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THE EFFECT OF POTASSIUM ON EXOCYTOSIS OF TRANSMITTER AT THE FROG NEUROMUSCULAR JUNCTION

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SUMMARY

1. Electrophysiology and morphology have been combined to investigate the time course of the exocytosis of quanta of neurotransmitter induced by elevated concentrations of K^+ at the frog neuromuscular junction.

2. Replicas of freeze-fractured resting nerve terminals fixed in the presence of 20 mM-K^+ showed images of fusion of synaptic vesicles with the presynaptic axolemma which were closely associated with the active zones. After 1 min in 20 mM-K^+ fusions appeared also outside the active zones, and by 5 min they became uniformly distributed over the presynaptic membrane.

3. The average total density of fusions was not significantly different at the various times examined since it decreased at the active zones while it increased over the rest of the membrane.

4. Resting terminals fixed in 20 mM-K^+ released 33000–45000 quanta after the addition of fixative; terminals stimulated by 20 mM-K^+ for 1–5 min released 50000–100000 quanta during fixation. The fixative potentiated K^+-induced transmitter release.

5. Fusions were uniformly distributed in terminals pre-incubated for 5 min in 20 mM-K^+ without added Ca^{2+}, stimulated by adding Ca^{2+} for 30 s, and then fixed. Conversely, after 5 min stimulation in hypertonic Ringer solution fusions remained predominantly located near the active zones. A similar distribution was observed after 15 min stimulation by a lower concentration of K^+ (15 mM).

6. At all concentrations of K^+ tested (10, 15, 20, 25 mM) miniature end-plate potential (MEPP) rate attained a steady-state value within 10–15 min. Values from a single junction were generally lower at higher concentrations of K^+, which indicates partial inactivation of the secretion–recycling process.

7. The data indicate that K^+ initially activates exocytosis at the active zones. Subsequently, ectopic exocytosis is activated while sites at the active zones appear to undergo partial inactivation. These phenomena are not related to the intensity or to the amount of previous secretion.

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INTRODUCTION

At the neuromuscular junction acetylcholine (ACh) is secreted by the nerve terminal in multimolecular packets, or quanta, which induce measurable electrical changes in the postsynaptic membrane (del Castillo & Katz, 1954). Most evidence indicates that quanta of transmitter are stored within the synaptic vesicles and released by exocytosis after the vesicle has fused with the axolemma (Heuser, Reese, Dennis, Jan, Jan & Evans, 1979; Ceccarelli & Hurlbut, 1980; Haimann, Torri-Tarelli, Fesce & Ceccarelli, 1985; Torri-Tarelli, Grohovaz, Fesce & Ceccarelli, 1985).

At the frog motor nerve terminal exocytosis of neurotransmitter normally occurs alongside the double rows of large intramembrane particles that mark the edges of the active zones (Heuser, Reese & Landis, 1974; Heuser *et al.* 1979; Ceccarelli, Grohovaz & Hurlbut, 1979*a*, *b*). These particles may represent the specialized sites for vesicle fusion hypothesized by Katz (1969), or they may represent the calcium channel itself, in which case the association of vesicle fusions with the active zones would follow the localized influx of Ca^{2+} ions at these sites (Heuser *et al.* 1974; Pumplin, Reese & Llinás, 1981).

An atypical distribution of fusion sites was reported in frog neuromuscular junctions stimulated by a high concentration of potassium (20 mM) for 15–60 min: under these conditions vesicle fusions were not preferentially located near the active zones, but were uniformly distributed all over the presynaptic membrane (Ceccarelli *et al.* 1979*b*). It was suggested that the prolonged depolarization might activate latent sites for vesicle fusion (Ceccarelli *et al.* 1979*b*), and that potassium-induced exocytosis might be localized near the active zones at early times (Fesce, Grohovaz, Haimann & Ceccarelli, 1982; Pecot-Dechavassine, 1982).

Although potassium has long been known to have complex effects on quantal secretion (Gage & Quastel, 1965; Cooke & Quastel, 1973), elevated concentrations of this ion have been widely employed as a substitute for the physiological stimulus, to induce secretion in a variety of neuronal systems. It is therefore important to clarify which particular mechanisms are involved in K^+ action and account for its complex effect on quantal secretion and vesicle fusion.

Our results suggest that K^+ initially activates and later inactivates exocytosis at the active zones, while it slowly activates fusion sites all over the presynaptic membrane.

METHODS

General procedures

Experiments were performed on frog (*Rana pipiens*) cutaneus pectoris nerve–muscle preparations mounted in Perspex chambers at room temperature (about 20 °C). The composition of the standard Ringer solution was: 116·2 mM-Na^+, 2·1 mM-K^+, 1·8 mM-Ca^{2+}, 116·9 mM-Cl^-, 3 mM-phosphate buffer, pH 7·0. When the ionic composition of the bathing solution was modified, the concentration of NaCl was adjusted to maintain isotonicity. Hypertonic Ringer solution contained 150 mM-sucrose added to the standard solution. To prevent fibrillation, tetrodotoxin (TTX, Sigma Chemical Co., St Louis, MO, U.S.A.) was used in all experiments at a final concentration of 5×10^{-7} g/ml (1·6 μM). The Perspex chamber was filled with a constant volume (4 ml) of solution, which was changed by emptying the experimental chamber and refilling it with an equal amount

of the new solution. The same procedure was followed to fix the muscles in the recording chamber.

All the experiments were performed in winter-time on animals from the same batch. When not otherwise specified, reagents were purchased from Merck (Bracco SpA, Milano).

Electrophysiology

Miniature end-plate potentials (MEPPs) were recorded by standard electrophysiological procedures from fibres of the first dorsal layer of the muscle. End-plate recordings were stored on magnetic tape both as a low-gain DC record (bandwidth 0–1250 Hz) and as a high-gain AC record (0·3–1250 Hz) using a Racal 4DS tape-recorder (Rank Precision Ltd, London) for subsequent acquisition and analysis by a DEC Micro-PDP 11/73 computer (Digital Equipment Corp., Maynard, MA, U.S.A.).

The entire time course of the MEPPs was well described by the product of an amplitude factor (h) times a dimensionless waveform factor $w(t)$, where: $w(t) = [\exp-(t/\theta_1)]-[\exp-(t/\theta_2)]$. The two time constants, θ_1 and θ_2, were deduced from the power spectrum of end-plate noise; MEPP rate and the amplitude factor 'h' were measured from the variance, skew and power spectra of end-plate noise (Segal, Ceccarelli, Fesce & Hurlbut, 1985). Variance and skew were corrected for non-linear summation of MEPPs; MEPP rate and amplitude were computed according to the following equations:

$$\text{MEPP rate} = (\text{var}^3/\text{skew}^2)\,(I_3^2/I_2^3), \quad h = (\text{skew}/\text{var})\,(I_2/I_3),$$

where I_2 and I_3 were the integrals of the square and the cube of $w(t)$, respectively. The procedure used is described in detail in Fesce, Segal, Ceccarelli & Hurlbut (1986*a*). A high-pass RC filter with 2 ms time constant was used to eliminate contributions of non-stationarity to the variance and skew (Fesce, Segal & Hurlbut, 1986*b*). In order to measure the rapidly varying MEPP rate during fixation the computations were performed on successive 5 s records (25000 samples at 5 kHz).

The adequacy of sample size and statistical procedures were verified on simulated end-plate recordings. Errors lower than 10% were obtained in the measured h, rate and total secretion when the most rapid changes observed in recordings during fixation were simulated, i.e. an MEPP rate rising exponentially (1 s time constant) to a peak over 5000/s and then declining exponentially to zero (5 s time constant).

Fixatives

Fixative solutions contained 1% glutaraldehyde (Fluka, Buchs, Switzerland), 0·5% formaldehyde (freshly prepared from paraformaldehyde) and either 20 mM-NaCl (control), or 20 mM-KCl, or 150 mM-sucrose, or 15 mM-KCl in 0·1 M-phosphate buffer, pH 7·2.

