NEUROTRANSMITTER RELEASE AND NERVE TERMINAL MORPHOLOGY AT THE FROG NEUROMUSCULAR JUNCTION AFFECTED BY THE DYE ERYTHROSIN B

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1. The quantal release of neurotransmitter and the fine structure of frog neuromuscular junctions has been examined in the presence of the xanthene dye Erythrosin B.

2. At concentrations of 10 μM or greater, Erythrosin B produced time- and dose-dependent increases in transmitter release from presynaptic nerve terminals.

3. Miniature end-plate potential (m.e.p.p.) frequency increased in an exponential manner during continuous exposure to the dye. The rate constant for this exponential was dose-dependent, increasing with concentrations from 10 μM to 1 mM.

4. The amplitude of evoked end-plate potentials (e.p.p.s) also increased exponentially during dye treatment, primarily due to an increase in quantal content. Rate constants for this effect were also dose-dependent, and were approximately 1/5 as large as those for m.e.p.p.s.

5. While the frequency of m.e.p.p.s was increasing, their amplitude distribution did not qualitatively change. Thus the dye has little effect on the size of individual quanta.

6. The presynaptic effects of Erythrosin B were irreversible under these experimental conditions. Brief exposure to the dye caused increases in m.e.p.p. frequency and e.p.p. amplitude which were maintained at steady levels during extensive rinsing with dye-free Ringer solution.

7. Prolonged exposure to the dye caused an eventual decrease in m.e.p.p. frequency and abolition of e.p.p.s. Coincident with this decline 'giant' m.e.p.p.s as large as 40 mV were observed.

8. At dye concentrations greater than approximately 200 μM, Erythrosin B rapidly and reversibly increased the membrane potential and input resistance of muscle fibres. This post-synaptic effect was small and variable in normal saline, but was pronounced in low potassium solutions.

9. During the period that release was enhanced by Erythrosin B, presynaptic nerve terminals contained the normal complement of synaptic vesicles and other organelles. Mitochondria were swollen in this condition.

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10. After m.e.p.p. frequency declined below normal levels and 'giant' m.e.p.p.s appeared, the number of synaptic vesicles within nerve terminals declined and dilated cisternae were present. Mitochondria were swollen further.

11. These results do not reveal any mechanism to explain the ability of Erythrosin B to increase transmitter release, but the decline in release may be caused by partial depletion of synaptic vesicles. The 'giant' m.e.p.p.s could be due to the discharge of acetylcholine from cisternae.

INTRODUCTION

In recent years much progress has been made in understanding chemical synaptic transmission, particularly with reference to the release of neurotransmitter from presynaptic nerve terminals (Katz, 1969). It is thought that during synaptic transmission a transient increase in ionized calcium occurs within the presynaptic terminal (Rahamimoff, Lev-Tov & Meiri, 1980) and that this increase in intracellular calcium concentration somehow provokes exocytotic release of transmitter from synaptic vesicles (Heuser, Reese, Dennis, Jan, Jan & Evans, 1979). The molecular means by which this exocytotic release is triggered remains unclear (Llinás & Heuser, 1977; Boyne, 1978; Kelly, Deutsch, Carlson & Wagner, 1979).

One approach to this problem has been to identify pharmacological agents which perturb transmitter release and to utilize these agents as tools in elucidating the processes underlying release. The approach, most fruitfully applied to nerve excitation and cholinergic receptor activation (see review of Narahashi, 1974), has recently been applied to the study of neurotransmitter release (Kelly *et al.* 1979; Howard & Gunderson, 1980). A number of substances have been found which produce alterations in release. These include bacterial neurotoxins such as botulinum (Harris & Miledi, 1971) and tetanus (Duchen & Tonge, 1973) toxins; venoms such as β-bungarotoxin (Kelly, Oberg, Strong & Wagner, 1975), taipotoxin (Chang, Su, Lee & Eaker, 1977), spider venoms (Longenecker, Hurlbut, Mauro & Clark, 1970; del Castillo & Pumplin, 1975) and *Glycera* venom (Manaranche, Thieffry & Israel, 1980); inorganic cations (Heuser & Miledi, 1971; Silinsky, 1978); and alcohols (Gage, 1965). These structurally diverse substances have, in several cases, proved helpful in understanding such things as the amount of transmitter stored in and released by presynaptic terminals (Ceccarelli, Hurlbut & Mauro, 1973; Katz & Miledi, 1979), the presynaptic sites of membrane fusion and exocytosis (Pumplin & Reese, 1977; Ceccarelli, Grohovaz & Hurlbut, 1979) and the dynamics of membrane retrieval (Heuser & Miledi, 1971; Lassignal & Heuser, 1977; Ceccarelli *et al.* 1973).

