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Calmodulin increases transmitter release by mobilizing quanta at the frog motor nerve terminal

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1 The role of calmodulin (CaM) in transmitter release was investigated using liposomes to deliver CaM and monoclonal antibodies against CaM (antiCaM) directly into the frog motor nerve terminal.

2 Miniature endplate potentials (MEPPs) were recorded in a high K^+ solution, and effects on transmitter release were monitored using estimates of the quantal release parameters *m* (number of quanta released), *n* (number of functional transmitter release sites), *p* (mean probability of release), and var_s *p* (spatial variance in *p*).

3 Administration of CaM, but not heat-inactivated CaM, encapsulated in liposomes (1000 units ml^{-1}) produced an increase in *m* (25%) that was due to an increase in *n*. MEPP amplitude was not altered by CaM.

4 Administration of antiCaM, but not heat-inactivated antiCaM, in liposomes (50 μl ml^{-1}) produced a progressive decrease in *m* (40%) that was associated with decreases in *n* and *p*. MEPP amplitude was decreased (15%) after a 25 min lag time, suggesting a separation in time between the decreases in quantal release and quantal size.

5 Bath application of the membrane-permeable CaM antagonist W7 (28 μM) produced a gradual decrease in *m* (25%) that was associated with a decrease in *n*. W7 also produced a decrease in MEPP amplitude that paralleled the decrease in *m*. The decreases in MEPP size and *m* produced by W7 were both reversed by addition of CaM.

6 Our results suggest that CaM increases transmitter release by mobilizing synaptic vesicles at the frog motor nerve terminal.

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Abbreviations: antiCaM, monoclonal antibodies against CaM; CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; *m*, number of transmitter quanta released; MEPP, miniature endplate potential; *n*, number of functional or operational transmitter release sites; *p*, probability of transmitter release; Tris, tris-(hydroxylmethyl)-aminomethane; var_s *p*, spatial variance in *p*; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide hydrochloride

Introduction

Calmodulin (CaM) is a ubiquitous 16.7 kD intracellular protein that undergoes a conformational change following cooperative binding of Ca^{2+} (Cheung, 1982). The resulting Ca^{2+}/CaM complex may in turn interact with several target proteins, e.g., CaM kinases, cAMP phosphodiesterase, and microtubule-associated protein kinases, to modify cell metabolism and function (Jurado *et al*., 1999; Soderling *et al*., 2001). The diversity of Ca^{2+}/CaM action is exemplified by its ability to also bind to the plasma membrane Ca^{2+}-pump (Falchetto *et al*., 1991), the P/Q-type calcium channel (Lee *et al*., 1999), and the inositol trisphosphate receptor type I (Hirota *et al*., 1999).

An involvement of CaM in the process of secretion was originally suggested based on results obtained in a number of different systems, e.g., insulin release (Schubart *et al*., 1980), amylase secretion (Heisler *et al*., 1981), neutrophil (Naccache *et al*., 1980) and mast cell degranulation (Douglas & Nemeth, 1982). A fundamental role of CaM in neurosecretion was proposed based on several lines of evidence from synaptosomes and synaptic vesicle preparations (DeLorenzo, 1982). However, despite its attractiveness, the notion that CaM might be involved in triggering neurosecretion appeared to be at odds with the rapid time course of the event (Hall, 1992).

One approach to examining this question was to block endogenous CaM with membrane-permeable antagonists such as chlorpromazine, trifluoperazine, calmidazolium, and N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide hydrochloride (W7). Results from these studies were highly equivocal (Duncan, 1983), as transmitter release was increased, decreased, or unaffected depending on experimental conditions (Cheng *et al*., 1981; Publicover, 1983; Sahaf & Publicover, 1985; Jinnai *et al*., 1986; Branisteanu *et al*., 1987; Singh & Prior, 1998). As indicated by Augustine *et al*. (1987), this disparity may have been due to lack of

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specificity of these agents or to the fact that CaM has multiple roles and that interfering with CaM may have had complex effects on the release process.

We have reexamined the role of CaM in transmitter release by introducing into the nerve terminal: (1) exogenous CaM to exaggerate its normal action, and (2) monoclonal antibodies against CaM to block its endogenous activity. The problem of 'gaining access to intracellular sites while maintaining essential cellular functions' (Augustine *et al*., 1987) was minimized by using liposomes to encapsulate these agents and transport them directly into the cytoplasmic compartment (de Paiva & Dolly, 1990; Silinsky *et al*., 1995). It has been shown that binomial statistics may be used reliably to study evoked transmitter release in high K^+ solutions (Provan & Miyamoto, 1993; Searl & Silinsky, 2002). Since a high K^+ solution was used in this study, transmitter release was assessed using the binomial analysis.

Methods

Isolated sciatic-sartorius nerve-muscle preparations from frogs (*Rana pipiens*) were used for these experiments. Experimental procedures were reviewed and approved by the University Committee for Animal Use and Care. Animals were decapitated followed by rapid double pithing, and muscles were removed and mounted in a 5-ml Sylgard-lined Petri dish bath. The bath was continuously perfused with Ringer solution using a dual-chambered roller pump. The $[K^+]$ in the control Ringer solution was increased (equimolar substitution of KCl for NaCl) to raise the basal frequency of miniature endplate potentials (MEPPs) and increase the likelihood of binomial (vs Poisson) release of transmitter. The control Ringer solution contained (mM): NaCl 100, KCl 12.5, $CaCl_2$ 1.8, tris-(hydroxylmethyl)-aminomethane (Tris) 2.0 to pH 7.2, and glucose 5.6. Preparations were equilibrated for 30 min before use.