In preliminary experiments without TTX all preparations fibrillated at the very addition of the fixative. This might be due to the ionic composition of the solution (possibly the very low concentration of Cl^-), since fibrillation was observed following application of 0·1 M-phosphate buffer alone (Clark, 1976). A similar effect on presynaptic membrane may be responsible for the high spontaneous release rates observed in aldehyde fixatives, especially without (Clark, 1976), but also with, TTX (Smith & Reese, 1980). It appears that the effects of different fixatives on MEPP frequency are very different, with regard to the peak rate as well as to the duration of the increase in rate (see Clark, 1976; Smith & Reese, 1980). Furthermore, the buffer used appears to be at least as important as the aldehyde chosen and its concentration, since very low increases in MEPP frequency were observed with 0·05 M-cacodylate buffer and 2·5% glutaraldehyde (Pecot-Dechavassine, 1982), and with phosphate buffer (present results).

Freeze fracture

Preparations were fixed for 1 h at room temperature and for an additional hour at 3–4 °C. Small bits of tissue from regions rich in end-plates were cut out from the fixed muscles. The specimens were glycerinated (final concentration 30% in 0·1 M-phosphate buffer), sandwiched between two copper holders and frozen in a mixture of 75% propane and 25% isopentane cooled at −196 °C (Jehl, Bauer, Doerge & Rick, 1981). They were stored in liquid nitrogen and finally fractured at −110 °C and 5×10^{-8} Torr, with a complementary replica device in a Reichert freeze-fracture apparatus (Cryofract 190, Reichert Jung S.A., Paris). Only superficially fractured specimens were considered. The platinum–carbon replicas were examined with a Hitachi H-600 electron microscope (Hitachi Ltd, Tokyo).

Morphometric analysis

Electron micrographs of replicas of neuromuscular junctions were printed at a final magnification of ×35000. Only P faces (the protoplasmic leaflets) of the fractured nerve terminal membranes were analysed because active zones are well defined on this face by the double rows of large particles. A set of lines, following the general contour of the closest double rows of particles, were drawn between successive active zones, at 90 nm intervals, on the exposed axolemma. In this way six regions were outlined: 0–90, 90–180, 180–270, 270–360, 360–450 and > 450 nm from the edge of the closest active zone. The axolemma underlying the Schwann cell digitations was always considered as part of the region > 450 nm.

The surface area and number of fusions contained within each region were measured with the aid of a Zeiss MOP 1 Digiplan (Zeiss, Oberkochen, F.R.G.). Counts and areas of symmetrical regions were combined to give single-sided distributions. Dimples ranging from 6 to 60 nm in diameter were counted as vesicle fusions (Heuser *et al.* 1974).

Unless otherwise indicated data are reported as mean ± standard deviation.

RESULTS

Distribution of vesicle fusions in high K^+

Freeze-fracture replicas of presynaptic membranes from resting preparations fixed with control fixative showed no (or very few) images of vesicle fusion (Fig. 1*A*). In replicas of presynaptic membrane from preparations fixed in 20 mM-K^+ (0 min K^+), vesicle fusions were confined to the active zones (Fig. 1*B*). Very small fusion images (< 30 nm) were often seen. Replicas from preparations exposed for either 1 or 5 min to 20 mM-K^+, and then fixed in the same concentration of K^+, are illustrated in Figs 2 and 3.

The morphometric analyses of the three conditions are illustrated in the histograms of Fig. 4. These data indicate that: (1) fusions outside the active zones (> 90 nm apart from the double rows of particles) are virtually absent at 0 min K^+, begin to appear at 1 min and reach by 5 min an average density close to $4/\mu m^2$; (2) fusions near the active zone are most numerous at 0 min K^+ ($\approx 17/\mu m^2$), and by 5 min their density is reduced to the value observed elsewhere (about $4/\mu m^2$).

Fig. 1. *A*, micrograph of a P face from a resting nerve terminal fixed in the presence of 20 mM-Na^+ (control). The Figure shows four active zones appearing as gentle ridges running perpendicular to the major axis of the nerve terminal and bordered by two parallel double rows of large particles (arrow-heads). Digitations of a Schwann cell (asterisks) embrace the terminal between the active zones. No images of vesicle fusion are evident on the presynaptic membrane. *B*, P face of a nerve terminal fixed in the presence of 20 mM-K^+ (0 min K^+). The membrane is marked by a large number of vesicle fusions (dimples) confined to the region immediately adjacent to the active zones. Very small dimples (< 30 nm), often present in this condition, can be noticed. On the complementary extracellular leaflet (E face, inset) the active zone appears as a furrow; the associated protuberances (arrows) show the crater typical of vesicle fusions. Scale markers, 0·5 μm.

Fig. 2. Micrographs of P faces from two nerve terminals exposed to 20 mM-K^+ for 1 min and then fixed in the presence of the same concentration of K^+ (1 min K^+). Numerous dimples are present, mostly associated with the active zones. Scale markers, 0·5 μm.

Fig. 3. P faces from terminals exposed to 20 mM-K^+ for 5 min and then fixed in the presence of the same concentration of K^+ (5 min K^+). In *A* many dimples are present, scattered over the whole presynaptic membrane. No infoldings of the axolemma can be seen. *B* and *C* show active zones at a higher magnification. Only a few images of fusions are associated with the double rows of particles. Scale markers, 0·5 μm.