A group of pharmacological agents potentially useful in extending this approach are xanthene dyes. These anionic compounds alter the membrane properties of molluscan neurones (Levitan, 1977) and sea urchin eggs (Carroll & Levitan, 1978), and thus might influence the membrane events associated with transmitter release. We have examined the effect of one of the more potent of these derivatives of fluorescein on the frog neuromuscular junction. In this paper, we describe the pronounced alterations in transmitter release and nerve terminal morphology produced by Erythrosin B, a derivative widely used as a food colourant (Marmion, 1979).

Possible modes of action of the dye are considered in the following paper (Augustine & Levitan, 1982). Brief reports of some aspects of this work have appeared (Augustine & Levitan, 1976, 1977, 1978, 1980).

METHODS

Saline and tissue preparation. Experiments were performed on the isolated cutaneous pectoris nerve–muscle preparation of the frog *Rana pipiens*. Specimens 4–5 cm in length were obtained from Carolina Biological Supply Co. (Burlington, NC) and maintained in a moist environment. The cutaneous pectoris was dissected according to the technique of Blioch, Glagoleva, Liberman & Nenashev (1968). Isolated preparations were pinned to a silicone resin (*Sylgard*, Dow Chemical Co.) lining the bottom of a plexiglass chamber (approximately 5 ml. volume), and were bathed in Ringer solution (120 mM-NaCl, 2 mM-KCl, 1·8 mM-CaCl$_2$, and 5 mM-Tris-HCl, pH 7·2). All experiments were performed at 18–22 °C, the temperature at which the animals had been maintained.
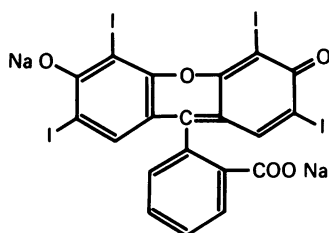

Fig. 1. Structure of Erythrosin B.

Erythrosin B (tetra-iodo fluorescein; Fig. 1) assayed at 93 % purity was obtained from the Stange Co. (Chicago, IL). The major impurities usually consist of trace amounts of several less iodinated fluorescein analogues (Marshall, 1976) which would be expected to have less biological activity than Erythrosin B (Levitan, 1977).

Electrophysiology. Transmembrane potentials of individual muscle fibres were measured with conventional intracellular techniques. Glass micro-electrodes (5–20 MΩ tip resistance) were filled with 3 M-KCl and inserted into fibres near end-plate regions. The electrodes were connected to a high input impedence d.c. pre-amplifier with negative capacitance compensation, and the output of this amplifier was displayed on an oscilloscope (Tektronix Model 5103) and pen recorder (Brush 220, Gould Instr. Co.), and also stored on magnetic tape (Hewlett–Packard FM Recorder Model 3960).

Spontaneous miniature end-plate potentials (m.e.p.p.s) and evoked end-plate potentials (e.p.p.s) recorded from post-synaptic muscle fibres were used as measures of transmitter release (Katz, 1969). M.e.p.p. frequency was determined manually by counting the number of m.e.p.p.s occurring every 30 or 60 s. High m.e.p.p. frequencies were difficult to determine, due to summation, but frequencies as high as 200 s^{-1} could be measured by searching for voltage inflections indicating the rising phase of individual m.e.p.p.s. Peak m.e.p.p. frequency also was occasionally estimated by measuring the d.c. depolarization produced by summation of m.e.p.p.s. (Heuser & Miledi, 1971), and yielded values similar to those obtained by counting discrete events.