Encapsulation of agents in liposomes is an accepted procedure for delivering small and large molecules (Brailoiu & van der Kloot, 1996; Brailoiu *et al*., 1999) into the motor nerve terminal (de Paiva & Dolly, 1990; Silinsky *et al*., 1995). Multilamellar liposomes were prepared with 60 mg ml^{-1} egg phosphatidylcholine (Sigma, type X-E), as previously described (Brailoiu *et al*., 2001). Agents to be incorporated into liposomes, i.e., 1000 units ml^{-1} CaM, heat-inactivated CaM, 50 μl ml^{-1} antiCaM or heat-inactivated antibody, were dissolved in 140 mM KCl solution at pH 6.9. Liposome batches were dialyzed (Sigma dialysis sacs) against control Ringer solution (1/600 v v^{-1}, 150 min) to remove non-incorporated agent, and the Ringer solution changed every 30 min. Control liposomes contained 140 mM KCl solution (pH 6.9) only. Liposome suspensions were administrated by continuous perfusion after 1/20 v v^{-1} dilution in control Ringer solution. Calmodulin and N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide hydrochloride (W7) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.), and anticalmodulin (mixture) antibody, clones 2D1, 1F11, 6D4 in 50 μl vials were obtained from Research Biochemicals International (Natick, MA, U.S.A.). Repeated (10 times) heating (75°C for 30 s) and cooling (4°C for 1 min) were used to inactivate CaM and antiCaM antibodies (Kim & Lisman, 2001).

MEPPs were recorded with standard intracellular microelectrode (3 M KCl, 10–15 MΩ resistance) techniques. Selection was made for impalements that showed focal recording, large MEPP size (>0.3 mV), moderately high frequency (>5 s^{-1}), and good signal-to-noise ratio (peak-to-peak noise level of 0.1 mV). Muscle resting potentials ranged between −50 and −60 mV and remained stable throughout the course of each experiment (<3 mV decrease). Only one single-junction experiment was carried out on each preparation. A typical experiment consisted of continually recording in control Ringer only (8 min), during perfusion with the liposomal suspension (10–20 min), and finally during wash with control Ringer only (40–60 min). Experiments were conducted at the ambient room temperature (21–23°C). MEPP frequency obtained under these conditions (12.5 mM K^+) was 24.3 ± 1.2 s^{-1} (mean ± s.e.mean) for 32 experiments. Bioelectric signals were fed into a high impedance preamplifier and viewed on a Tektronix oscilloscope. Signal-to-noise ratio was enhanced with a band-pass filter (0–1 kHz). Results were recorded on magnetic tape using a modified videocassette recorder. Signals were boosted 20 fold by a rear-output amplifier of the oscilloscope to allow interfacing with an A-D data acquisition unit. Results were stored on magnetic tape for off-line analysis. MEPP amplitudes were measured with a grid template on a flat screen monitor and 100 measurements made for each point in time.

Our working model of quantal release was based on the definitions of Brown *et al*. (1976) that '*n* represents the number of sites at which transmitter release can occur, and ... *p* is the probability that a quantum of transmitter is released at a given site'. These authors noted that *p* may vary from one release site to the next, so that there may be 'spatial' variance in *p* ($var_s\ p$). Similarly, *n* and *p* may vary with time, so that there may be 'temporal' variance in *n* and *p*. Use of simple binomial statistics under these circumstances leads to overestimates of *p* and underestimates of *n* and *p*, respectively (Brown *et al*., 1976). On the other hand, use of a compound binomial model that incorporates these variances will provide unbiased estimates of *n* and *p* (McLachlan, 1978). The problem is that this involves five unknowns, none of which is directly measurable. One approach is to set up five simultaneous equations using the first to fifth moments of *m* (*m* being the only measurable variable), but this leads to very large errors due to sampling fluctuations with the higher moments. We instead took a pragmatic approach of reducing sampling time to minimize temporal variation and thereby eliminate two of the variables (temporal variance in *n* and *p*). This left us with only three unknowns, which could be handled by solving simultaneously the first three moment equations for *m* (Miyamoto, 1986).

In practice, the reduction in sampling time could be effected using MEPP frequencies in place of a long series of nerve-evoked endplate potentials. Thus, the number of quanta released by one nerve impulse (*m*) was replaced by the number of MEPPs in a 50 ms interval (bin), and 500 sequential bins used for each quantal estimate. Data were divided into subgroups of 100 before analysis to minimize nonstationarity, and results that were nonstationary according to statistical tests were discarded. Unbiased estimates of *m*, *n*, *p*, and $var_s\ p$ were then computed using equations previously reported (Miyamoto, 1986). The slightly negative *estimates* obtained for $var_s\ p$ were due to a systematic underestimation from the use of 50 ms bins, due to the presence of a small amount of temporal

variance (Brown *et al*., 1976; Provan & Miyamoto, 1993). Use of shorter duration (5–10 ms) bins eliminated the negativity (Provan & Miyamoto, 1993) but usually resulted in very few counts (MEPPs) per bin. For this reason, we elected to retain the use of 50 ms bins and use the slightly negative estimates as the baseline from which to monitor changes in var_s p. As expected, absolute values of the quantal parameters obtained with MEPPs were not the same as values obtained with the traditional method. To avoid possible confusion, we presented effects on n, m and p as simple per cent changes from control. Data for each experiment were expressed as per cents of the value at time zero, and results from four to six experiments averaged (points indicate means ± s.e.mean). Analysis of statistical differences was made by comparing each point with all points obtained in control Ringer, with $P<0.05$ indicating significant differences (Student's paired t-test).