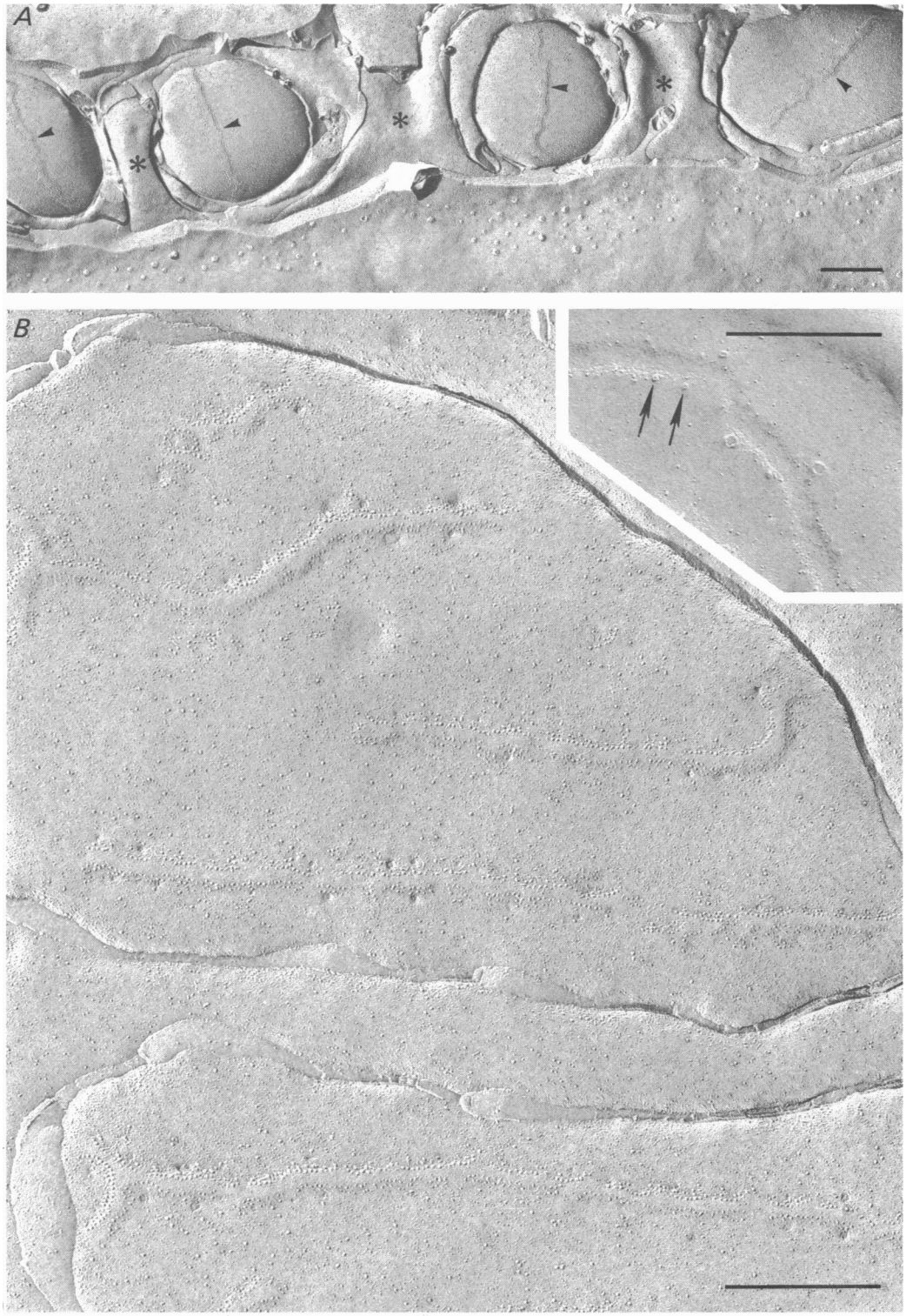
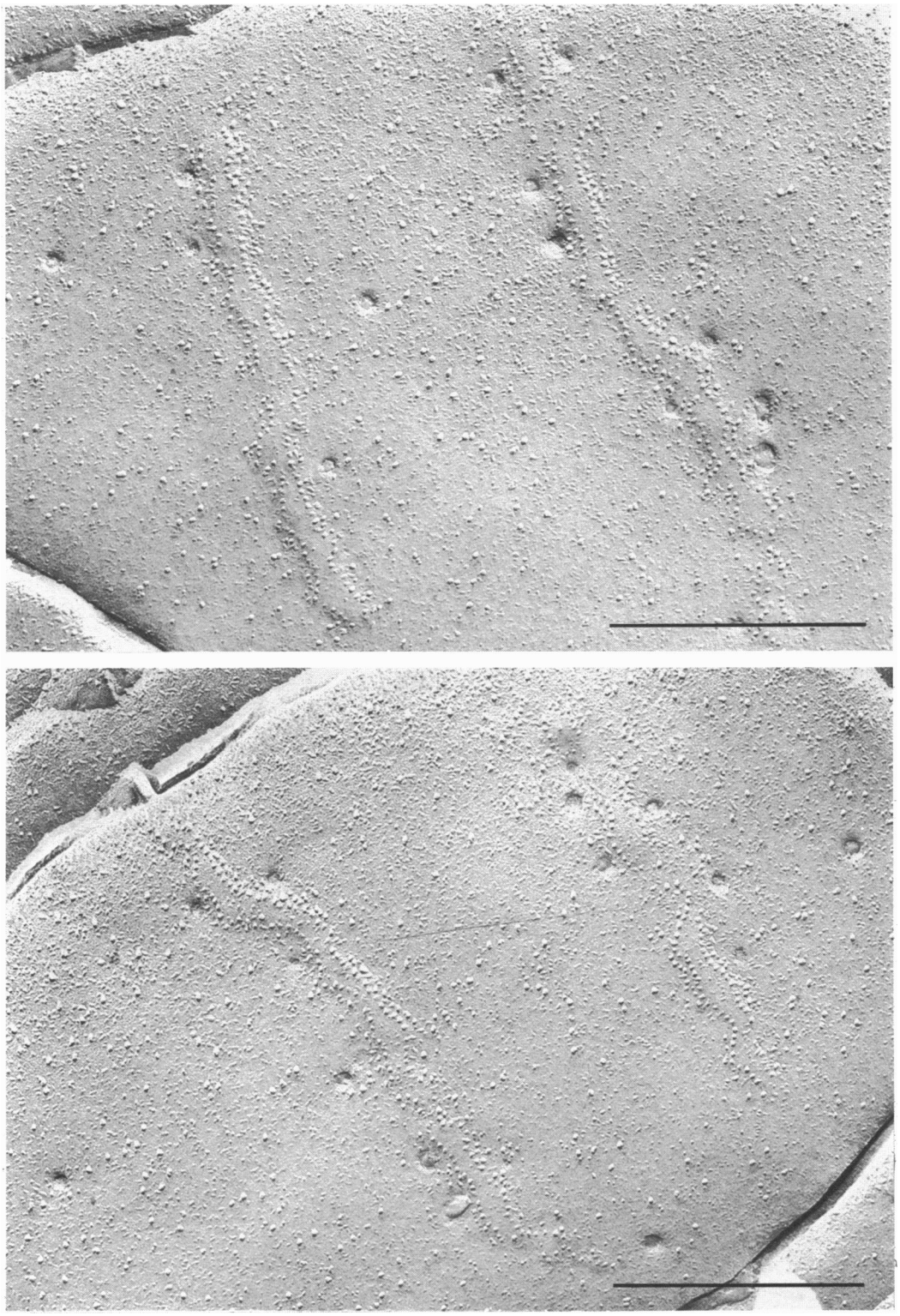


Fig. 1. For legend see opposite.



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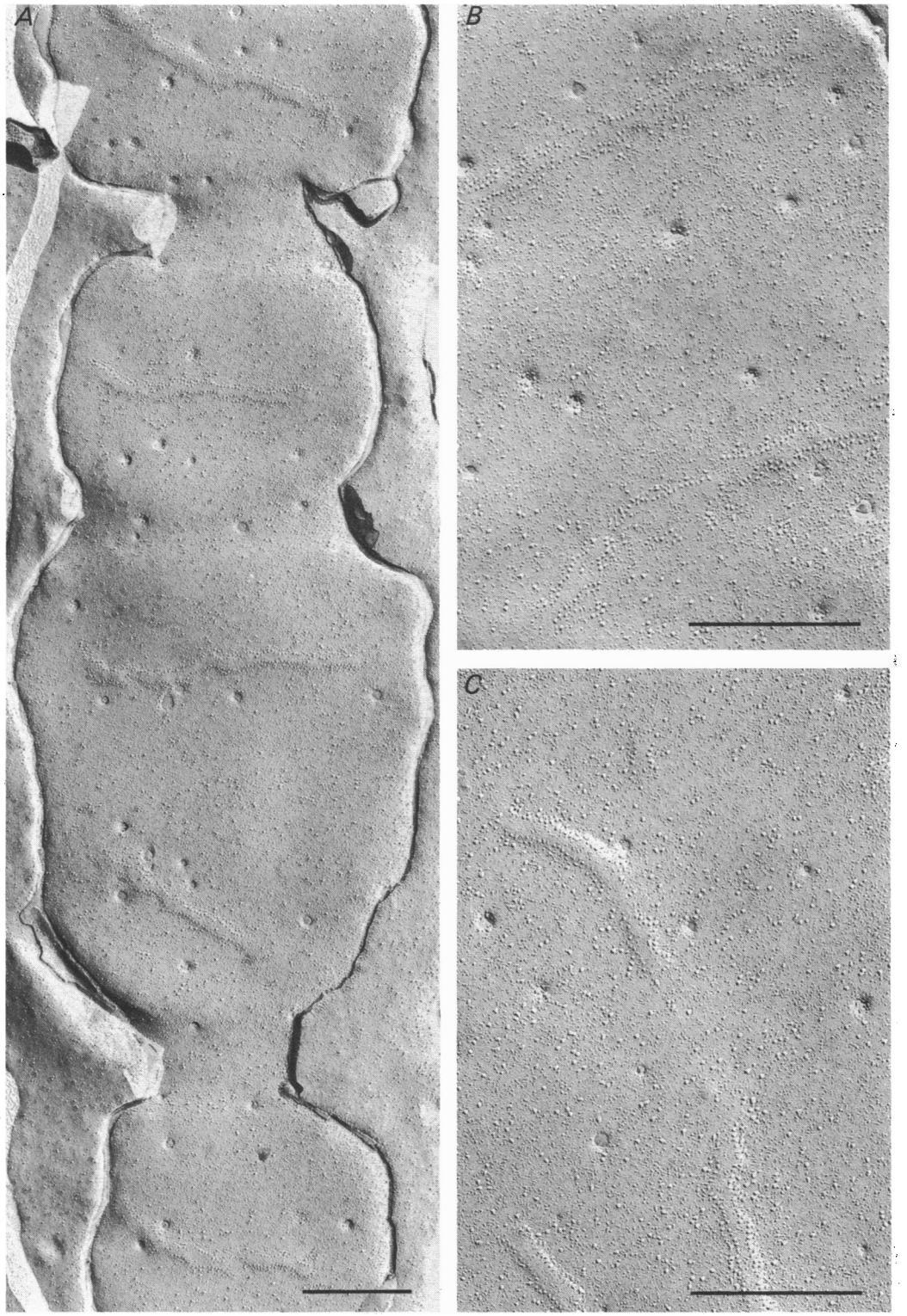

Fig. 3. For legend see p. 166.