M.e.p.p. amplitude were measured and grouped into 'bins' of width approximately equal to the background noise level (peak to peak) of the recording system (Kriebel, Llados & Matteson, 1976). End-plate potentials were evoked in the muscle by stimulating its innervating nerve every 2 s with 0·1 ms current pulses (Grass Model S-44 stimulator) delivered via a polyethylene suction electrode. E.p.p. amplitude was reduced presynaptically by bathing the preparation in Ringer solution with a low calcium/magnesium ratio (0·3 mM-CaCl$_2$, 1 mM-MgCl$_2$) to reduce non-linear summation (Martin, 1955). Mean e.p.p. amplitude was determined with a signal averaging computer (Nicolet,

Model 1072). Either 128 or 256 e.p.p.s. were averaged to allow accurate determination of mean amplitudes despite fluctuations in membrane potential caused by increasingly frequent m.e.p.p.s. Quantal content (m) was calculated by the method of del Castillo & Katz (1954):

$$m = \frac{\text{mean e.p.p. amplitude}}{\text{mean m.e.p.p. amplitude}}$$

Mean m.e.p.p. amplitude was measured at the 5 or 10 min intervals.

In some experiments the cumulative number of m.e.p.p.s which occurred after dye treatment was determined. The total number of quanta released was calculated by determining the area under plots of log m.e.p.p. frequency *versus* time, considering the area under these curves to be trapezoidal.

Electron microscopy. Paired cutaneous pectoris nerve–muscle preparations were isolated and one muscle of the pair was immersed in Ringer solution containing 100 μM-Erythrosin B, while the other was placed in normal Ringer solution. Both muscles were incubated at room temperature for either 60 or 150 min, times when dye-induced release approaches its peak (60 min in 100 μM), or when release has virtually ceased except for occasional events which are often much larger than normal (150 min in 100 μM).

Muscles were fixed with 3 % glutaraldehyde in 100 mM-Millonig's (1976) phosphate buffer (pH 7·2) to which 0·04 mM-CaCl$_2$ had been added. Glutaraldehyde apparently requires less than 10 min to fix this tissue (Ceccarelli *et al.* 1973; Heuser, Reese & Landis, 1974) and at this concentration produces minimal increases in transmitter release (Smith & Reese, 1980). After 3 h of fixation, rectangular pieces of tissue (1 × 0·5 mm) were excised from end-plate rich regions of the muscle and were placed in fresh fixative solution for a total fixation time of 4 h. The tissue was then rinsed in 130 mM-buffer solution and post-fixed in 1 % osmium tetroxide for 60 min. Tissue was dehydrated with ethanol (15 min each in 50, 75, 95 and 100 % and 20 and 30 min in 100 %) and immersed in a propylene oxide intermediate solvent. Finally the tissue was embedded in Epon 812 epoxy resin. Nerve terminals were first located in tissue samples by examining thick (0·5 or 1 μm) sections stained with 1 % trichrome solution (Sato & Shamoto, 1973) under a compound light microscope. Suitable blocks were oriented to produce cross-sections of nerve terminals and were sectioned into silver-grey (60–80 nm) thin sections using a Sorvall MT2-B ultramicrotome and a DuPont diamond knife.

Sections were positively stained with both 0·2 % lead citrate (Venable & Coggeshall, 1965) and 1 % uranyl acetate and examined on a Hitachi HU-12 electron microscope at 50 kV. All terminals located were photographed at 15000 × magnification and printed at a final magnification of 100,000 × for subsequent morphometric analysis.

Because of the the pronounced alterations in the morphology of some nerve terminals caused by prolonged exposure to Erythrosin B (Pl. 2), criteria were established to permit unambiguous identification of nerve terminals and their contents. Nerve terminals were defined as membrane-bound structures appearing roughly oval in cross-section, with a largest dimension less than 3 μm. Terminals were required to be surrounded by a Schwann cell process over most of their perimeter and to be 50–100 nm away from a muscle fibre with the junctional folds and post-synaptic densities characteristically present at neuromuscular junctions (Birks, Huxley & Katz, 1960). Structures were counted as synaptic vesicles only if their membranes were roughly circular in cross-section and had a maximum diameter between 25 and 100 nm.