Results obtained with the above approach led us to the following working model. The parameter n (number of sites at which transmitter release can occur) is associated with the number of functional release sites. Although the number of anatomical sites is of course *fixed*, the number of *functional* release sites is *variable* and will depend on the fraction that is operational. Because occupation by a synaptic vesicle is necessary to be operational, it is suggested that n is associated with the number of occupied release sites and that changes in n reflect mobilization or demobilization of vesicles to the release sites (an analogy might be the variable number of agonist-bound receptors in a large, fixed population of receptors). Our results suggest that p is associated with $[Ca^{2+}]$ at the active zones, since the calcium ionophore A23187 causes an increase in p but no increase in n or var_s p (Brailoiu *et al*., 2001). Our results also suggest that var_s p is associated with variations in $[Ca^{2+}]$ at the active zones, as might occur with release of Ca^{2+} from distal organelles, since agents that promote Ca^{2+} release from mitochondria cause increases in var_s p (Provan & Miyamoto, 1993; 1995).

Results

Effect of exogenous CaM on quantal transmitter release

Administration of CaM encapsulated in liposomes (1000 unit ml^{-1}) produced a transient increase in quantal release

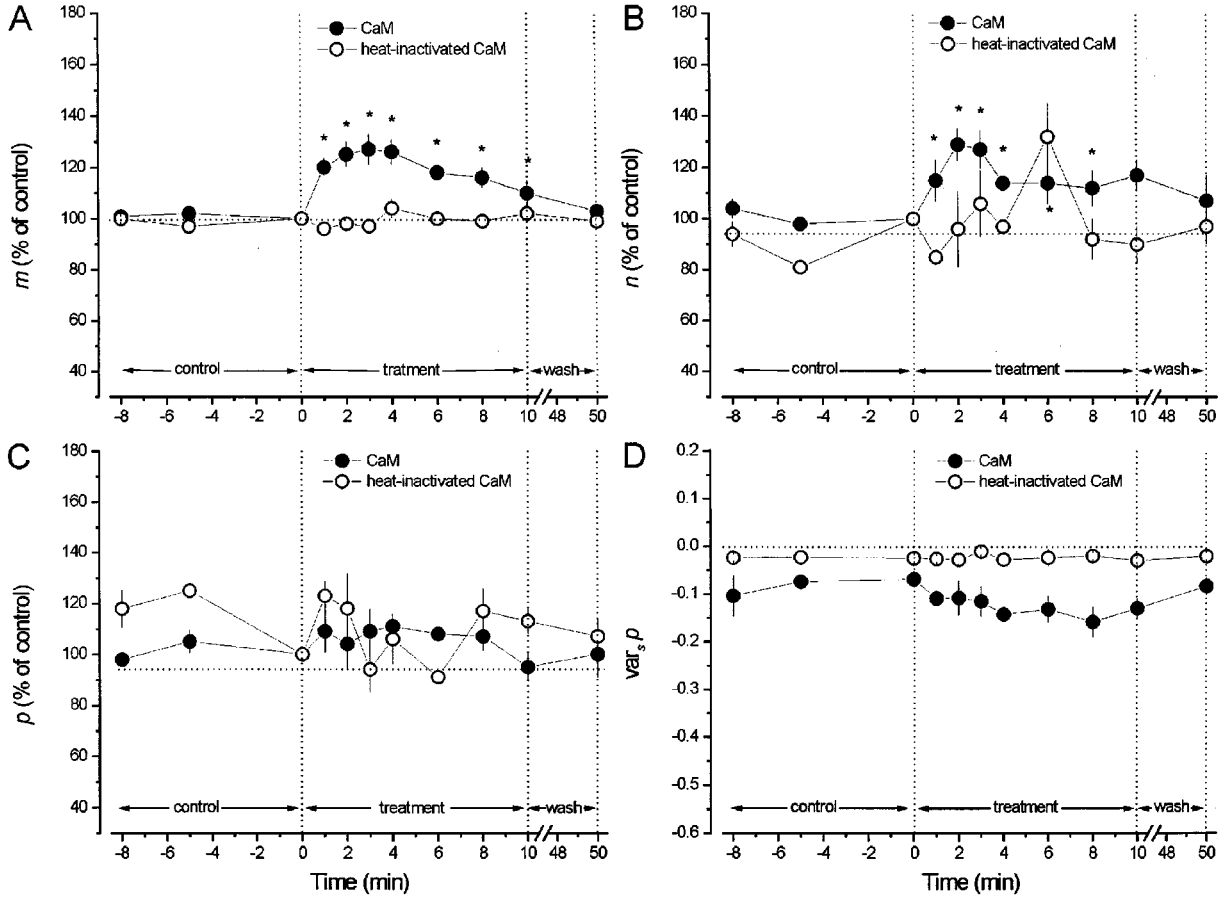

Figure 1 Effects of calmodulin (CaM) and heat-inactivated CaM on quantal release parameters at the frog neuromuscular junction. CaM and heat-inactivated CaM were encapsulated in liposomes (1000 units ml^{-1}) and the solution perfused into the nerve-muscle chamber. Graph shows effect of CaM (filled circles) and heat-inactivated CaM (open circles) on: (A) number of quanta released (m); (B) number of functional transmitter release sites (n); (C) mean probability of release (p); (D) spatial variance in p (var_s p). Single asterisks indicate a significant difference compared to control ($P<0.05$). Stated concentration refers to CaM in the aqueous phase; the concentration in the nerve terminal is about 10^{-2} of the encapsulated concentration. Estimates of quantal release parameters were derived as described in the Methods. Control MEPP frequencies at time 0 are (means ± s.e.mean): 21.8 ± 2.4 s^{-1} ($n=6$) for CaM; 19.9 ± 3.0 s^{-1} ($n=4$) for heat-inactivated CaM.

