo & Katz, 1954). Recordings of EPPs and miniature endplate potentials (MEPPs) were made at neuromuscular junctions from isolated cutaneous pectoris nerve-muscle preparations of the frog (*Rana pipiens*). Animals were killed by ether anaesthesia, followed by double pithing. The mean number of quanta of ACh released synchronously in response to an action potential (m) is proportional to the size of the EPPs and was generally quantified as the ratio of the mean EPP amplitude to the mean MEPP amplitude. In some experiments, the method of failures was employed to determine m (del Castillo & Katz, 1954). Evoked MEPP frequency in Ba^{2+} solutions was estimated from the amplitude of the slow depolarization of the postjunctional membrane (see Silinsky, 1984). For details of the averaging techniques and statistical methods see Silinsky (1984).

Preparations were continually superfused in Ringer solution (115 mM NaCl, 2 mM KCl, with the indicated concentrations of Ca^{2+} and Mg^{2+} as chloride salts, 2 mM Hepes; pH 7.2–7.4). Normal Ringer solution contained 1.8 mM CaCl_2 and no added

Mg^{2+} . Most solutions were modified as described below. Ca^{2+} liposomes were suspended in Ringer solution of identical composition to the extracellular solution and superfused by one of two alternative methods. In most experiments, Ca^{2+} liposomes were locally superfused using 'fast-flow' delivery from 300 μm diameter glass flow pipes. The flow pipes were gravity fed from a syringe reservoir. The solution in the flow pipes was of identical composition to the superfusion solution but also contained the Ca^{2+} liposomes. The latency from the opening of the syringe tap to the beginning of a postjunctional depolarization was 50–100 ms when tested with 100 μM ACh in the flow pipe. Recovery of the membrane potential to the resting level after fast-flow ACh delivery occurred with a similar time course to depolarization. The other method of liposome application was global bath superfusion using a peristaltic pump (Watson-Marlow or Masterflex). Apart from the more rapid kinetics and the smaller volumes of solution required for the local superfusion, both methods produced similar results.

Preparation of liposomes

Liposomes were prepared using conventional methods, which rely on the fact that when a dispersion of phospholipid in aqueous medium is subjected to high power ultrasonic irradiation, small (25–50 nm) unilamellar lipid vesicles with an entrapped aqueous core are formed (see Mellow *et al.* 1982 for citations). Specific details follow.

Egg phosphatidylcholine (PC) in hexane (Sigma Chemical Co., St Louis, MO, USA) in 83.5 mg (or 167 mg) aliquots was evaporated to dryness in a 25 ml (or 50 ml) round-bottomed flask using a vacuum pump. The flask with its thin film of dried lipid was then flushed with N_2 gas and used immediately or stored at 0 °C until needed. Next, 5–10 ml of the solution to be entrapped within liposomes (formation solution) was added to the flask. Generally the PC concentration at this stage was 16.7 mg ml⁻¹ but concentrations as high as 60 mg ml⁻¹ were used in early studies. The suspension was vortexed at high speed for 5–10 min; this procedure generates large, multilamellar liposomes. The formation solution used to produce Ca^{2+} -containing liposomes (Ca^{2+} liposomes) generally contained 80 mM CaCl_2 and 2 mM Hepes (pH 7.2–7.4). The resulting lipid dispersion was transferred to a plastic vial (pre-cooled in ice) and then subjected to intermittent ultrasonic irradiation (Branson Model 200 sonicator, Branson Sonic Power Company, Danbury, CT, USA). The sonicator was set at high power (settings ranged from 8 to maximum) and the duty cycle was set at 0.3 s sonication s⁻¹. After 15–20 min of treatment, the lipid suspensions had a transparent amber colour and further sonication failed to alter their appearance. This is indicative of the formation of small (25–50 nm) unilamellar liposomes with an entrapped core of 80 mM CaCl_2 (Mellow *et al.* 1982). Because of the high concentration of lipid employed in these studies (60 mg ml⁻¹ in early studies), neither a bath sonicator nor a microprobe attachment to the Branson sonicator was capable of producing unilamellar liposomes within the 20 min sonication period (authors' unpublished observations). After sonication, the liposomes were centrifuged at 5000 g for 30 min at 0–4 °C to remove particles of titanium from the sonicator probe, and then diluted with Ringer solution. The amount of dilution varied, the liposomes were diluted to 1:7 (experiments in 1982) or 1:3 (our present method).

The final stage of preparation involved separating the liposomes (with their entrapped 80 mM CaCl_2) from the 80 mM CaCl_2 formation solution bathing the liposomes. This was

accomplished by ultrafiltration with an Amicon MMC ultrafiltration unit, in which the liposomes were filtered with approximately five sample volumes of Ringer solution generally containing 1 mM Mg^{2+} and no added Ca^{2+} , i.e. Ca^{2+} -free Ringer solution. The ultrafiltration was performed using either He gas (experiments in 1982) or N_2 gas (20–25 lb in $^{-2}$). When this method was employed using XM 100 or YM 100 membranes, extraliposomal Ca^{2+} was removed from the fluid bathing the liposomes leaving unilamellar liposomes containing 80 mM CaCl_2 suspended in Ca^{2+} -free Ringer solution (for justification see Fig. 1 in Mellow *et al.* 1982). The filtered liposomes in the most recent experiments were usually at a final lipid composition of 4.2 mg ml $^{-1}$. In some instances, when large levels of ACh release were generated, liposomes were diluted further (e.g. see Mellow *et al.* 1982). The filtered liposomes were gently warmed to room temperature and applied either with a peristaltic pump or fast-flow superfusion (see above). In some experiments, the formation solution entrapped within PC liposomes contained 15 mM calcein (4',5'-bis((carboxymethyl)aminomethyl)fluorescein; GSS Chemicals, Powell, OH, USA) + 5 mM CoCl_2 and in others the PC contained 0.5% phosphatidylethanolamine conjugated with rhodamine (RhPE) (see below).