The qualitative observations presented reflect results obtained on eight paired preparations treated with Erythrosin B (or normal saline) for 60 min, and three preparations treated similarly for 150 min. Two paired preparations from each treatment group were used for quantitative analysis. The perimeter and area of various presynaptic structures were determined using an electronic planimeter (Tektronix 4956). To determine the relative distribution of membrane within presynaptic terminals, the perimeters of the presynaptic plasma membrane, synaptic vesicles, and other intraterminal organelles were converted into total membrane area per terminal. The perimeter of plasma membrane and pooled cisternae–smooth endoplasmic reticulum (per section) was multiplied by the total linear length of cutaneous pectoris terminals, 360 μm (Letinsky & Morrison-Graham, 1980), to determine their total area. The total area of synaptic vesicle membrane was determined by multiplying the amount of vesicle membrane per section (calculated from the number of vesicles/section and mean vesicle diameter), by the number of 70 nm sections per terminal. A mean vesicle diameter of 45 nm was used in these calculations because of the similarity of our measurements of vesicle external diameters to those of Heuser & Reese (1973), and the constancy of the vesicle diameter in our various treatment groups.

The statistical significance of differences between measurements from control and experimental groups was determined by performing Student's t test, using $P \leqslant 0.05$ as the significance limit.

RESULTS

Addition of Erythrosin B to the bathing medium altered both transmitter release and the presynaptic morphology of isolated neuromuscular junctions.
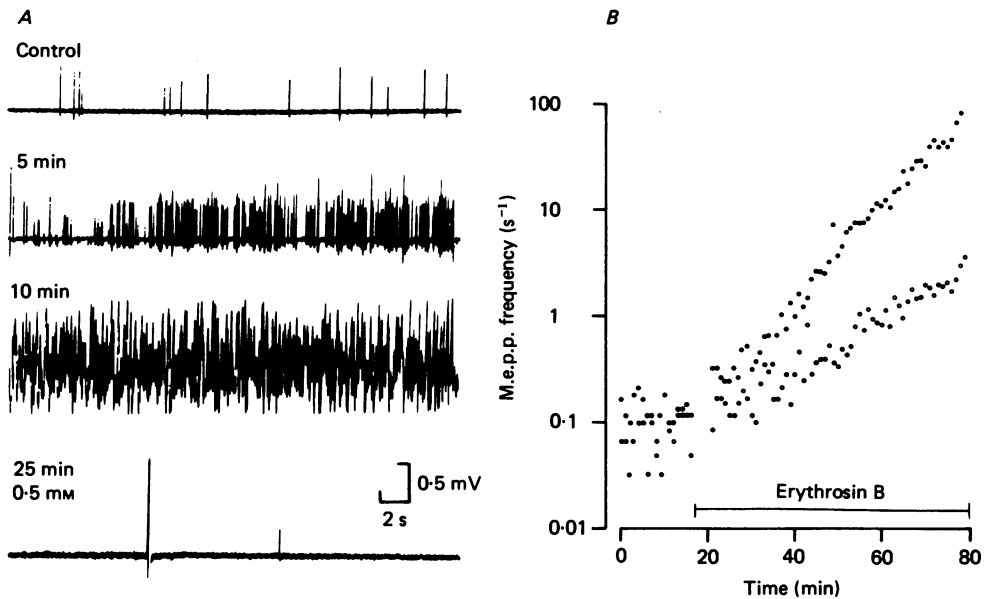

Fig. 2. Effect of Erythrosin B on m.e.p.p. frequency. A, in normal Ringer solution (control) m.e.p.p.s occur at a frequency of approximately 0.4 s^{-1}. 5 min after adding 500 μM-dye solution to the bathing medium, m.e.p.p. frequency has increased to 10 s^{-1}, and to much higher than 100 s^{-1} after 10 min. After 25 min in this dye solution, m.e.p.p. frequency is reduced to less than 0.1 s^{-1}. The spontaneous potential at the left in the bottom record is a 'giant' m.e.p.p. which is off-scale at this gain (see text). Calibration in control, 5 min and 25 min traces: 0.5 mV, 2 s; calibration for 10 min record: 1 mV. B, semi-logarithmic plots of m.e.p.p. frequency *versus* time; upon addition of dye m.e.p.p. frequency increases exponentially. Points represent m.e.p.p. frequency averaged over 1 min intervals. Open circles, 20 μM-Erythrosin B; closed circles, 100 μM.

Alterations in transmitter release produced by Erythrosin B

Effects on m.e.p.p.s. The frequency of m.e.p.p.s increased progressively during exposure to the dye and then declined to rates lower than that measured before dye exposure (Fig. 2A).