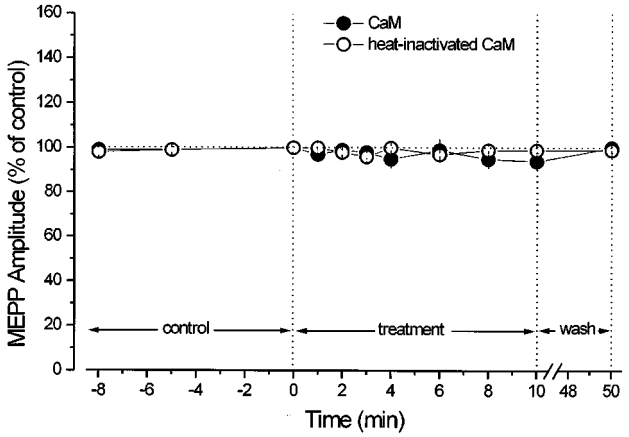

Figure 2 Effects of calmodulin (CaM, filled circles) and heat-inactivated CaM (open circles) delivered by liposomes on miniature endplate potential (MEPP) amplitude. Data were converted to per cent of the values at time 0, and the results from 4–6 experiments expressed as means ± s.e.mean. There was no change in muscle resting potential in either instance.

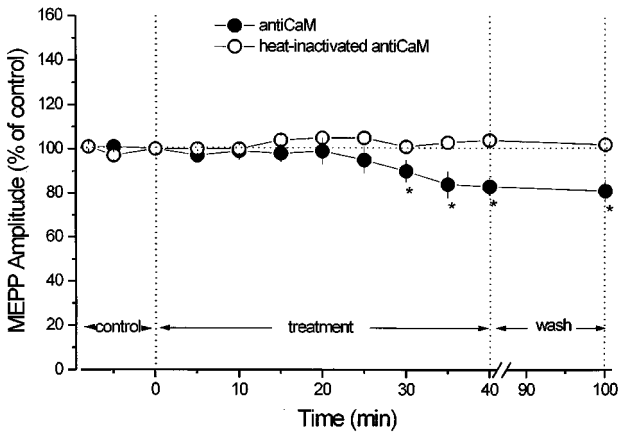

Figure 4 Effects of liposome-encapsulated monoclonal antibodies against calmodulin (antiCaM, filled circles) and heat-inactivated antiCaM (open circles) on MEPP amplitude. Data were converted to per cent of the values at time 0, and the results from 4–6 experiments expressed as means ± s.e.mean. Note that antiCaM causes a small but significant decrease in MEPP amplitude (15%), but only after a 25 min lag period. By contrast, antiCaM causes a decrease in quantal output (*m*) after the first 5 min (Figure 3A). Note also that the decrease in MEPP amplitude with antiCaM is not reversed on prolonged wash. There is no change in muscle resting potential in either instance.