Microscopy

A confocal laser scanning microscope (BIO-RAD MRC-600) was used for imaging neuromuscular junctions after treatment with fluorescent indicators. We employed the A1/A2 filter set for detecting simultaneous emission from two different fluorescent indicators. In each of the sets of studies, one probe was green emitting and fluorescence was recorded on the green channel (through which the photomultiplier tube receives light at 525–555 nm), the other was red emitting and recorded on the red channel (> 600 nm). Both fluorophores were excited by the 514 nm line of the argon ion laser. The general protocol was to compare the distribution of fluorescence using a probe known to stain motor nerve endings with a probe used to tag either the liposome membrane or the solute entrapped within liposomes. Prior to exposure to fluorescent indicators, preparations were bathed in 0.1–0.01% collagenase (Sigma type I) in normal Ringer solution for 20 min, followed by a 1 h wash in collagenase-free Ringer solution.

In both sets of confocal microscope experiments to be described below, unambiguous identification of nerve terminals was made by treating the preparation with fluorescent derivatives of peanut agglutinin (PNA, Sigma Chemical Co.). Peanut agglutinin is a lectin that binds to carbohydrates located primarily in the extracellular matrix near the Schwann cell, and selectively and irreversibly stains the junctional region of motor nerve endings in this frog preparation without affecting the myelinated or preterminal unmyelinated axons (Ko, 1987).

The first set of confocal microscope experiments involved a fluorescent lipid which allowed us to examine the disposition of the liposomal membranes. In these experiments, the nerve-muscle preparation was treated with a suspension of vesicles prepared from egg PC containing 0.5 mol per cent of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine b sulphonyl) (RhPE) (Avanti Polar Lipids, Alabaster, AL, USA) in a final lipid composition of about 5 mg ml $^{-1}$. Since RhPE is firmly embedded in the bilayer (Struck, Hoekstra & Pagano, 1981), the Amicon filtration step is unnecessary with these liposomes and it was only necessary to sonicate them. The protocol for these experiments was as follows. First, nerve terminals were localized visually and a background level of

fluorescence was viewed in the absence of liposomes. The preparation was then treated with RhPE liposomes and fluorescence observed on the red channel. To ascertain that the fluorescent structures were indeed motor nerve endings, preparations were subsequently stained with fluorescein-labelled peanut agglutinin (F-PNA) and the fluorescence observed on the green channel. This protocol was necessitated by the weak fluorescence of the liposome staining in comparison with that of the F-PNA. Indeed if the F-PNA was used prior to the liposomes, there was too great a leakage of the F-PNA into the RhPE channel to identify unambiguously the regions marked by the liposomes.

The second set of confocal microscope experiments used liposomes loaded with an aqueous solution of the fluorescent dye calcein (Kendall & MacDonald, 1983). After first localizing nerve endings using tetramethylrhodamine isothiocyanate-conjugated PNA (TMR-PNA, Sigma Chemical Co.) using the red channel, preparations were superfused with PC liposomes containing 15 mM calcein + 5 mM CoCl_2 (calcein liposomes). Calcein fluorescence is self-quenched at the high concentrations incorporated into the liposomes (Kendall & MacDonald, 1983), so if released into the nerve terminal it becomes diluted in the cytoplasm, the self quenching is relieved, and the nerve fluoresces. In preliminary experiments, the residual calcein fluorescence of the vesicle suspensions was quite intense and it was necessary to add an additional quenching agent, CoCl_2 , to the liposome formation solution (Perin & MacDonald, 1989). Calcein fluorescence was recorded on the green channel. Whilst this combination of detection wavelengths was less than optimal due to constraints inherent in the excitation wavelength of the argon ion laser, it was sufficient to observe selective fluorescence due to calcein within the motor nerve ending cytoplasm (e.g. Fig. 6).

RESULTS

General observations on ACh release in Ca^{2+} -free solutions

Ca^{2+} liposomes, suspended in Ringer solution containing no added Ca^{2+} and 1 mM Mg^{2+} (' Ca^{2+} -free' solution), promoted evoked ACh release in a total of twenty experiments. Figure 1A–D presents the results of four such experiments. Continuous motor nerve stimulation at 1 Hz failed to elicit ACh release in all experiments in Ca^{2+} -free Ringer solution without Ca^{2+} liposomes (Fig. 1A, 'No liposomes'). In the experiment shown in Fig. 1A, several seconds after the beginning of local superfusion with Ca^{2+} liposomes in Ca^{2+} -free solution, EPPs appeared in response to individual action potentials (e.g. Fig. 1A, EPP). The EPPs continued to increase in amplitude until a suprathreshold action potential was generated 22 s after the beginning of superfusion of this nerve ending with Ca^{2+} liposomes (Fig. 1A, AP). The effects of Ca^{2+} liposomes were reversible; evoked ACh release disappeared within 10 s after the superfusion solution was changed to Ca^{2+} -free Ringer solution without Ca^{2+} liposomes. Figure 1B shows an experiment in which large EPPs were generated by Ca^{2+} liposomes without muscle action potentials, allowing us to quantify the level of neurotransmitter release. In this cell, after a baseline was obtained by nerve stimulation in Ca^{2+} -

free solution, superfusion with Ca^{2+} liposomes was begun and eventually a steady-state level of ACh release (m) of 19.4 quanta was obtained (see elapsed time indicated on the graph). Figure 1C shows superimposed EPPs in response to 128 stimuli from another experiment and illustrates the more typical level of ACh release observed in these experiments ($m \approx 0.7-0.9$). Note the quantal fluctuations including failures of response (flat baseline). This level of ACh release is similar to that observed with 0.2–0.3 mM extracellular Ca^{2+} in 1 mM Mg^{2+} solutions. The experiment of Fig. 1D shows the lowest level of ACh release obtained in successful experiments, namely an occasional single quantum of ACh release phase locked to the nerve impulse ($m \approx 0.1$).

It might be argued that a very small amount of Ca^{2+} is entering the nerve terminal via Ca^{2+} channels in the Ca^{2+} -free solution containing the Ca^{2+} channel blocker Mg^{2+} . Such entry might not be detectable as EPPs unless additional Ca^{2+} is provided (e.g. via liposomes). We performed the following experiments in an attempt to dispel this argument.