After 5 min K^+ the distribution of fusions was similar to that previously reported after 15 and 60 min in 20 mM-K^+ (Ceccarelli *et al.* 1979*b*). However, infoldings of the presynaptic membrane, described following longer periods of stimulation, were not observed.

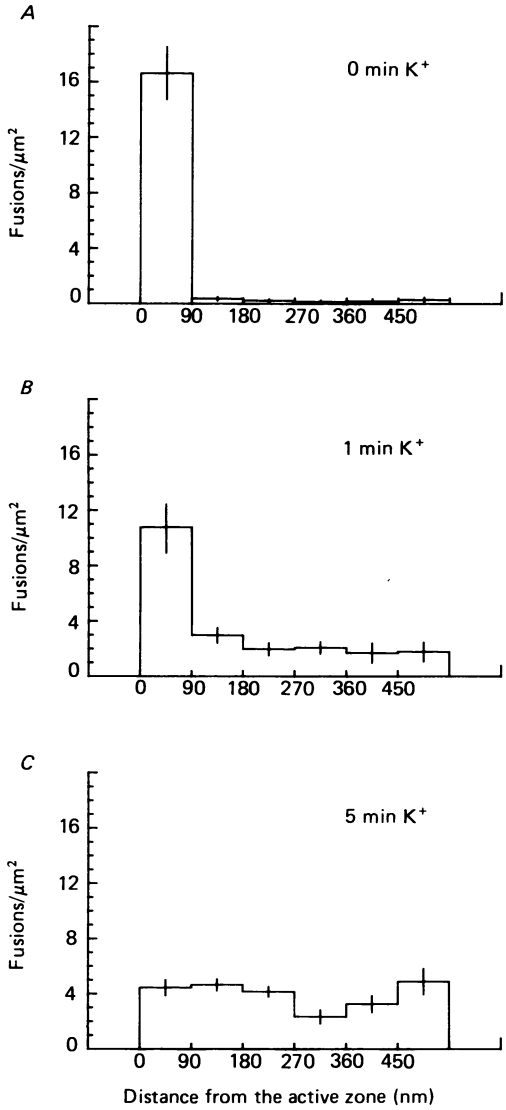

Fig. 4. Time course of the distribution of fusion images on the presynaptic membrane during exposure to 20 mM-K^+. Columns in the histograms represent average density within each region (number/μm^2, $\pm$S.E.M.). *A*, resting terminals fixed in the presence of 20 mM-K^+ (0 min K^+); 785 fusions were counted on 186 μm^2 of presynaptic membrane comprising 140 active zones (eighteen terminals). *B*, terminals fixed after 1 min in 20 mM-K^+; 383 fusions, 126 μm^2, 70 active zones (thirteen terminals). *C*, terminals fixed after 5 min in 20 mM-K^+; 718 fusions, 182 μm^2, 173 active zones (twenty-three terminals).

Measurement of quantal secretion during fixation in high K^+

Following the application of a control fixative to resting neuromuscular preparations, MEPP rate only slightly increased. As illustrated in Fig. 5*A*, MEPPs

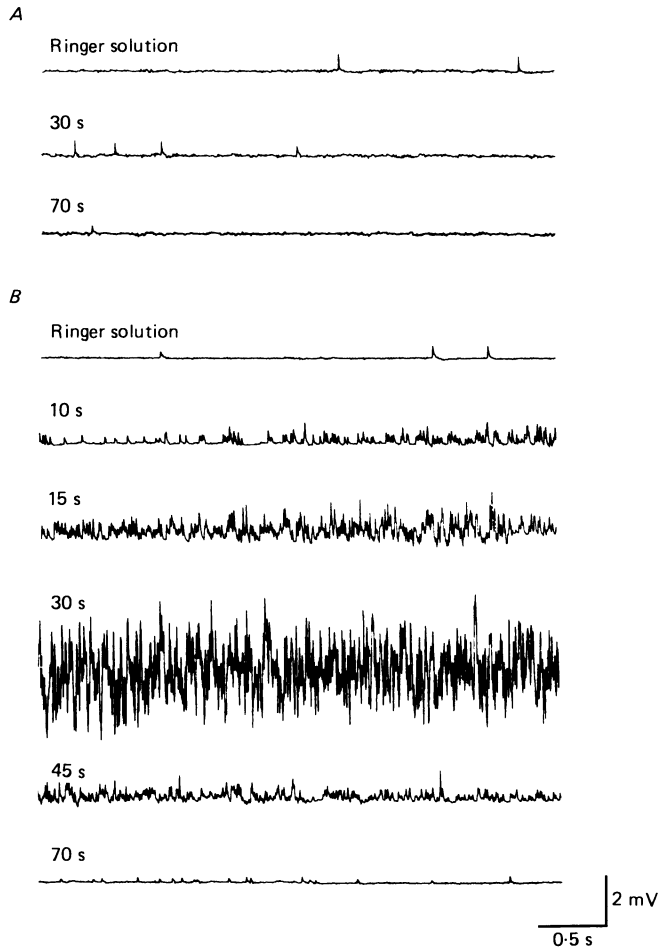

Fig. 5. Effect of control and high-K^+ fixatives on MEPP rate. *A*, recordings from a single end-plate before (top) and during exposure to control fixative (middle and bottom traces). The membrane potential of the muscle fibre was: −86 mV in Ringer solution, −73 mV at 30 s and −64 mV at 70 s in fixative. MEPP rate is only slightly increased by the fixative. *B*, recordings of MEPPs before (top trace) and during exposure to 20 mM-K^+ fixative (all other traces). The membrane potential at the different times shown was: −83 mV (Ringer solution), −63 mV (10 s), −58 mV (15 s), −40 mV (30 s), −50 mV (45 s) and −50 mV (70 s). MEPP rate declines to zero while single MEPPs are still recognizable from the baseline noise.

could be clearly recognized for 1–2 min after the addition of fixative. The MEPP amplitude did not decline much more than expected from the concomitant depolarization of the muscle fibre suggesting that the secretory machinery is impaired and blocked by the fixative before the sensitivity of the ACh receptor is