Initially, m.e.p.p. frequency increased with time in a non-linear fasion during dye treatment. Semi-logarithmic plots of m.e.p.p. frequency *versus* time yield a more linear relationship (Fig. 2B), suggesting that the dye produced an exponential increase in spontaneous release. An exponential increase in spontaneous release can be designated by a rate constant, α (Hurlbut, Longenecker & Mauro, 1971), which

is the slope of semilogarithmic plots of the exponential as a function of time. The rate of increase in m.e.p.p. frequency (α) increased with increasing dye concentrations, and thus provided a convenient means of quantifying the effect of different dye concentrations on spontaneous release.

Significant increases in the rate of increase in m.e.p.p. frequency occurred with dye concentrations as low as 10 μM. Higher dye concentrations produced greater rates of increase and at 1 mM, close to the limit of the dyes solubility in Ringer solution, the dose–response curve for this effect (Fig. 3) did not appear to plateau.
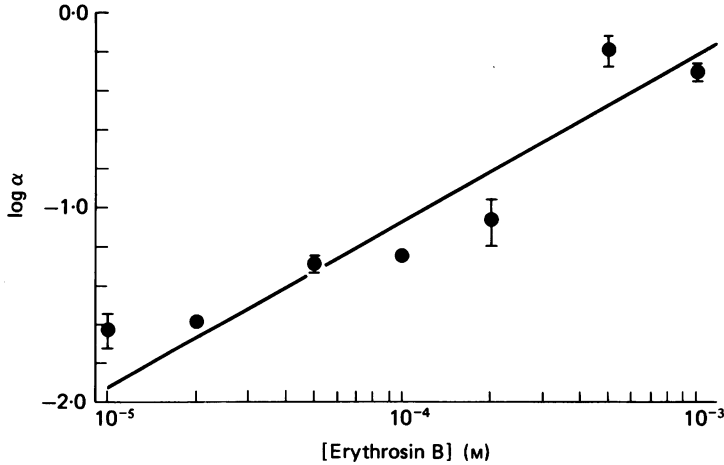

Fig. 3. The rate of increase in spontaneous release, represented by the mean rate constant $\bar{\alpha}$ in min^{-1}, is a function of the dye concentration. Linear regression analysis of this double logarithmic plot yields a line (correlation co-efficient [r^2] = 0·82) with a slope of 0·70. Bars represent ± 1 S.E. of mean.

The ability of Erythrosin B to stimulate spontaneous release was irreversible under these experimental conditions. Brief exposure to the dye produced an increase in m.e.p.p. frequency which was maintained even after rinsing with dye-free Ringer solution for more than 2 h. A second application of the dye at the same concentration and for a similar period of time produced a second, comparable increase in m.e.p.p. frequency (Fig. 4).

During extended exposure to Erythrosin B, m.e.p.p. frequency plateaued briefly at very high levels (greater than 100 s^{-1}) and then declined. This decline had a roughly exponential time course, and occurred earlier with higher dye concentrations (Fig. 5). The area under curves representing m.e.p.p. frequency as a function of time after dye treatment provides a measure of the total number of quanta released by Erythrosin B (Heuser & Miledi, 1971; Ceccarelli *et al.* 1973; Crawford, 1975) and yielded similar values for all dye concentrations considered (Table 1).