External Co^{2+} does not block ACh release evoked by Ca^{2+} liposomes

In the first series of experiments, we added 1 mM Co^{2+} , a potent Ca^{2+} channel blocker at motor nerve endings (Weakly, 1973; Silinsky & Solsona, 1992) to the superfusion solution. Figure 2 shows that even in solutions containing

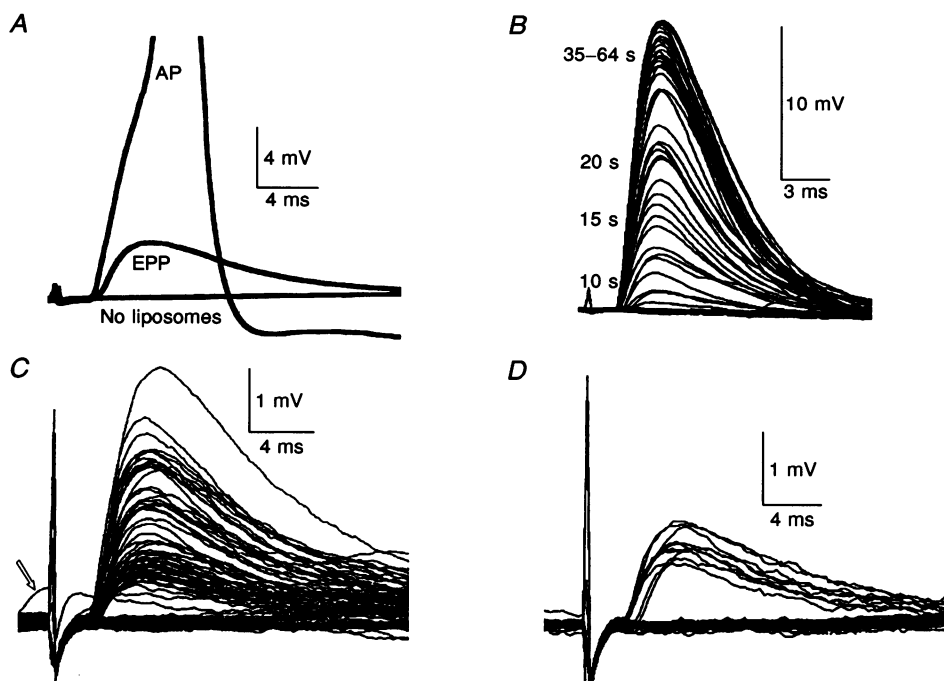
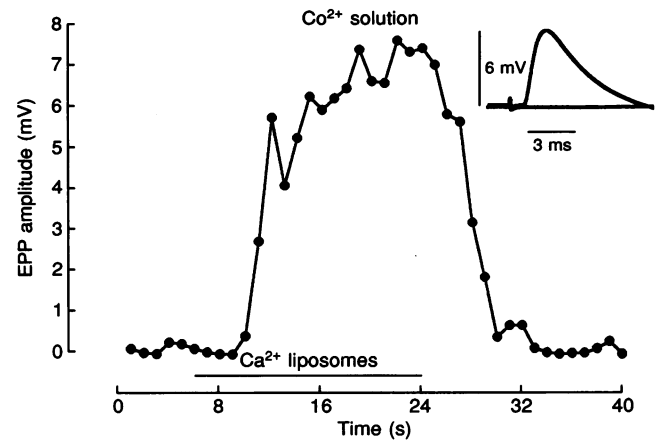


Figure 1. Generation of neurally evoked neurotransmitter release (EPPs) by intracellular Ca^{2+} delivered in Ca^{2+} -containing liposomes (Ca^{2+} liposomes)

The extracellular solution contained 1 mM Mg^{2+} and no added Ca^{2+} (' Ca^{2+} -free' solution). In this and the experiments shown in Figs 2 and 3, nerve stimulation was delivered at a frequency of 1 Hz. *A*, results from a cell in which Ca^{2+} liposomes promoted sufficient acetylcholine (ACh) release to generate suprathreshold muscle action potentials. Ca^{2+} -free solution without Ca^{2+} -containing liposomes failed to support neurotransmitter release (no liposomes, average of 16 stimuli). EPP shows averaged EPP ($n = 5$ stimuli) made 16–20 s after the beginning of superfusion with Ca^{2+} -containing liposomes. AP shows a suprathreshold EPP and superimposed action potential, which was generated 22 s after the addition of Ca^{2+} -containing liposomes in this cell. The effects of liposome addition were fully reversible. *B*, results from another cell in which substantial levels of ACh release were generated. Note the progressive increase in evoked ACh release to a stable level of $m \approx 19.4$ between 35 and 64 s after the beginning of superfusion with Ca^{2+} liposomes. Numbers to the left of the EPPs show elapsed time after the beginning of superfusion with Ca^{2+} liposomes. The frequency of occurrence of MEPPs was also accelerated approximately 5-fold; mean control MEPP frequency (± 1 s.e.m.), $1.21 \pm 0.06 \text{ s}^{-1}$, $n = 30$; MEPP frequency in Ca^{2+} liposomes, $6.17 \pm 0.44 \text{ s}^{-1}$, $n = 30$ (see Mellow *et al.* 1982 for further details). *C*, more typical levels of release observed with Ca^{2+} liposomes (128 superimposed EPPs are shown). Note the fluctuation of ACh release during continuous application of Ca^{2+} liposomes ($m = 0.9$ by direct method; $m = 0.7$ by the method of failures). Arrow shows spontaneous MEPP. *D*, the lowest level of evoked ACh release, in effect, a phase-locked single quantum of ACh every 6–7 stimuli ($m = 0.13-0.16$).

Figure 2. Ca^{2+} -containing liposomes generate ACh release in Ca^{2+} -free Ringer solution containing 1 mM Co^{2+}

Liposomes were superfused during the time indicated by the bar. Inset shows both the average EPP ($n = 11$) obtained near the peak level of ACh release ($m = 14.4$) and the absence of evoked release prior to the addition of liposomes.



Co^{2+} , Mg^{2+} and no added Ca^{2+} (Co^{2+} solution), the presence of Ca^{2+} liposomes (indicated by bar) supports synchronous ACh release evoked by action potentials (m , 14.4; EPPs shown as inset). In a total of eleven experiments, Ca^{2+} liposomes in Co^{2+} solution supported EPPs in response to nerve stimulation.