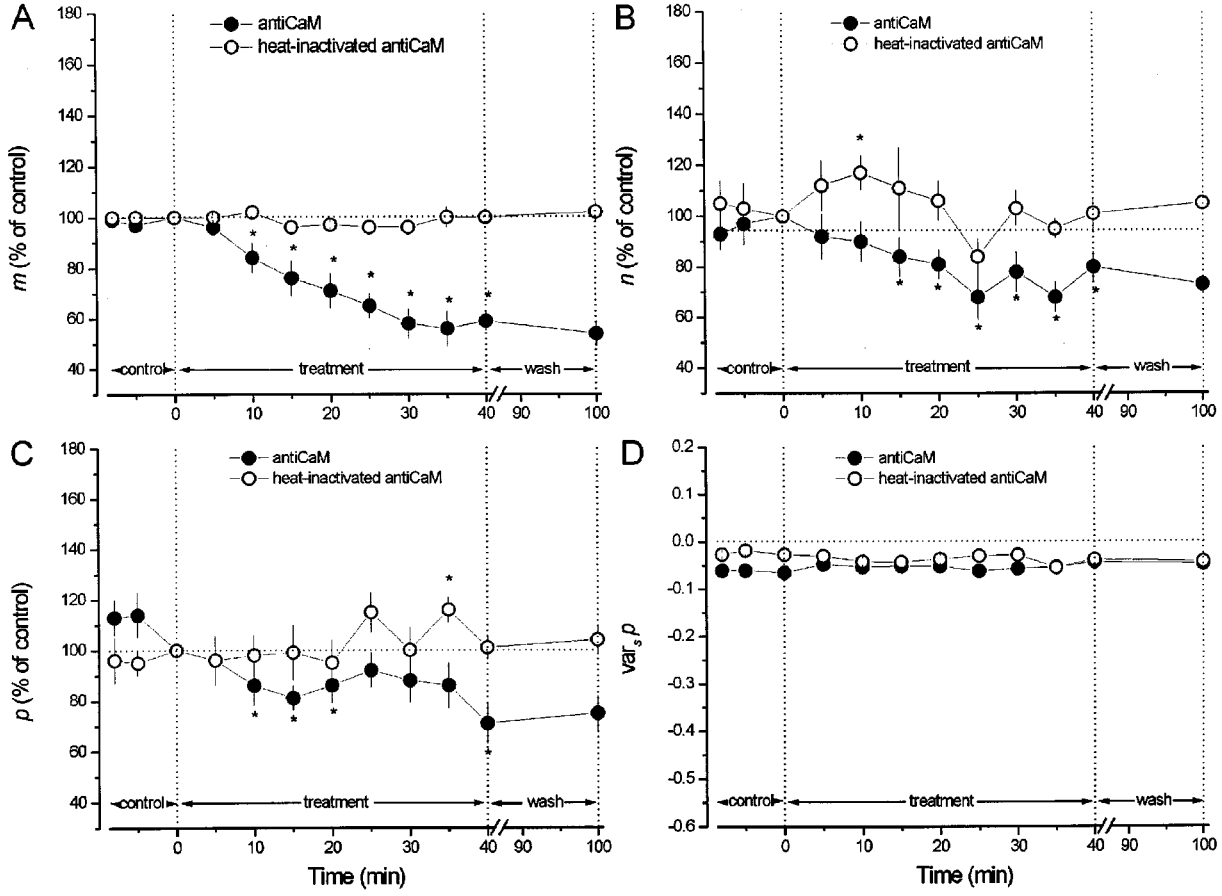

Figure 3 Effects of monoclonal antibodies against calmodulin (antiCaM) and heat-inactivated antiCaM on quantal release parameters. AntiCaM (filled circles) and heat-inactivated antiCaM (open circles) were encapsulated in liposomes (50 μl ml^{-1}), and the liposomal solution perfused into the organ bath at time zero. Shown are the effects on: (A) number of quanta released (*m*); (B) number of functional transmitter release sites (*n*); (C) mean probability of release (*p*), (D) spatial variance in *p* (var_s *p*). Single asterisks denote significant differences from control ($P<0.05$). Note that the effects of antiCaM were not reversed after 60 min wash. Control MEPP frequencies at time 0 are (means ± s.e.mean): 24.5 ± 2.5 s^{-1} ($n=6$) for antiCaM; 21.6 ± 3.7 s^{-1} ($n=4$) for heat-inactivated antiCaM.

(m) that peaked at 125% of control after 3 min and subsided to 108% of control after 10 min (filled circles in Figure 1A). The increase in m was associated primarily with an increase in n, the number of functional transmitter release sites (to 130% of control; filled circles in Figure 1B), as there were no significant changes in p, the mean probability of release (filled circles in Figure 1C) or var_s p, the spatial variance in p (filled circles in Figure 1D). The effects were reversed after 40 min of wash. For a molecule the size of CaM, the final concentration attained in the nerve terminal was estimated to be about 10^{-2} of the concentration contained in the liposomes (Brailoiu & van der Kloot, 1996; Brailoiu *et al*., 1999). Heat-inactivated CaM (1000 units ml^{-1}) had no significant effects on any of the quantal release parameters (open circles in Figure 1A–D). There was no change in muscle resting potential or mean MEPP amplitude during the administration of CaM (filled circles in Figure 2) or heat-inactivated CaM (open circles in Figure 2). Control liposomes filled with 140 mM KCl also had no effect on MEPP frequency or amplitude (results not shown).

Effect of antibody against CaM

Administration of liposomes that contained antibodies against CaM (50 μl ml^{-1}) produced a progressive, significant decrease in m (filled circles in Figure 3A). The maximal decrease in m was to 57% of control after 40 min and was associated with significant decreases in n (to 60% of control; filled circles in Figure 3B) and p (to 70% of control; filled circles in Figure 3C). There was no effect on var_s p (filled circles in Figure 3D). The effects of antiCaM were not reversed, even after 1 h of wash. Again, it was estimated that the concentration attained in the cytoplasm for large molecules such as antibodies was about 10^{-2} of the concentration encapsulated in the liposomes (Brailoiu & van der Kloot, 1996; Brailoiu *et al*., 1999). Liposomes