The decline in m.e.p.p. frequency is accompanied by a change in the size of individual spontaneous events. In normal Ringer solution the amplitude of m.e.p.p.s is generally described by a normal (Gaussian) distribution (Fatt & Katz, 1952; Fig. 6*A*). The distribution of m.e.p.p. amplitudes had a similar appearance while m.e.p.p.
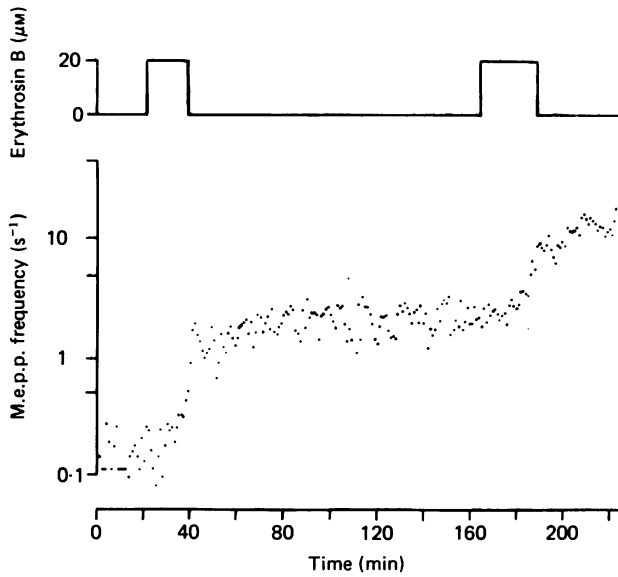
Fig. 4. Exposure of 20 μM-Erythrosin B for 17 min produced approximately a ten-fold increase in m.e.p.p. frequency which was undiminished by subsequent continuous perfusion with dye-free Ringer solution for 2 h. A second exposure (for 22 min) produced a second, similar elevation of spontaneous release.
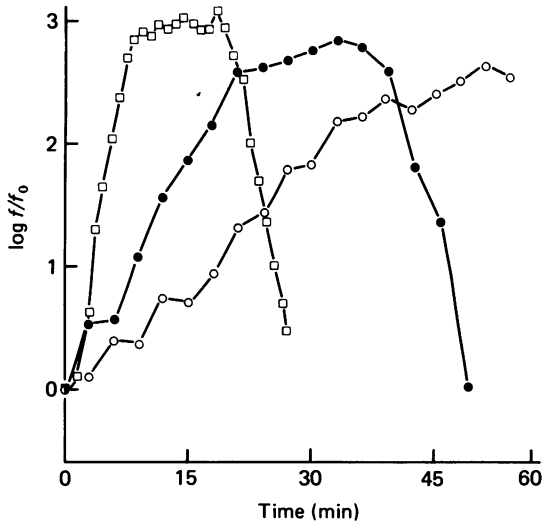
Fig. 5. With prolonged dye treatment m.e.p.p. frequency (f) initially increases relative to control level (f_0), plateaus, and then declines with time. The decline in frequency occurs sooner with higher dye concentrations. Dye concentrations are: squares, 1 mM; filled circles, 200 μM; open circles, 50 μM. Abscissa: time after dye addition; Ordinate: logarithm of ratio of m.e.p.p. frequency at any time (f) to that prior to adding dye, f_0.
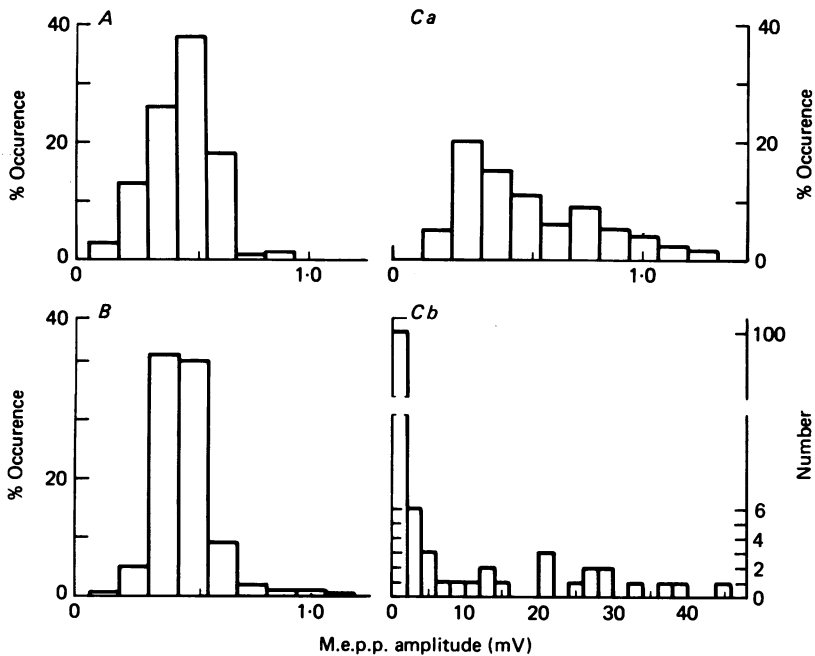
Fig. 6. M.e.p.p. amplitude distributions. *A*, in normal saline (mean frequency = 0·10 s⁻¹, for 144 events); *B*, 3–5·5 min after adding 500 μM-Erythrosin B (mean frequency = 3·70 s⁻¹ for 459 events); *C*, 25–60 min after adding dye (mean frequency = 0·06 s⁻¹ for 128 events), showing 'giant' and 'sub-miniature' m.e.p.p.s. *Ca* scale same as *A* and *B*. Events larger than 1·5 mV omitted. *Cb* abscissa compressed to include 'giant' m.e.p.p.s. Saline also contained 2×10^{-6} g/ml. tetrodotoxin to prevent muscle twitches during large spontaneous events.