To confirm that Co^{2+} was indeed blocking Ca^{2+} entry, we examined the effects of Co^{2+} on damaged liposomes in a few experiments; extracellular Co^{2+} (1 mM) completely blocked ACh release evoked by liposomes damaged by prolonged (24 h) exposure to room temperature ($n = 3$). Such results suggest that, if Ca^{2+} were leaking from intact liposomes to the extracellular fluid and then entering via Ca^{2+} channels, then 1 mM Co^{2+} would block such release. A problem we did encounter with Co^{2+} solutions, however, was the apparent

failure of the motor nerve ending to sustain ACh release after a period of time in Ca^{2+} liposomes suspended in Co^{2+} solutions. This effect may be due to conduction failure in solutions containing the divalent cation antagonists Co^{2+} and Mg^{2+} without added Ca^{2+} (authors' unpublished observations). As regards the batch-to-batch variability in responses to liposomes (e.g. Fig. 1), the explanation is unknown. It could be due to variations in the efficiency by which Ca^{2+} liposomes deliver their entrapped cation, differences in nerve terminal Ca^{2+} buffering capacities, diffusion barriers and/or some as yet undetermined process.

Ca^{2+} liposomes promote ACh release in EGTA solutions

To eliminate further the possibility that a small residuum of extracellular Ca^{2+} was being delivered via Ca^{2+} channels

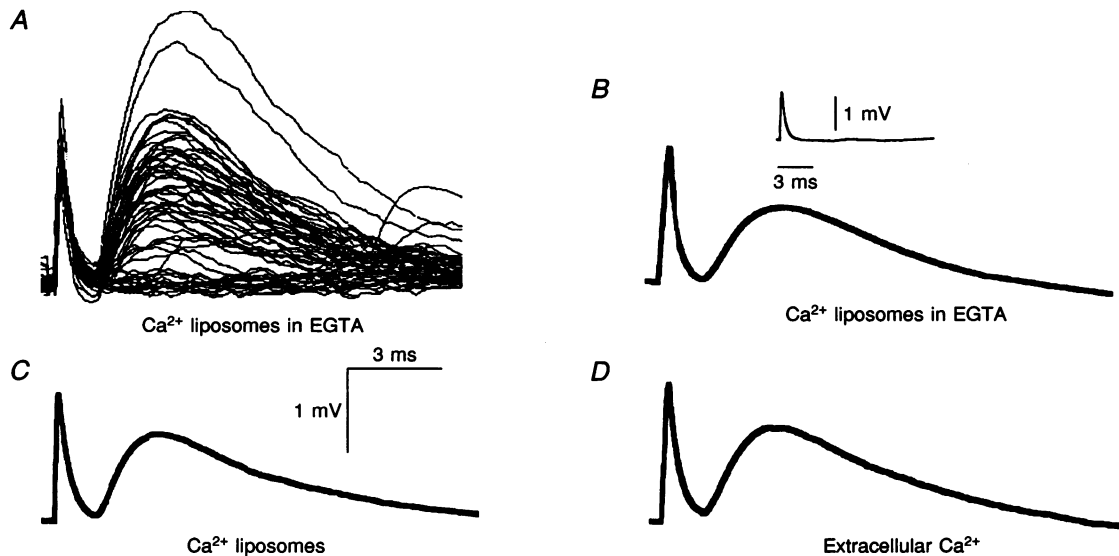


Figure 3. Ca^{2+} liposomes generate ACh release in Ca^{2+} -free Ringer solution containing the Ca^{2+} chelator EGTA (2 mM)

A, the raw data obtained in the presence of Ca^{2+} liposomes in 1 mM Mg^{2+} solutions containing 2 mM EGTA (MgEGTA solution). *B*, the averaged EPP in MgEGTA Ringer solution from the traces shown in *A* ($m = 1.36$; $n = 45$ stimuli). Absence of ACh release in the absence of Ca^{2+} liposomes is shown as an inset (average of 20 stimuli). *C*, the effect of Ca^{2+} liposomes in 1 mM Mg^{2+} solutions without EGTA ($m = 1.34$; $n = 31$ stimuli). *D*, ACh release in the presence of 0.2 mM extracellular Ca^{2+} (1 mM Mg^{2+}) in the absence of Ca^{2+} liposomes ($m = 1.49$; $n = 15$ stimuli).

in the extracellular solution containing Co^{2+} or Mg^{2+} , we performed experiments in solutions containing 1 mM Mg^{2+} and the Ca^{2+} chelator EGTA (2 mM MgEGTA solution). MgEGTA solution contains such low levels of extracellular Ca^{2+} ($< 10^{-9}$ M; Rotshenker, Erulkar & Rahimimoff, 1976; Shimoni, Alnaes & Rahimimoff, 1977) that the gradient for Ca^{2+} is actually reversed, i.e. nerve stimulation should drive Ca^{2+} out of the nerve ending via Ca^{2+} channels (see also Zucker & Lando, 1986; Silinsky & Solsona, 1992). Hence, any release generated by nerve stimulation in MgEGTA solutions could not be due to Ca^{2+} entry via Ca^{2+} channels as Ca^{2+} would be exiting the nerve ending in such a reverse gradient. Figure 3 shows that Ca^{2+} liposomes support ACh release evoked by action potentials in MgEGTA Ringer solution. Figure 3A shows the raw data collected in the presence of Ca^{2+} liposomes in MgEGTA solutions. Figure 3B shows the average EPP in response to sixty-four stimuli in MgEGTA (m , 1.36; the control tracing in the absence of Ca^{2+} liposomes is shown as an inset above). The level of ACh release in MgEGTA is similar to that produced by Ca^{2+} liposomes in the absence of EGTA (Fig. 3C; m , 1.34) and to a solution containing 0.2 mM extracellular Ca^{2+} with 1 mM Mg^{2+} (Fig. 3D; m , 1.49). Ca^{2+} liposomes in MgEGTA supported EPPs in a total of twenty-one experiments, producing a range of ACh release levels similar to that shown in Fig. 1.