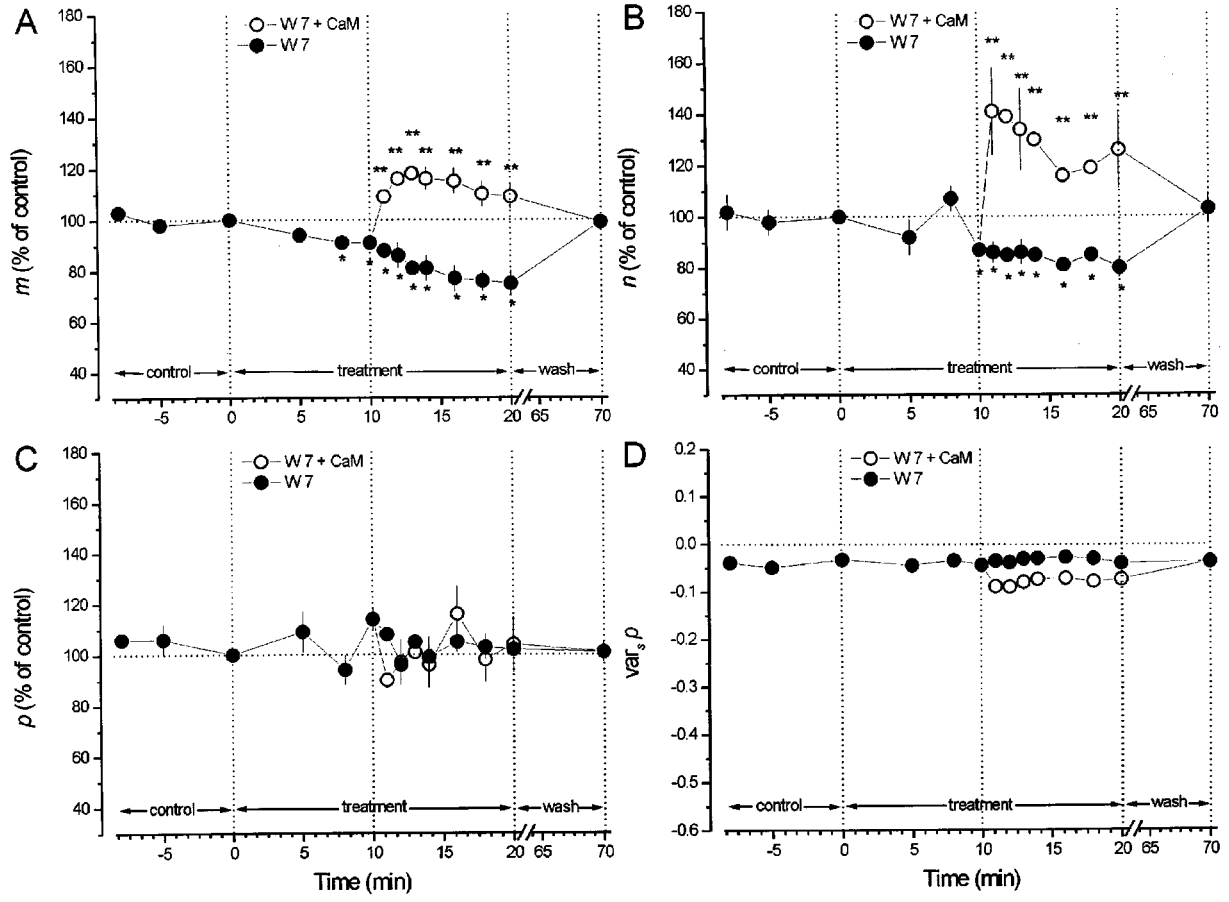

Figure 5 Effects of the CaM antagonist W7 on quantal release parameters and the reversal of these effects by CaM. Graph shows effects of bath application of 28 μM W7 (filled circles) on: (A) number of quanta released (m); (B) number of functional release sites (n); (C) mean probability of release (p); (D) spatial variance in p (var_s p). Note that the decrease in m is due to a decrease in n and that the effects are reversed with wash. Single asterisks denote significant differences from control ($P<0.05$). Superimposed on this graph are results from a parallel set of experiments in which CaM is added to preparations treated with 28 μM W7. Application of liposome-encapsulated CaM (1000 units ml^{-1}) at time = 10 min causes an increase in m (open circles in panel A) that reverses the decrease produced by W7. The increase in m is due to a large increase in n (open circles in panel B) that reverses the decrease caused by W7. Double asterisks indicate that points are significantly different from control and from results for W7 alone (filled circles). There is a small, non-significant decrease in var_s p after application of CaM (open circles in panel D) that is similar to the effect seen with CaM alone (filled circles in Figure 1D). One explanation may be that the CaM synchronously activates voltage-gated Ca^{2+} channels, which leads to a more uniform $[Ca^{2+}]$ at the active zones. Control MEPP frequencies at time 0 are (means ± s.e.mean): 24.8 ± 2.5 s^{-1} ($n=6$) for W7; 29.98 ± 3.3 s^{-1} ($n=6$) for W7 + CaM.

containing heat-inactivated CaM antibodies (50 μl ml^{-1}) had no consistent effect on any of the quantal release parameters (open circles in Figure 3A–D). There was again no change in muscle resting potential. Administration of antiCaM did however produce a small (15%) but significant decrease in MEPP amplitude that was not reversed by 1 h of wash (filled circles in Figure 4). This effect on quantal size was separated in time from the effect on quantal release, as the decrease in MEPP amplitude began after 25 min (filled circles in Figure 4), whereas the decrease in m began after only 5 min (filled circles in Figure 3A). As expected, administration of heat-inactivated CaM produced no change in MEPP amplitude (open circles in Figure 4).

Effect of the CaM antagonist W7

While the primary aim was to document the effect of CaM and antiCaM on transmitter release, it was important to compare these results with those obtained with a membrane-permeable CaM inhibitor, using the same conditions and methods. We chose W7, which was reportedly one of the better CaM antagonists (Hidaka & Tanaka, 1985). Bath application of W7 (to a final IC_{50} concentration of 28 μM) produced a gradual decrease in m to 73% of control after 20 min (filled circles in Figure 5A). This effect was associated primarily with a decrease in n (filled circles in Figure 5B), as there were no significant changes in either p (filled circles in Figure 5C) or var_s p (filled circles in Figure 5D). The effects were reversed after 50 min of wash.

Effect of exogenous CaM after inhibition of endogenous CaM with W7

If the decrease in transmitter release with W7 (Figure 5A) were due to a prejunctional inhibition of CaM, then supplementation with exogenous CaM would be expected to reverse the decrease. Accordingly, muscles were exposed to W7 (28 μM) for 20 min as before, but CaM (1000 units ml^{-1} in liposomes) added after the first 10 min, with W7 still present in the perfusate. The results (superimposed on the W7 data) showed that CaM again caused an increase in m (open circles in Figure 5A) that was associated primarily with an increase in n (open circles in Figure 5B), as there was no marked change in p (open circles in Figure 5C) and only a slight, non-significant decrease in var_s p (open circles in Figure 5D).

W7 also caused a gradual decrease in MEPP amplitude to 42% of control after 17 min (filled circles in Figure 6). Liposomal administration of CaM after 10 min in W7 caused a reversal of the decrease in MEPP amplitude (open circles in Figure 6), even though W7 was still present in the perfusate.

Interestingly, the time course of effect of W7 on MEPP amplitude appeared similar to the time course of effect of W7 on m (*cf.* Figure 5A). This raised the possibility that W7 might have reduced postjunctional sensitivity and caused an 'apparent' decrease in MEPP frequency (m) due to loss of MEPPs in the noise. To test this, we plotted the cumulative MEPP amplitude-frequency distributions in the presence and absence of W7 (six experiments). In each case, there was a shift to the left with W7 (filled circles in Figure 7) from the control sigmoidal curve (open circles in Figure 7), indicating that W7 caused a decrease in MEPP amplitude. There was, however, no truncation at the low end of the W7 curve, as would be expected if MEPPs were being lost in the noise. These results thus indicated that the decrease in m (MEPP frequency) with W7 was not an artifact due to the decrease in MEPP amplitude.