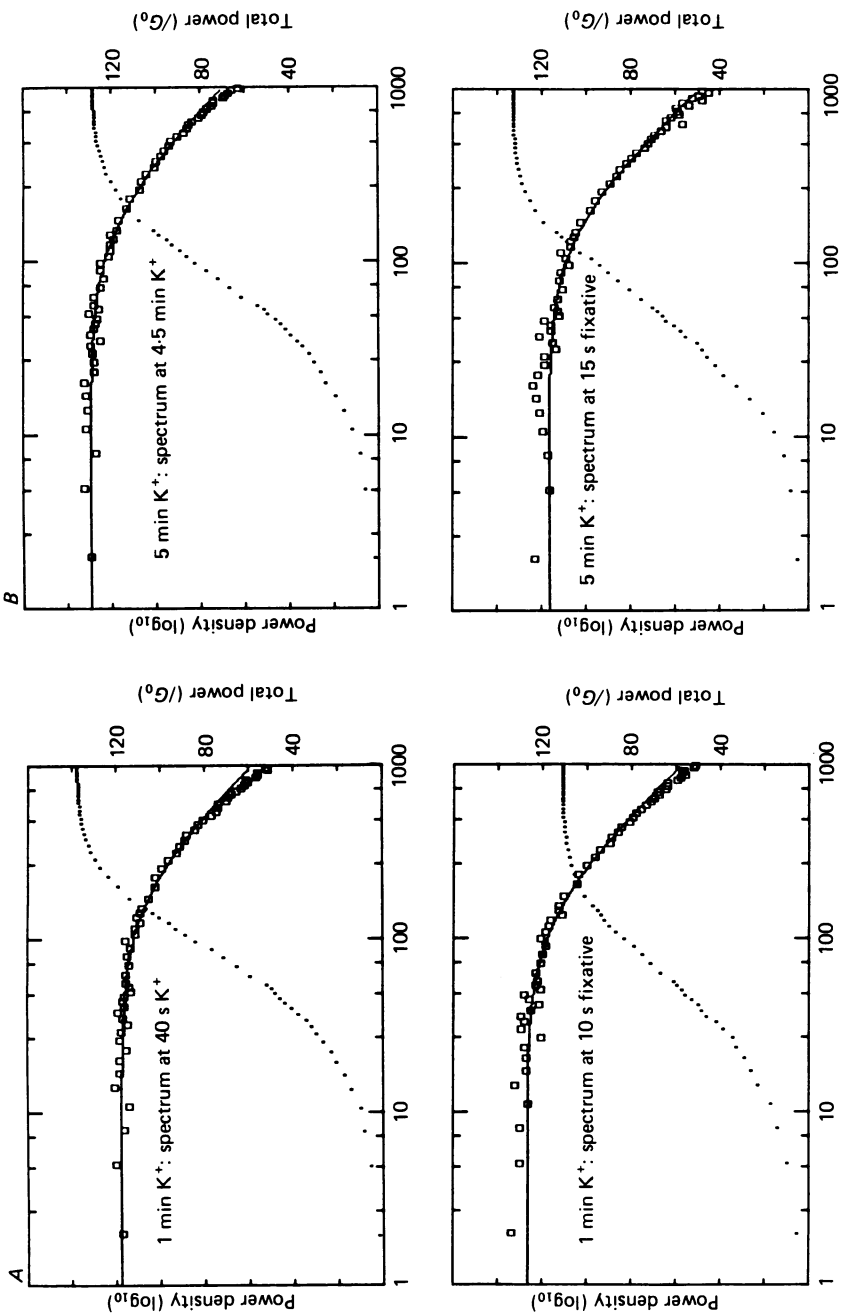

Fig. 6. Power spectra of the fluctuations in membrane potential at two end-plates during active secretion. *A*, preparation exposed to 20 mM-K^+ for 1 min and then fixed in the same concentration of K^+ (top, 40 s K^+; bottom, 10 s fixative). *B*, preparation exposed to 20 mM-K^+ for 5 min and then fixed in the same concentration of K^+ (top, 4·5 min K^+; bottom, 15 s fixative). The experimental spectrum in each panel (□) is the average of eight spectra from records of 2048 points (0·82 s), further smoothed by frequency averaging. The spectrum of the end-plate noise in the absence of MEPPs was subtracted as a baseline before plotting. Continuous lines are analytical spectra for the double-exponential fit to the data. The time constants for the fit were: 1·4 and 0·5 ms (top), 2·0 and 0·7 ms (bottom) in *A*; 1·6 and 0·5 ms (top), 1·5 and 0·8 ms (bottom) in *B*. The integrals of the spectra are shown by dotted lines to emphasize the contribution of the deviations, in the low-frequency region, to the variance (= total power, given by the rightmost point). The integrals are expressed as multiples of the power density at the low-frequency asymptote (G_0).

abolished. The baseline noise usually increased during the second minute of fixation (possibly due to increased membrane impedance).

When fixative containing 20 mM-K^+ was applied to resting preparations (0 min K^+) MEPP rate rapidly increased to such high levels that fluctuation analysis was necessary to measure it (Fig. 5*B*).

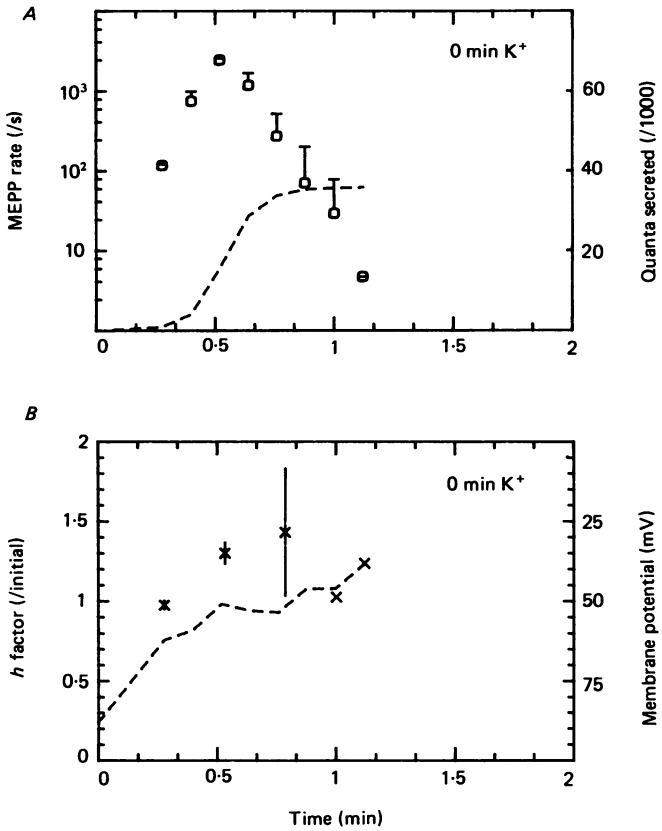

Fig. 7. Time course of the effect of 20 mM-K^+ fixative, added at rest (0 min K^+), on MEPP rate and amplitude and on the membrane potential of the muscle fibre. *A*, MEPP rate (□, logarithmic scale, mean ± S.D. of four experiments) and average of the cumulative quantal secretion (dashed line, linear scale). Measurements from different fibres are synchronized on the time of the peak MEPP rate. *B*, MEPP amplitude factor *h* (×, mean ± S.D. of four experiments) and average membrane potential (dashed line). Values of *h* for each experiments were normalized to the first measurement after addition of the fixative. Notice the increase in *h* notwithstanding the depolarization.

The power spectra of end-plate noise during fixation in 20 mM-K^+ retained the general shape corresponding to a double-exponential waveform for the MEPP (Fig. 6), thus permitting the application of fluctuation analysis. Slight distortions were often present at the low-frequency end, mainly due to rapid time variations in MEPP rate. Such distortions remained confined to a narrow band ($\leqslant$ 10–20 Hz) and could easily be eliminated by a high-pass filter (Haimann *et al.* 1985; Fesce *et al.* 1986*a*, *b*). The rise time constant of MEPPs, as deduced from the power spectra, was often increased by the application of the fixative.

The average time course of MEPP rate at 0 min K^+ is illustrated in Fig. 7*A*. In this Figure the measurements from the different fibres are synchronized on the peak of MEPP rate (2414 ± 107 MEPPs/s), which occurred 31 ± 21 s after the application of the fixative. MEPP rate declined to zero within about 1·5 min fixation, and totals of 33000–45000 quanta were secreted.

The membrane potential rapidly decreased after the fixative was added and reached a minimum at the peak of MEPP rate. The values of MEPP amplitude factor *h* (normalized to the first value measured in fixative) usually increased with time notwithstanding the marked depolarization (Fig. 7*B*).

TABLE 1. Number of quanta secreted during fixation and densities of vesicle fusion

	0 min K^+	1 min K^+	5 min K^+	*F**	*P**
Quanta secreted during fixation (thousands)	17·3 ± 4·3	28·1 ± 11·9	33·2 ± 19·8	1·51	0·26
Fusions/μm^2 nerve terminal axolemma	4·73 ± 1·9	3·48 ± 1·6	4·00 ± 1·3	2·30	0·11

* Analysis of the variance. No significant differences are evident in either fusion density or cumulative secretion during fixation among the times examined.

In preparations exposed to 20 mM-K^+ for either 1 or 5 min, and then fixed with a solution containing the same concentration of K^+, MEPP rate quickly rose to a peak, started declining by about 30 s, and slowly vanished within 1–1·5 min. Again isolated MEPPs could be clearly distinguished from the baseline noise before the recording became silent. Peak MEPP rate of 3500 ± 1500 (six fibres) and 4400 ± 3000 MEPPs/s (four fibres) were attained during fixation after 1 and 5 min K^+, respectively. A total of 50000–100000 quanta were secreted after addition of fixative at 1 and 5 min. The MEPP *h* factor increased in a few cases, but not consistently.