Physiological controls to test for the leakage of Ca^{2+} from Ca^{2+} liposomes

This next series of experiments acts as a control for Ca^{2+} leakage from the interior of the liposomes to the extracellular

solution by exploiting an earlier electrophysiological observation that small amounts of extracellular Ca^{2+} can actually antagonize ACh release produced by nerve stimulation in solutions containing extracellular Ba^{2+} (Silinsky, 1977, 1978). The experiment shown in Fig. 4 illustrates such an electrophysiological control made on the cell studied in Fig. 3. The solution contained 1 mM Ba^{2+} and 1 mM Mg^{2+} (Ba^{2+} solution). Brief repetitive nerve stimulation in Ba^{2+} solution (20 Hz for 1.4 s; see bars below each trace in Fig. 4) elicited an asynchronous avalanche of MEPPs of such magnitude that an underlying slow depolarization of the muscle membrane was generated (vertical arrow in Fig. 4A). The depolarization is produced by the entry of Ba^{2+} through Ca^{2+} channels and its amplitude is directly related to asynchronous ACh release reflected as increases in evoked MEPP frequency (evoked MEPP frequency, 1355 s^{-1} in Fig. 4A). Whilst Ca^{2+} liposomes (i.e. intracellular Ca^{2+}) in Ba^{2+} solution had a small but significant stimulatory effect on evoked MEPP frequency in this experiment (Fig. 4B; MEPP frequency, 1532 s^{-1}), the addition of 0.2 mM extracellular Ca^{2+} to the Ba^{2+} solution (Fig. 4C) profoundly antagonized the evoked MEPP discharge (139 s^{-1}). If small amounts of Ca^{2+} were leaking from the liposomes to the extracellular fluid, then Ca^{2+} liposomes should have antagonized the effects of Ba^{2+} on asynchronous ACh release in a manner similar to 0.2 mM extracellular Ca^{2+} , which in this experiment produced similar EPPs to Ca^{2+} liposomes (see Fig. 3). This was indeed not the case. In a total of six experiments, Ca^{2+} liposomes failed to antagonize asynchronous evoked ACh release. The stimulatory effects of Ca^{2+} liposomes is thus

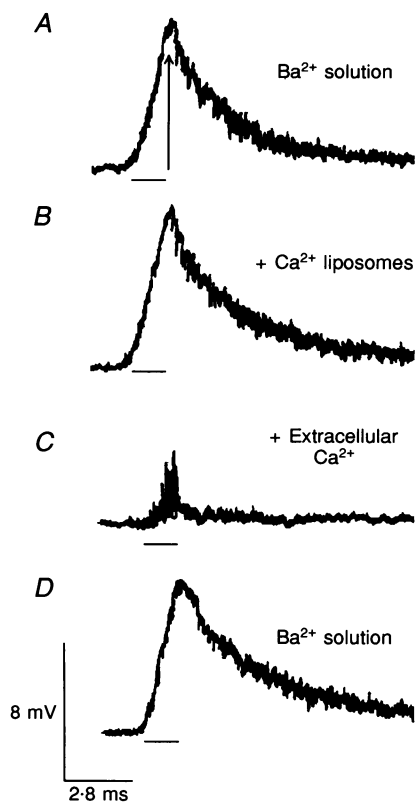


Figure 4. Electrophysiological control for the leakage of extracellular Ca^{2+} from Ca^{2+} -containing liposomes suspended in Ca^{2+} -free solutions

Figures 3 and 4 show results from the same experiment. The motor nerve was stimulated at 20 Hz for 1.4 s (bars) in Ba^{2+} solution (1 mM Ba^{2+} , 1 mM Mg^{2+}). A, control evoked MEPP frequency ($1355 \pm 35 \text{ s}^{-1}$, $n = 4$). Slow depolarization (vertical arrow in A) is directly related to evoked MEPP frequency and was calculated as in Silinsky, 1984. In B, inclusion of Ca^{2+} liposomes in the Ba^{2+} solutions produced a small but highly significant augmentation of Ba^{2+} -mediated ACh release (MEPP frequency, $1532 \pm 45 \text{ s}^{-1}$; $n = 4$; $P < 0.05$). In C, addition of 0.2 mM extracellular Ca^{2+} to the Ba^{2+} solution profoundly antagonized the evoked MEPP discharge (MEPP frequency, $139 \pm 16 \text{ s}^{-1}$; $n = 4$). The effect of 0.2 mM Ca^{2+} on Ba^{2+} -mediated ACh release was reversible (D).

unlikely to be due to leakage of the contents of Ca^{2+} -containing liposomes to the fluid bathing the extracellular surface of the nerve ending.

Given the results thus far, it would appear of interest to provide direct evidence for the interaction of liposomes with the membrane of the nerve ending and for the delivery of the aqueous contents of the liposomes to the nerve terminal cytoplasm. To this end we used confocal laser microscopy in conjunction with fluorescent double labelling methods.

Morphological evidence for the interaction of liposomes with the nerve ending and the delivery of entrapped contents to the cytoplasm

Figures 5 and 6 illustrate, respectively, the interaction of the liposomal membrane with the nerve terminal membrane and the delivery of solute encapsulated within liposomes to the nerve terminal cytoplasm. To confirm that the fluorescent structures observed running longitudinally along the muscle surface were indeed motor nerve endings, we used peanut agglutinin (PNA) conjugated with either

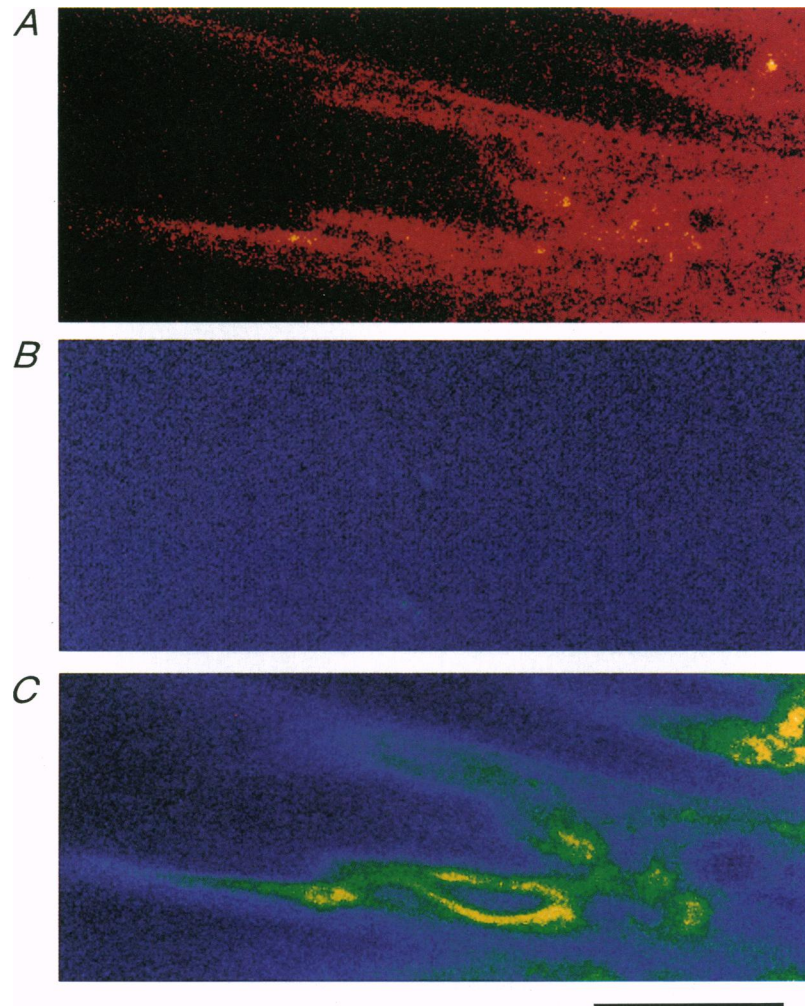


Figure 5. Nerve terminal fluorescence induced by PC liposomes containing 0.5% lissamine rhodamine conjugated to PE (RhPE liposomes)