TABLE 1. Cumulative number of quanta released by nerve terminals as a function of erythrosin B concentration

[Erythrosin B] (μM)	Number of quanta released
1000	3.65×10^5
200	7.10
	2·46
	1·20
50	5·35
	1·69
	Mean = 3.58×10^5 quanta
	Standard error = 0.93×10^5

frequency was increasing (Fig. 6*B*). During the decline in frequency, 'giant' m.e.p.p.s (up to 40 mV in amplitude) and spontaneous events below the usual amplitude range occurred (Fig. 2*A*). This resulted in a distorted amplitude distribution, as shown in Figs. 6*Ca* and 6*Cb*.

Effects on e.p.p.s. The mean amplitude of evoked e.p.p.s also increased transiently

during treatment with Erythrosin B. Calculation of e.p.p. quantal content revealed that Erythrosin B increased mean e.p.p. amplitude presynaptically, by increasing e.p.p. quantal content (Fig. 7A).

The increase in quantal content (m) produced by Erythrosin B was both time- and dose-dependent. During exposure to the dye, m initially increased, but declined during prolonged exposure (Fig. 7A). Graphs of the logarithm of m as a function of time
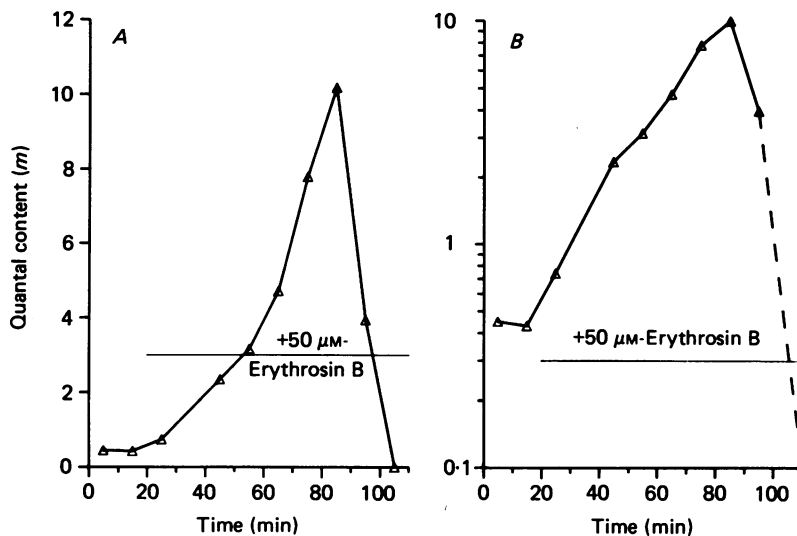

Fig. 7. Kinetics of dye-induced changes in e.p.p. quantal content (m). Linear (A) and semi-logarithmic (B) graphs of m *versus* time, after adding 50 μM-dye.

yielded a more linear function for the dye-induced increase in m (Fig. 7B). Similar to the analysis used for spontaneous release, we refer to the rate constant for the dye-induced increase in evoked release as α_m. This parameter was determined for several experiments and used to produce the dose–response curve of Fig. 8. Like the dose–response curve for the dye's effect on spontaneous release (Fig. 3), this curve is approximately linear on double logarithmic coodinates and does not appear to reach a plateau over the range of concentrations considered. However, the rate constant (α_m) for dye effects on evoked release was consistently smaller than that for spontaneous release by a factor of approximately 5.

Post-synaptic effects. Dye concentrations greater than 100 μM also hyperpolarized post-synaptic muscle fibres. This effect was quite variable in normal Ringer solution, but was more pronounced in solutions with reduced potassium concentrations. For example, 1 mM-Erythrosin B hyperpolarized fibres by 11·3 mV ($\pm$6·4 mV s.e. of mean, $n = 3$) in saline containing 0·5 mM-potassium while in normal saline this dye concentration had no significant effect (mean depolarization = 0·6$\pm$5·6 mV, $n = 4$). Thus while the dye would be expected to increase m.e.p.p. amplitude in low potassium saline it had little effect in normal Ringer solution (Fig. 6B). The hyperpolarization produced by Erythrosin B was rapid and reversible, similar to the dye's effect on the

membrane potential of molluscan neurones (Levitan, 1977) and sea urchin eggs (Levitan & Carroll, 1977).