Correlation between quantal secretion and vesicle fusions

If one assumes that fixation of the nerve terminal begins when MEPP rate starts to decline (about 30 s) and is complete by the time MEPPs disappear from electrophysiological recordings (about 1–1·5 min), then the number of quanta secreted during fixation can be assessed. The numbers measured in this way during fixation at 0, 1 and 5 min K^+ are reported in Table 1, together with the corresponding densities of vesicle fusions.

In all conditions studied the decline in MEPP rate at the end of fixation was about exponential, with a time constant of 5–7 s. If the release of one quantum corresponds to the fusion of one vesicle, then the figures of 3·5–4·7 fusions/μm^2 observed in these

Fig. 8. Replicas of nerve terminals pre-depolarized for 5 min in 20 mM-K^+ without added Ca^{2+}, exposed to Ca^{2+} (1·8 mM) for 30 s and then fixed in the presence of the same concentration of K^+. *A* and *B*, micrographs of P faces showing several active zones and numerous fusions dispersed over the presynaptic membrane. *C*, micrograph of an E face. Many protuberances are seen, most of which are located more than 90 nm away from the closest active zone. Scale markers, 0·5 μm.

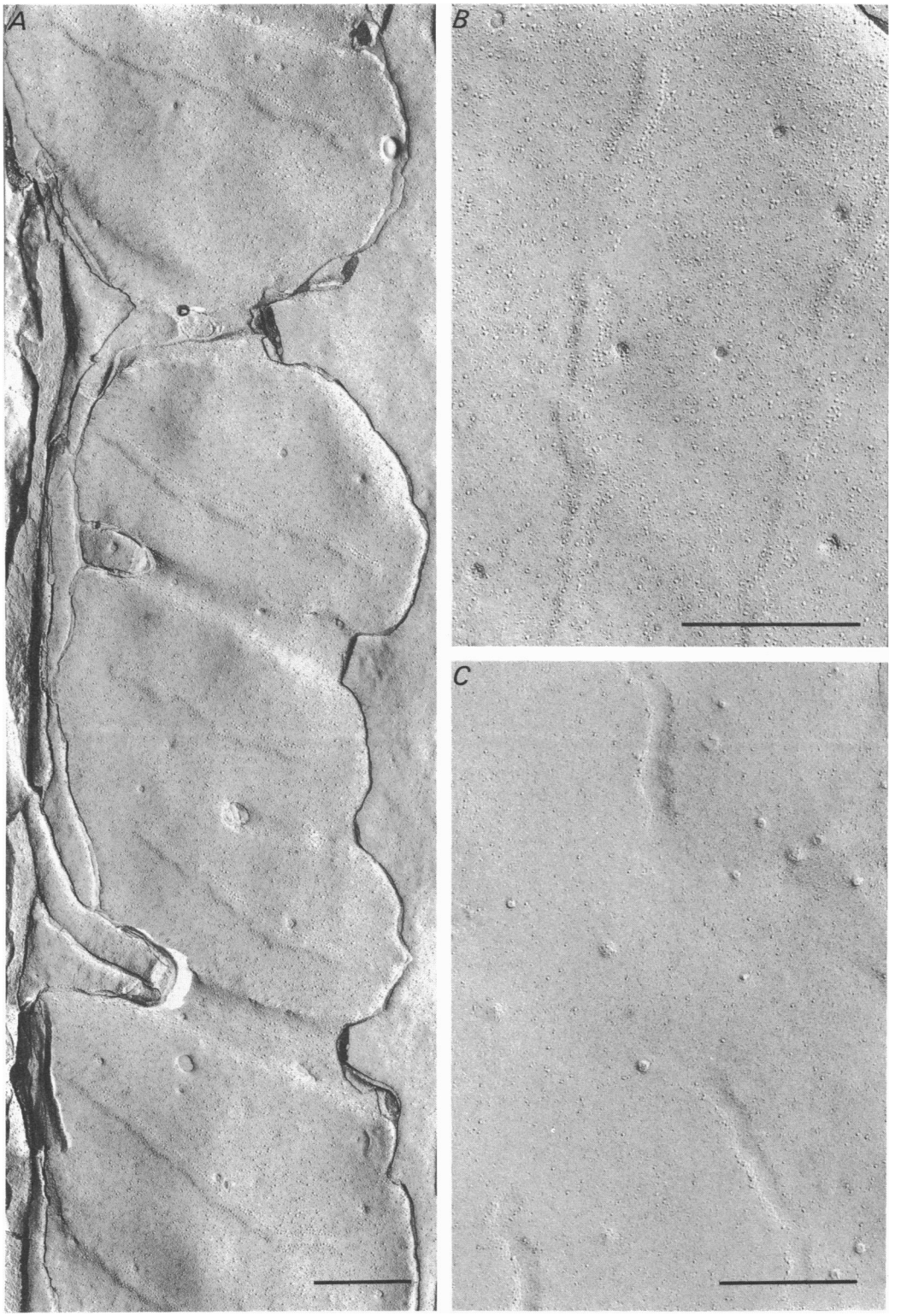

Fig. 8. For legend see opposite.

preparations yield about 2500 fusions per terminal, considering a 600 μm total arborization (Valtorta, Madeddu, Meldolesi & Ceccarelli, 1984), and correspond to the number of quanta secreted by nerve terminals from about 45 s fixation on.

Effect of pre-depolarization in Ca^{2+}-free solution

The MEPP rate did not increase when K^+ concentration was raised in the absence of Ca^{2+}. It increased when Ca^{2+} was restored, reached a peak 30 s later, when the fixative was added, and then declined to zero within 1–2 min.

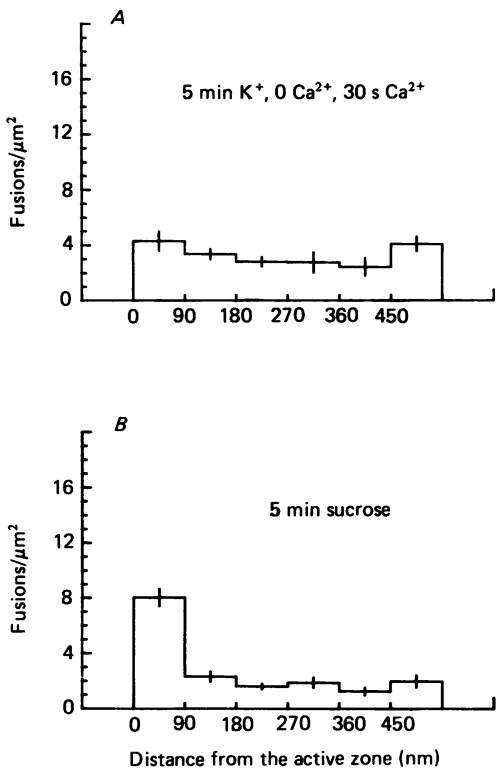

Fig. 9. Histograms of the distribution of fusions on the presynaptic membrane. *A*, preparations pre-depolarized for 5 min in 20 mM-K^+ without Ca^{2+}, exposed to Ca^{2+} for 30 s and then fixed in the same concentration of K^+; 617 fusions were counted on 186 μm^2 of presynaptic membrane comprising 101 active zones (twenty-three terminals). *B*, preparations stimulated for 5 min by hypertonic solution (150 mM-sucrose); 496 fusions were counted on 148 μm^2 of presynaptic membrane comprising ninety-four active zones (sixteen terminals). Columns in the histograms represent average density within each region (number/μm^2, $\pm$S.E.M.).

Fig. 10. P faces from two terminals exposed to hypertonic Ringer solution (150 mM-sucrose) for 5 min and then fixed in the presence of the same concentration of sucrose. Numerous dimples are evident; they are mainly located at the active zones, though some are present in other regions of the presynaptic membrane. Scale markers, 0·5 μm.