A, fluorescence recorded on the green channel. Image was recorded after first superfusing for 30 min with RhPE liposomes followed by a 10 min wash (in the absence of liposomes, the field appears black – data not shown). Note the apparent outline of motor nerve endings. *B*, same field as *A* recorded on the red channel prior to bathing the tissue in fluorescein-conjugated peanut agglutinin (F-PNA). *C*, same field as *A* recorded on the red channel 5 min after F-PNA treatment. Scale bar, 20 μ m. Figures 5 and 6 were made using a $\times 40$ water immersion lens and a $\times 2$ zoom was implemented by the software. Caution is required in interpreting these results, since the staining by RhPE vesicles could be due to adherent vesicles or to the lipid of vesicles that have fused or otherwise transferred to the cell membrane. For further details, see text and Methods

tetramethylrhodamine (TMR-PNA) or fluorescein (F-PNA) as described in Methods; PNA selectively stains motor nerve endings in this frog preparation (Ko, 1987). Figure 5A shows a preparation after treatment with RhPE liposomes recorded on the red channel (see Methods). Note the apparent staining of nerve endings (in the absence of RhPE, the image appears black – data not shown).

Confirmation of the identity of the fluorescent structures was made by removing the RhPE vesicles and switching the channel to the green channel sensitive to F-PNA (Fig. 5B and C). Whilst in the absence of F-PNA, no fluorescence is observed (Fig. 5B), after addition of F-PNA a characteristic staining pattern of motor nerve ending results. Comparison of Fig. 5A and C demonstrates that

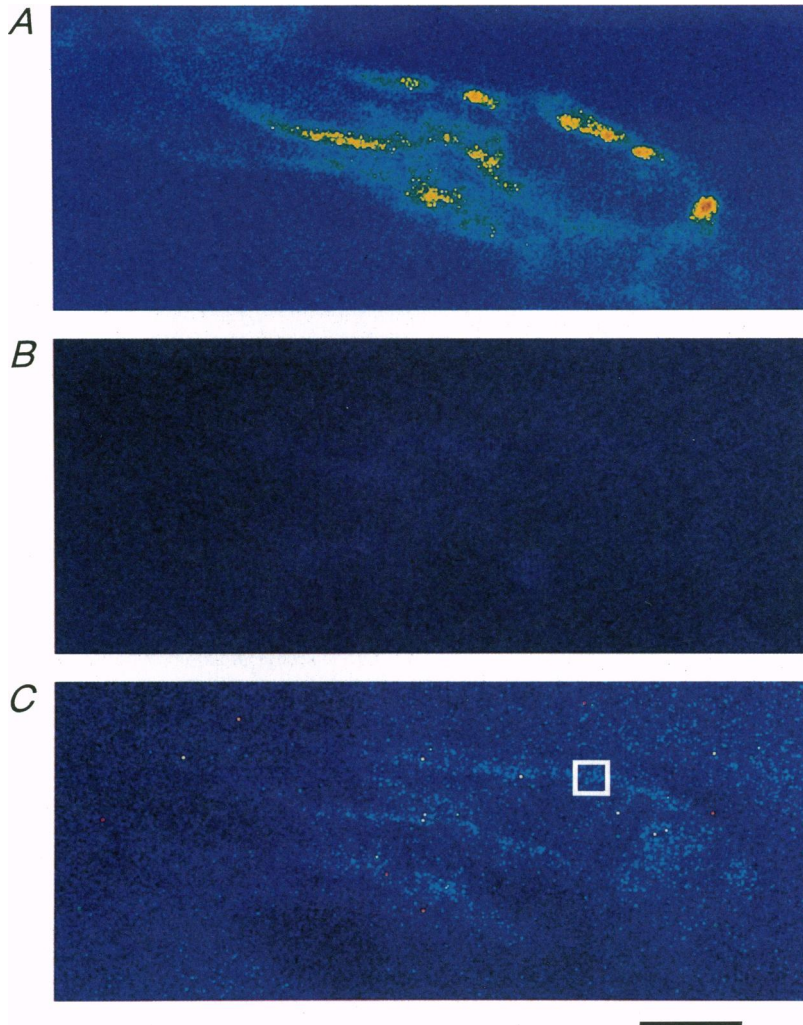


Figure 6. Nerve terminal fluorescence induced by calcein-containing liposomes

PC liposomes were prepared as described in Methods and contained 15 mM calcein + 5 mM CoCl_2 (calcein liposomes). *A*, control image of nerve terminal stained with TMR-PNA and recorded in the red channel. *B*, fluorescence of the same field recorded in the absence of calcein liposomes on the green channel. *C*, fluorescence recorded as in *B* but after treatment with calcein liposomes. For the image shown in *C*, the preparation was exposed to liposomes for 25 min and then returned to normal solution for 18 min prior to taking the photograph. Scale bar, 10 μm . In other experiments, similar results were observed within 7 min after superfusion with calcein liposomes. CoCl_2 (10 μM) was added to the liposome suspension to quench any extracellular calcein (Kendall & MacDonald, 1983; Perin & MacDonald, 1989). A pixel intensity comparison of the nerve terminal region enclosed in the square reveals a 2.4-fold increase in intensity produced by calcein liposomes. The fact that calcein from the vesicles is observed in the cells does not preclude the possibility that fusion is rare relative to adhesion or transfer. Indeed the two sets of experiments illustrated in Figs 5 and 6 could represent different aspects of the interaction between the liposome and the surface membrane of the nerve ending.