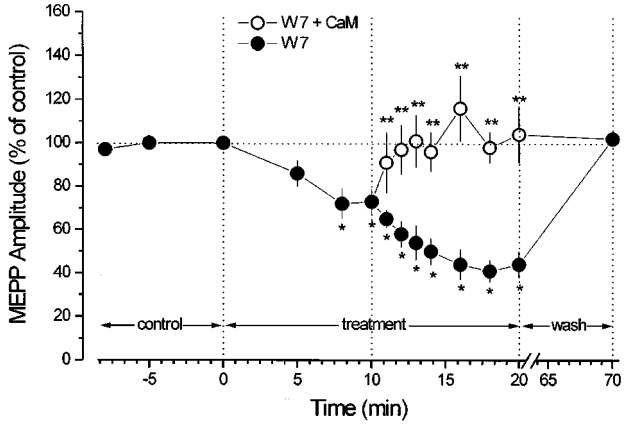

Figure 6 Effects of W7 on MEPP amplitude in the absence and presence of CaM. MEPP amplitudes are converted to per cent of the values at time 0 and results from six experiments graphed (mean ± s.e.mean) as a function of time. Bath application of W7 causes a marked decrease in MEPP amplitude (filled circles), which is reversed with wash. Single asterisks indicate significant difference from control. Superimposed on this plot are results from a parallel set of experiments ($n=6$) in which CaM is added to preparations treated for 10 min with W7 (28 μM). Note that the decrease in MEPP amplitude with W7 is reversed by CaM in a relatively brief period (3 min). Double asterisks indicate significant difference from control and from points representing W7 only. There was no change in muscle resting potential in any of the muscle fibres tested.

Discussion

The hypothesis that CaM is important in neurosecretion (DeLorenzo, 1982) was based on results obtained in neural systems (DeLorenzo, 1981; Burke & DeLorenzo, 1982) and on the possibility that the binding of four Ca^{2+} by CaM might be linked to the fourth power cooperativity between Ca^{2+} and transmitter release (Dodge & Rahamimoff, 1967). Subsequent studies, however, failed to demonstrate an obligatory role of CaM in neurosecretion, which agreed with the *a priori* argument that the rapidity of the event would preclude a mechanism involving activation of a kinase (Hall, 1992). Nonetheless, the possibility remained that CaM might have a regulatory action or help to transport vesicles to the release site (Hall, 1992). This was supported by studies showing that Ca^{2+}/CaM-dependent protein kinase II (CaM-KII) or synapsin I enhanced transmitter release when either was injected into the presynaptic digit of the squid giant synapse (Lin *et al.*, 1990). The implication was that CaMKII acted by phosphorylating synapsin I, which reduced vesicle binding to actin and increased vesicle availability (Llinás *et al.*, 1991).

The present results show that administration of CaM directly into a vertebrate nerve terminal produces an increase in quantal transmitter release and that administration of antibodies against CaM produces the opposite effect. Analysis of the effect on quantal release parameters shows that the increase in m with CaM is due primarily to an