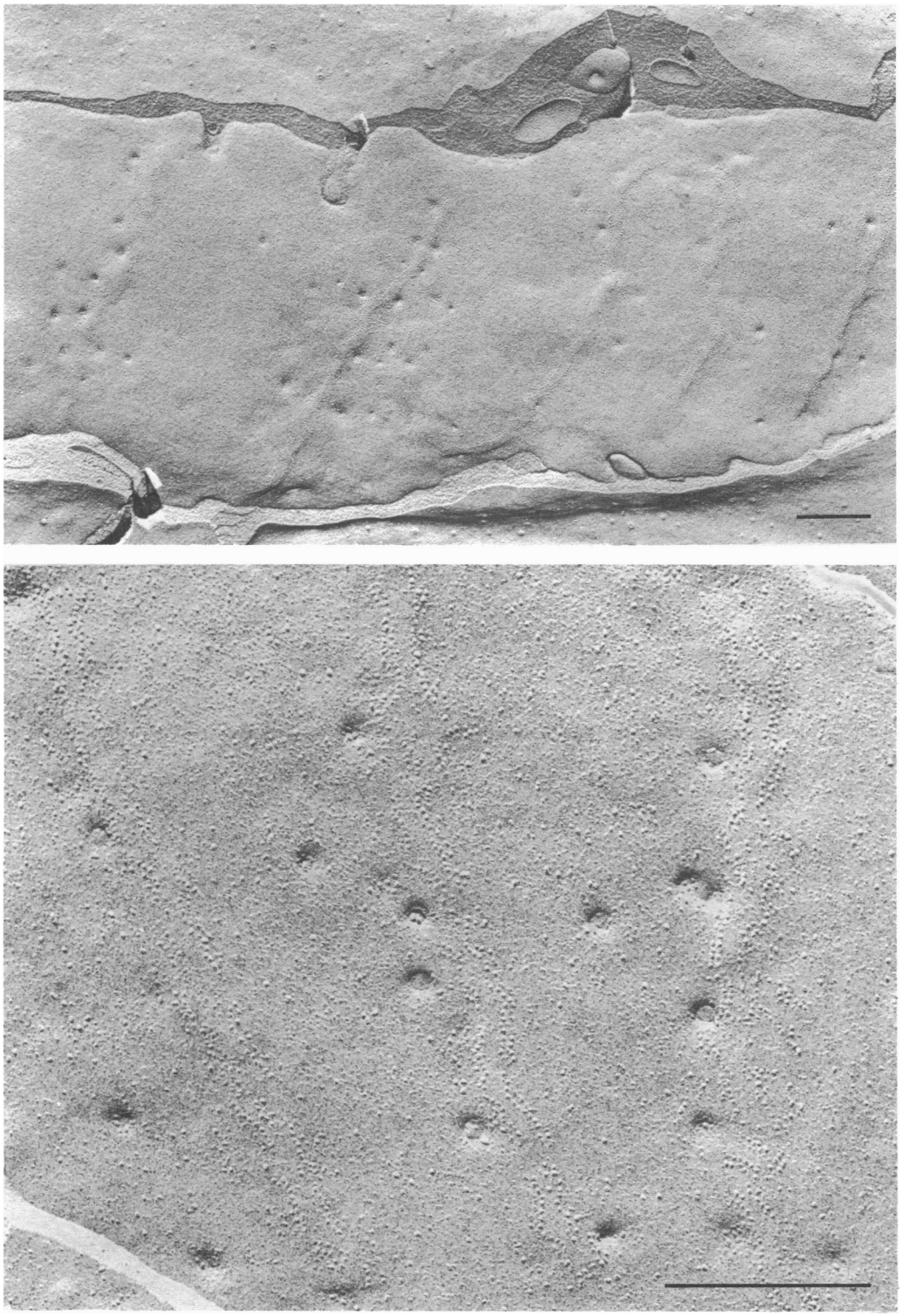

Fig. 10. For legend see opposite.

In replicas of presynaptic membranes from these preparations many vesicle fusions were observed, uniformly distributed over the membrane (Figs 8 and 9*A*). Fusion density was not significantly higher near the active zones than in other regions of the membrane (Student's t test, $P > 0{\cdot}2$).

Exocytosis of quanta induced by hypertonic solution

In order to assess whether the appearance of ectopic fusions in 20 mM-K^+ was a specific effect we studied the action of hypertonicity, a different condition which strongly activates asynchronous quantal secretion (Fatt & Katz, 1952; Furshpan, 1956; Shimoni, Alnaes & Rahamimoff, 1977; Kita & Van der Kloot, 1977).

Application of hypertonic Ringer solution (150 mM-sucrose added) to resting preparations produced an increase in MEPP rate which gradually reached 610 ± 186/s (four fibres) within 2–4 min. The time courses of the rise in MEPP frequency were similar among the various fibres examined. After 5 min in hypertonic solution, when hypertonic fixative (150 mM-sucrose) was added, the membrane potential of the muscle fibre generally decreased but, contrary to what was observed with high K^+, MEPP rate did not rise. It began to decrease after about 30 s and then fell to zero within 1·5–2 min. A total of 2000–9000 quanta were secreted after the application of the fixative.

Micrographs of freeze-fracture replicas of preparations exposed for 5 min to hypertonic solutions and fixed also in hypertonic conditions are shown in Fig. 10 and the corresponding morphometric results are presented in Fig. 9*B*. Most fusions were located within 90 nm from the active zones ($8{\cdot}1/\mu m^2$), but some were present in remote regions as well ($2{\cdot}0/\mu m^2$).

Inactivation of K^+-induced quantal secretion

The density of vesicle fusions at the active zones remains high during quantal secretion induced by prolonged electrical stimulation, black widow spider venom (Ceccarelli *et al.* 1979*a*, *b*) and hypertonicity, whereas it declines with time in 20 mM-K^+. This raises the possibility of a time-dependent inactivation of K^+ effect on quantal secretion. Such a phenomenon, which has not been described before, might be detected studying the kinetics of quantal secretion induced by different concentrations of K^+.

At a single neuromuscular junction a steady-state rate of secretion is attained only if the rate of quantal secretion equals the rate of recycling. A higher steady-state rate is expected if either secretion or recycling or both, are activated more intensely. It directly follows that a stronger stimulus for secretion must produce a higher MEPP rate; if it fails to do so, inactivation of some steps of synaptic transmission machinery must be assumed.

We performed a series of experiments measuring the effects of different concentrations of K^+ (10, 15, 20 and 25 mM) on MEPP rate at single junctions. The time courses and absolute values of MEPP rate induced by a fixed concentration of K^+ were different at different neuromuscular junctions (see also Takeuchi & Takeuchi, 1961), but the steady-state rate at a given junction was highly reproducible for a fixed concentration of K^+, and after adequate rest in Ringer solution the whole

time course of MEPP rate could be reproduced (see e.g. Fig. 11*A*). At least two concentrations were tested at each junction, with a period of rest (20–30 min) in Ringer solution between successive treatments.

Examples of the time courses of MEPP rates during exposure to different concentrations of K^+ are illustrated in Fig. 11. The kinetic analysis was performed

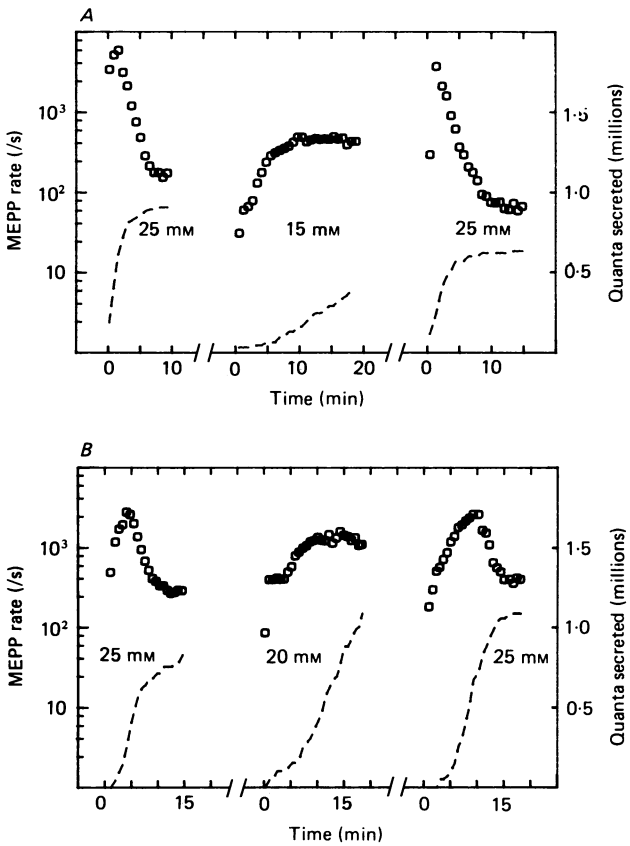

Fig. 11. Time courses of MEPP rates (□, logarithmic scales) during exposures to different concentrations of K^+ at single neuromuscular junctions. Notice breaks in the time scales. *A*, end-plate exposed to 25, 15 and again 25 mM-K^+. Steady-state MEPP rates were 200 and 80/s in 25 mM-K^+ *versus* 450/s in 15 mM-K^+. *B*, end-plate exposed to 25, 20 and again 25 mM-K^+. Steady-state rates were 300 and 400/s in 25 mM-K^+ *versus* 1200/s in 20 mM-K^+. In the Figure the cumulative counts of quantal secretion induced during each treatment are indicated by the dashed lines (linear scales). Notice that higher cumulative secretion was produced in *B* by the second treatment (and third) than by the first one.

only when it was possible to apply a final treatment equal to the first one, which produced a superimposable time course of MEPP rate, indicating that neither irreversible depletion nor relevant fatigue in synaptic transmission had occurred. This is evident in Fig. 11*B* where the total number of quanta secreted is higher during the second treatment (20 mM-K^+) than during the first (25 mM-K^+).