RhPE stained large portions of the motor nerve terminals. As rhodamine conjugated with lipid does not exchange across the aqueous milieu (Struck *et al.* 1981), the persistence in staining pattern when coupled with the similar staining pattern between the stain selective for nerve endings (Fig. 5C) and RhPE (Fig. 5A) suggests that RhPE liposomes are in direct membrane communication (via adsorption or fusion) with nerve terminal membranes. (For discussion of other regions of fluorescence, see legend to Fig. 5 and Discussion).

Figure 6 presents a typical experiment made with calcein liposomes to illustrate the delivery of the liposomal contents to the nerve terminal cytoplasm. Figure 6A shows the TMR-PNA staining pattern of the nerve ending recorded on the red channel. On the calcein channel (green), no fluorescence is observed either in the absence of calcein or shortly after calcein addition when calcein remains in the liposomes in high concentration and is thus self-quenched (Fig. 6B). In Fig. 6C, note that after exposure to calcein liposomes, a fluorescent pattern similar in distribution to Fig. 6A is observed, suggesting that dilution and subsequent elimination of calcein self-quenching has occurred as a consequence of the delivery of the entrapped calcein to the nerve terminal cytoplasm. (For further details and quantitation of the difference in fluorescence of the indicated nerve terminal region after treatment with calcein liposomes, see legend to Fig. 6.)

DISCUSSION

The results suggest that action potentials in vertebrate nerve endings promote physiologically functional ACh release in the absence of Ca^{2+} entry provided that the cytoplasmic Ca^{2+} levels are elevated by Ca^{2+} released from Ca^{2+} -containing liposomes into the interior of the cell. Such liposomes appear to deliver their encapsulated contents to a local cytoplasmic region near the sites of ACh secretion, much as is the case with Ca^{2+} delivered via Ca^{2+} channels (see also Hirsh *et al.* 1990 for discussion).

The behaviour of extracellular Ca^{2+} as an antagonist of Ba^{2+} in Fig. 4 merits further discussion. Extracellular Ca^{2+} is a very weak partial agonist for the process of neurally evoked asynchronous MEPP discharges (Silinsky, 1977, 1978). Because of the great difference in efficacy between Ba^{2+} and Ca^{2+} for this asynchronous release process, Ca^{2+} acts as a potent competitive inhibitor of neurally evoked MEPPs mediated by extracellular Ba^{2+} (e.g. Fig. 4C). This effect is due to the block of Ba^{2+} entry from the extracellular fluid into the nerve ending by Ca^{2+} (Silinsky, 1977, 1978). Indeed, the affinity of extracellular Ca^{2+} as an antagonist of Ba^{2+} is greater than the apparent affinity of Ca^{2+} to activate the ACh release process itself (Silinsky, 1977, 1978, 1981). Hence the failure of Ca^{2+} liposomes to antagonize Ba^{2+} -mediated ACh release provides further confirmation of earlier results (see Fig. 1 in Mellow *et al.* 1982) that liposomes prepared in this manner are

delivering their entrapped contents to the cytoplasmic milieu of the nerve ending.

Even given these results, it might still be argued that liposomes burst locally upon contact with the surface membrane and expose circumscribed regions of the nerve ending to extremely high concentrations of extracellular Ca^{2+} . Several lines of evidence argue against this contention. Firstly, if this were true, then liposomes loaded with Ca^{2+} and Mg^{2+} should be less effective in promoting ACh release than Ca^{2+} liposomes alone, as the extracellular Mg^{2+} derived from the ruptured liposomes would antagonize the action of concomitantly leaked Ca^{2+} . The published results reveal the opposite effect, namely that Mg^{2+} in liposomes actually enhances the effects of intracellular Ca^{2+} , possibly by blocking an extrusion mechanism (see Figs 2 and 3 in Kharasch *et al.* 1981). Secondly, when the ability of Sr^{2+} -containing liposomes to increase ACh release is compared with that mediated by extracellular Sr^{2+} , it was observed that Sr^{2+} delivered in liposomes was able to increase evoked ACh release severalfold above the maximum that can be produced with extracellular Sr^{2+} , however high the Sr^{2+} concentration in the bathing fluid (Silinsky, 1981; M. Watanabe & E. M. Silinsky, unpublished data). Finally, the frequency of MEPPs in the absence of nerve stimulation is accelerated by Ca^{2+} or Sr^{2+} liposomes in a manner that would not occur if evoked release were produced by the local leakage of cation to the extracellular fluid (see e.g. Figs 5 and 6 in Mellow *et al.* 1982; and legend to Fig. 1B in this paper).

The confocal fluorescence micrographs (Figs 5 and 6) show that PC liposomes interact with nerve terminals and both outline the nerve terminal surface and dilute their contents in the cytoplasm of the cell. As RhPE is not known to dissociate from bilayers to any significant extent (Struck *et al.* 1981) the staining of the nerve ending with RhPE liposomes must reflect the direct association of the vesicles with the cell surface membranes. It is possible, however, that some RhPE could transfer from the bilayer of an adherent vesicle to the membrane. Indeed, our results do not distinguish adsorption to the cell membrane from fusion with the membrane. Consequently, the presence of calcein in the cells could be a result of endocytosis followed by efflux into the cytoplasm or of direct fusion with the cell membrane.

The effects of liposomes illustrated in Figs 5 and 6 appear relatively selective to nerve terminals and to regions of the muscle membrane parallel to the striations (possibly t-tubule membranes, e.g. Fig. 6). The reason for this is not clear, although in the case of the nerve terminal membrane the regulated synaptic vesicle fusion, exocytosis and endocytosis that occur at a highly organized secretory apparatus represent clear departures from the activities of most cells. Such behaviour associated with transmitter secretion and the subsequent retrieval of synaptic vesicle membranes might make the nerve ending a more favourable target for liposomes.

With respect to the relevance of these results to physiologically functional neurotransmitter release, there is little doubt that optimal levels of neurotransmitter

secretion require Ca^{2+} entry through voltage-gated Ca^{2+} channels. The vertebrate motor nerve ending appears to possess an additional property to improve the fidelity of information transfer from nerve to muscle, namely a voltage-sensitive Ca^{2+} binding protein that confers cation selectivity on the secretory apparatus (Silinsky, 1981, 1985). This protein is likely to control the kinetics of the release process (Silinsky, 1985; Parnas & Parnas, 1988; Hochner *et al.* 1989; Dudel, 1990) and thus allow for temporal harmony between each nerve terminal action potential and the synchronous secretion of neurotransmitter (for models of this process, see Parnas & Parnas, 1988; Silinsky, 1985).