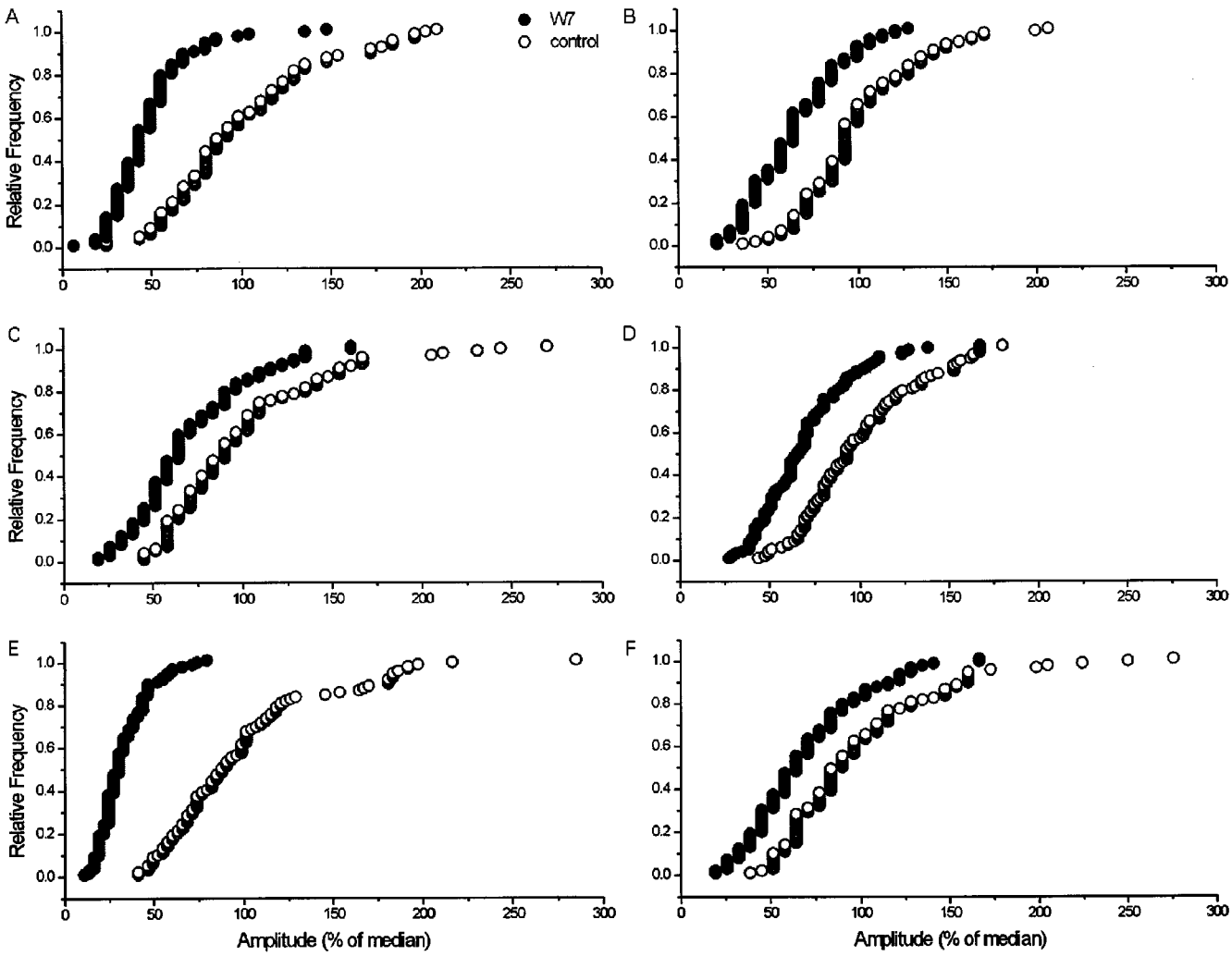

Figure 7 Cumulative MEPP amplitude-frequency plots in the absence (open circles) and presence (filled circles) of W7 (28 μM). Panels 1 through 6 show results from the six single junction experiments with W7. Abscissas represent MEPP amplitudes as per cent of the median MEPP amplitude obtained at time = 0 min in control solutions. Ordinates represent the relative frequency of 100 measurements for each curve. Results in the presence of W7 were obtained during the maximal effect of W7 at time = 18 min. In all cases, there is a leftward shift from the control curves, which indicates a decrease in MEPP amplitude with W7. There is no truncation at the lower end of the W7 curves (filled circles), which indicates that MEPPs are not being obscured in the noise. Medians for each experiment are: 0.372, 0.317, 0.352, 0.414, 0.428, and 0.353 mV, respectively.

increase in n. According to our working hypothesis (see Methods), this means that CaM increases the number of functional release sites, i.e., sites occupied by synaptic vesicles. Since changes in the number of occupied release sites could occur with mobilization or demobilization of vesicles to the active zone, our results are compatible with the proposal of Llinás *et al.* (1991) that CaM may increase vesicle availability in the releasable pool by reducing the attachment of vesicles to actin filaments (see also discussions by DeLorenzo, 1982; Singh & Prior, 1998). In keeping with this scenario, the inability of CaM to sustain a constant level of increase in m and n over the 10 min of exposure (Figure 1A,B) may be due to a gradual depletion of vesicles in the reserve pool.

According to our model, p is associated with $[Ca^{2+}]$ at the active zones and var_s p is associated with variations in $[Ca^{2+}]$ at that site, due to release of Ca^{2+} from distal organelles. The absence of effect of CaM on p and var_s p (Figure 1C,D) thus suggests that exogenous CaM does not alter $[Ca^{2+}]$ at the active zones or promote Ca^{2+} release from organelles distal to the active zone.

Administration of liposomes that contain antiCaM produces a decrease in m that is associated with decreases in n and p (Figure 3B,C). The decrease in n is the converse of the effect with CaM and consistent with a decrease in vesicle migration resulting from decreased activation of CaMKII (Llinás *et al.*, 1991). Again, the absence of effects on var_s p suggests that CaM does not normally promote Ca^{2+} release from distal organelles. However, the decrease in p implies a reduction in $[Ca^{2+}]$ at the active zone. One explanation for this might be that endogenous CaM has a role at voltage-gated Ca^{2+} channels (Lee *et al.*, 1999; DeMaria *et al.*, 2001), such that inhibition with antiCaM leads to a decrease in the steady-state influx of Ca^{2+} at the plasmalemma.

The results with W7 show a progressive decrease in *m* that is associated primarily with a decrease in *n* (Figure 5B). CaM reverses the W7-induced decrease in *m* by increasing *n* (Figure 5B). The opposing effects of CaM and W7 on *n* again support the idea that CaM and inhibitors of CaM alter transmitter release by modulating vesicle migration and thereby altering the number of transmitter release sites occupied by synaptic vesicles.

W7 also produces a decrease in MEPP amplitude that can be reversed by CaM (Figure 6). Since the effect of CaM is presumably prejunctional, i.e., increase in MEPP frequency with no change in MEPP amplitude, the reversal by CaM of the W7-induced decrease in MEPP amplitude would appear to have a prejunctional locus. However, it is possible that W7 also has a postjunctional effect, as reported for trifluoperazine (Cheng *et al*., 1981). A concurrent postjunctional action could explain the larger per cent decrease in MEPP amplitude with W7 (60%) compared to that with antiCaM (15%). In fact, a dual action of W7 would be consistent with the divergence of results found with the membrane-permeable CaM antagonists (Duncan, 1983). This would further underscore the importance of using direct delivery of CaM and antiCaM into the nerve terminal.

In conclusion, this is the first report to demonstrate that delivery of exogenous CaM into a vertebrate nerve terminal enhances neurosecretion and that delivery of antiCaM antibodies decreases neurosecretion. The changes are due primarily to alterations in the number of functional transmitter release sites, i.e., the variable fraction of release sites that are occupied by synaptic vesicles. The results support the proposal that CaM regulates neurosecretion through a chain of biochemical events that lead to mobilization of vesicles in the releasable pool (Sakaba & Neher, 2001). The increase in the releasable pool may be the basis for the dependence of post-tetanic potentiation on CaMKII but not on protein kinase C (Augustine *et al*., 1994).

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