The maximum MEPP rate attained was always higher in the higher concentration of K^+, as expected. Conversely, much higher steady-state rates (more than double) were obtained at two junctions with 15 *vs.* 25 mM-K^+ and at two junctions with 20 *vs.* 25 mM-K^+. In two experiments 15 mM-K^+ produced steady-state rates 25 and 50% higher than 20 mM-K^+, and in only two experiments (10 *vs.* 20 mM and 15 *vs.* 25 mM-K^+) were the steady-state rates lower (by 30 and 40%) in the lower concentration of K^+. The higher steady-state rates observed in most fibres with lower concentrations of K^+ indicate that either secretion, or recycling, or both are impaired by prolonged exposure to high K^+, to such an extent that the relation between steady-state MEPP rate and K^+ concentration becomes inverted in the range between 15 and 25 mM.

The time-dependent decline in vesicle fusions along the active zones might be related to the partial inactivation of K^+ effect reported above. Since the latter phenomenon seems not to be prominent at K^+ concentrations as low as 15 mM we examined whether the fusion density near the active zones remained high in preparations stimulated for 15 min by 15 mM-K^+ and then fixed in the presence of the same concentration of K^+. An average density of $5{\cdot}3 \pm 0{\cdot}7$ (S.E.M.) fusions/μm^2 was observed near the active zones as opposed to $1{\cdot}76 \pm 0{\cdot}25$ fusions/μm^2 over the rest of the presynaptic axolemma (207 fusions on 83 μm^2 of presynaptic membrane comprising sixty active zones, from thirteen terminals; $P < 0{\cdot}001$ according to Student's t test for paired data).

DISCUSSION

Distribution of vesicle fusions in high K^+

Vesicle fusions induced by 20 mM-K^+ are mostly associated with the active zones at early times and become uniformly distributed on the presynaptic membrane by 5 min.

Fusions outside the active zones might represent a delayed onset of endocytosis following the collapse of vesicle membrane and translocation of its components in the axolemma (Heuser & Reese, 1973; Smith & Reese, 1980). Against this interpretation argues the extreme paucity of ectopic fusions observed under different conditions of intense recycling (Ceccarelli *et al.* 1979*b*).

A correlation between the number of quanta secreted during fixation and the density of vesicle fusions on the presynaptic axolemma could help in solving this issue; this is now feasible using a stochastic method to measure reliably MEPP rates over 200–300/s. Significant comparisons between the numbers of quanta and fusions can be performed in different experimental conditions, even though a one-to-one correlation is difficult, because images of chemically fixed preparations show the integration of the events occurring during the fixation process (Heuser *et al.* 1974; Ceccarelli *et al.* 1979*b*; Smith & Reese, 1980), and the limits of the integration period ('functional fixation time') are not known.

Our measurements with fluctuation analysis indicate that comparable numbers of quanta were secreted during fixation after 0, 1 and 5 min stimulation by K^+. In the presence of similar amounts of exocytosis, a hypothetical delayed onset of endocytosis should induce an increase in the total density of fusions, which was not

observed. Besides, ectopic fusions were numerous after only 30 s stimulation in preparations pre-depolarized without Ca^{2+}. Thus ectopic fusions seem to be due to a relocation of exocytosis which is independent of the number of quanta secreted previously.

These data are consistent with the view that endocytosis does not occur as a separate process at short stimulation times, the vesicle being immediately interiorized after a transient opening to the synaptic cleft (Ceccarelli, Hurlbut & Mauro, 1973; Torri-Tarelli *et al.* 1985; Torri-Tarelli, Haimann & Ceccarelli, 1987).

Intracellular recordings during chemical fixation

The effects of the fixative were monitored by intracellular recordings. MEPPs could be clearly recognized from the baseline noise, indicating that the ACh receptor was still functioning, until quantal secretion ceased. Although the muscle fibres were markedly depolarized, MEPP amplitude was little affected. Indeed, MEPP amplitude and rise time were often increased as if cholinesterase activity were inhibited early by the fixative. It is likely that the presynaptic membrane was also depolarized by the fixative. Such depolarization could account for the increased activity in control and high-K^+ fixatives and for the lack of effect in hypertonicity where depolarization does not increase MEPP rate (Shimoni *et al.* 1977).

The number of fusions observed in our preparations corresponded to the number of quanta secreted after 45 s fixation and before the electrophysiological 'death' of the neuromuscular junction. Based on the hypothesis that each fusion represents one exo-endocytotic event, this interval constitutes a tentative delimitation of the 'functional fixation time'.

The mechanism of 'ectopic exocytosis'

Calcium-dependent exocytosis is generally localized near the active zones. This might be due to selective localization of calcium channels (Heuser *et al.* 1974; Pumplin *et al.* 1981). Ectopic fusions, observed following incubation in 20 mM-K^+, occurred even when exposure to Ca^{2+} was very brief and therefore were not due to a slow, diffuse increase of Ca^{2+} concentration in the axoplasm. Rather, ectopic fusions may reflect either a time-dependent displacement of sites of exocytosis (possibly calcium channels) or the slow activation of a different population of sites.

Hypertonicity stimulates quantal secretion of ACh independently of extracellular Ca^{2+}, presumably by reducing the volume of the terminal and thereby increasing intracellular Ca^{2+} concentration (Shimoni *et al.* 1977; Van der Kloot, Cohen & Barton, 1986). Fusions still predominate near the active zones, possibly due to a locally higher concentration either of vesicles or of sites for exocytosis.

The inactivation of K^+-induced secretion

As fusion density rises outside the active zones the density along the zones declines. Depletion of vesicles near the active zones would not account for this phenomenon since it is independent of the amount of previous secretion and is not observed with means of stimulation other than 20 mM-K^+ (see also Ceccarelli *et al.* 1979*a*, *b*). Rather, fusion sites associated with the active zones may become displaced

or inactivated with time. Partial inactivation of K^+ effect was indeed evident when MEPP rates were measured by fluctuation analysis. In fact the inverse relation between steady-state MEPP rate and K^+ concentration, observed in the range 15–25 mM, indicates concentration-dependent inactivation of K^+ effect. Inactivation of K^+ effect and the reduced exocytosis at the active zones are presumably related. Consistently, fusions were more concentrated near the active zones after 15 min in 15 mM-K^+, a condition where no major inactivation occurs.

Partial impairment of recycling and indirect effects of the depolarization (like ion redistribution and volume changes) might occur, but are not likely to mediate the effects of K^+ on exocytosis, since block of recycling and massive ion redistribution accompany the action of black widow spider venom, ultimately leading to impressive swelling of the terminals, but exocytosis remains localized at the active zones under this conditions (Ceccarelli *et al.* 1979*a*).

In conclusion it appears that two populations of fusion sites for quantal secretion of ACh are activated by high K^+ with differential time courses. Furthermore, fusion sites at the active zones, traditionally implied in the physiological exocytosis, appear to undergo partial inactivation with time. Thus high K^+ only initially mimics the pattern of quantal secretion induced by single action potentials, even though its effects are also Ca^{2+} dependent and presumably mediated by the depolarization of the presynaptic membrane.

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