- AUGUSTINE, G. J., ALDER, E. M. & CHARLTON, M. P. (1991). The calcium signal for transmitter secretion from presynaptic nerve terminals. *Annals of the New York Academy of Science* **635**, 365–381.
- AUGUSTINE, G. J., CHARLTON, M. P. & SMITH, S. J. (1987). Calcium action in synaptic transmitter release. *Annual Review of Neuroscience* **10**, 633–693.
- AUGUSTINE, G. J. & ECKERT, R. (1984). Divalent cations differentially support transmitter release at the squid giant synapse. *Journal of Physiology* **346**, 257–271.
- DEL CASTILLO, J. D. & KATZ, B. (1954). Quantal components of the end-plate potential. *Journal of Physiology* **124**, 560–573.
- DUDEL, J. (1990). Depolarizing pulses to neuromuscular terminals of frogs can elicit graded, phasic transmitter release in the absence of Ca^{2+} influx. *Neuroscience Letters* **116**, 94–100.
- GUTMAN, Y., LICHTENBERG, D., COHEN, J. & BOONYAVIROJ, P. (1979). Increased catecholamine release from adrenal medulla by liposomes loaded with sodium or calcium ions. *Biochemical Pharmacology* **28**, 1209–1211.
- HIRSH, J. K., SILINSKY, E. M. & SOLSONA, C. S. (1990). The role of cyclic AMP and its protein kinase in mediating acetylcholine release and the action of adenosine at frog motor nerve endings. *British Journal of Pharmacology* **101**, 311–318.
- KATZ, B. (1969). *The Release of Neurotransmitter Substances. The Sherrington Lectures X*. Liverpool University Press, Liverpool, UK.
- HOCHNER, B., PARNAS, H. & PARNAS, I. (1989). Membrane depolarization evokes neurotransmitter release in the absence of calcium entry. *Nature* **342**, 433–435.
- KENDALL, D. A. & MACDONALD, R. C. (1983). Characterization of a fluorescence assay to monitor changes in the aqueous volume of lipid vesicles. *Analytical Biochemistry* **134**, 26–33.
- KHARASCH, E. D., MELLOW, A. M. & SILINSKY, E. M. (1981). Intracellular magnesium does not antagonize calcium-dependent acetylcholine secretion. *Journal of Physiology* **314**, 255–263.
- KO, C.-P. (1987). A lectin, peanut agglutinin, as a probe for the extracellular matrix in living neuromuscular junctions. *Journal of Neurocytology* **16**, 567–576.
- MELLOW, A. M., PERRY, B. D. & SILINSKY, E. M. (1982). Effects of calcium and strontium in the process of acetylcholine release from motor nerve endings. *Journal of Physiology* **328**, 547–562.
- MULKEY, R. M. & ZUCKER, R. S. (1991). Action potentials must admit calcium to evoke transmitter release. *Nature* **350**, 153–155.
- PARNAS, I. & PARNAS, H. (1988). The ' Ca^{2+} -voltage' hypothesis for neurotransmitter release. *Biophysical Chemistry* **29**, 85–93.
- PERIN, M. S. & MACDONALD, R. C. (1989). Fusion of synaptic vesicle membranes with planar bilayers. *Biophysical Journal* **55**, 973–986.
- RAHAMIMOFF, R., MEIRI, H., ERULKAR, S. D. & BARENHOLZ, Y. (1978). Changes in transmitter release induced by ion-containing liposomes. *Proceedings of the National Academy of Sciences of the USA* **75**, 5214–5216.
- ROTSHENKER, S., ERULKAR, S. D. & RAHAMIMOFF, R. (1976). Reduction in the frequency of miniature end-plate potentials by nerve stimulation in low calcium solutions. *Brain Research* **101**, 362–365.
- SHIMONI, Y., ALNAES, E. & RAHAMIMOFF, R. (1977). Is hyperosmotic neurosecretion from motor nerve endings a calcium-dependent process? *Nature* **267**, 170–172.
- SILINSKY, E. M. (1977). Can barium support the release of acetylcholine by nerve impulses? *British Journal of Pharmacology* **59**, 215–217.
- SILINSKY, E. M. (1978). On the role of barium in supporting the asynchronous release of acetylcholine quanta by motor nerve impulses. *Journal of Physiology* **274**, 157–171.
- SILINSKY, E. M. (1981). On the calcium receptor that mediates depolarization–secretion coupling at cholinergic motor nerve terminals. *British Journal of Pharmacology* **73**, 413–429.
- SILINSKY, E. M. (1984). On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings. *Journal of Physiology* **346**, 243–256.
- SILINSKY, E. M. (1985). The biophysical pharmacology of calcium-dependent acetylcholine secretion. *Pharmacological Reviews* **37**, 81–132.
- SILINSKY, E. M. & SOLSONA, C. S. (1992). Calcium currents at motor nerve endings: absence of effects of adenosine receptor agonists in the frog. *Journal of Physiology* **457**, 315–328.
- STRUCK, D. K., HOEKSTRA, D. & PAGANO, R. E. (1981). Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20**, 4093–4099.
- THEOHARIDES, T. C. & DOUGLAS, W. W. (1978). Secretion in mast cells induced by calcium entrapped within phospholipid vesicles. *Science* **201**, 1143–1145.
- WEAKLY, J. N. (1973). The action of cobalt ions on neuromuscular transmission in the frog. *Journal of Physiology* **234**, 597–612.
- ZUCKER, R. S., DELANEY, K. R., MULKEY, R. M. & TANK, D. W. (1991). Presynaptic calcium in transmitter release and posttetanic potentiation. *Annals of the New York Academy of Science* **635**, 191–207.
- ZUCKER, R. S. & LANDO, L. (1986). Mechanism of transmitter release: voltage hypothesis and calcium hypothesis. *Science* **231**, 574–579.

Acknowledgements

This work was supported by research grants from the NIH (NS12782, NS30795) and a training grant (NS07140). We are grateful to Dr R. W. Berry for comments on the manuscript.

Received 2 December 1993; accepted 6 July 1994.