Alterations in nerve terminal morphology produced by Erythrosin B

Appearance during increased release. Presynaptic terminals of junctions bathed in normal Ringer solution (Pl. 1 *A*) were similar to those typically seen in this preparation (Heuser, 1976). Cross-sectioned terminals had an oval shape, were surrounded over most of their perimeter by Schwann cell processes, and contained synaptic vesicles, mitochondria, tubular cisternae, smooth endoplasmic reticulum, and occasionally microtubules, neurofilaments, or glycogen particles.
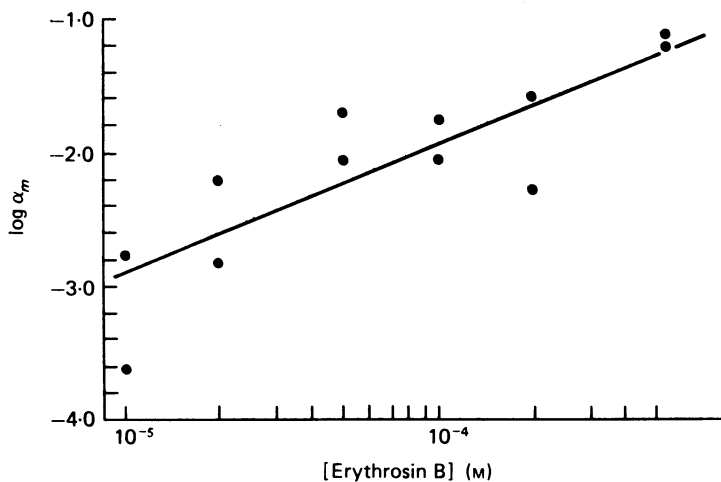

Fig. 8. Dose–response curve for the effect of various concentrations of Erythrosin B on e.p.p. quantal content. Points represent time constants for the increase in m (α_m) for individual experiments. Linear regression analysis of these points yields a slope of 0·95 (correlation coefficient [r^2] = 0·70).

The morphology of terminals treated for 60 min with 100 μM-Erythrosin B (Pl. 1 *B*) was similar to that of terminals not treated with the dye, despite the fact that these terminals were releasing quanta hundreds of times more frequently than the control preparations. These terminals contained the usual complement of organelles, apparently in normal numbers. Sections of dye-treated terminals contained $91\cdot3 \pm 19\cdot0$ synaptic vesicles per section (mean $\pm$ S.E. of mean, $n = 16$) which was not significantly different ($0\cdot60 > P > 0\cdot40$) from $76\cdot1 \pm 10\cdot1$ ($n = 25$) vesicles per section found in untreated terminals. The size of synaptic vesicles was similar in both groups, with mean (maximum) vesicle diameters of $56\cdot7 \pm 1\cdot9$ nm in normal saline and $56\cdot6 \pm 2\cdot4$ nm in 100 μM-Erythrosin B ($P > 0\cdot80$). Vesicles were more dispersed in dye-treated terminals.

The most obvious structural change in dye-treated terminals was the increased size and decreased number of mitochondria per section. The mean cross-sectional area of mitochondria in terminals treated with 100 μM-Erythrosin B for 60 min was $8\cdot6 \times 10^{-2} \pm 1\cdot3 \times 10^{-2}$ μm^2 (twenty-five mitochondrial profiles in sixteen sections),

compared to $2.8 \times 10^{-2} \pm 0.2 \times 10^{-2}\ \mu m^2$ in control terminals (107 mitochondrial profiles in twenty-five sections). These means are significantly different ($P < 0.01$). The increased cross-sectional area and decreased number per section may indicate that mitochondria became more spherical (Lynch, 1982).

Heuser & Reese (1973) have proposed that the membrane of synaptic vesicles fuses with the presynaptic membrane during exocytosis, and is retrieved via incorporation into other intracellular organelles such as coated vesicles and cisternae. Calculations
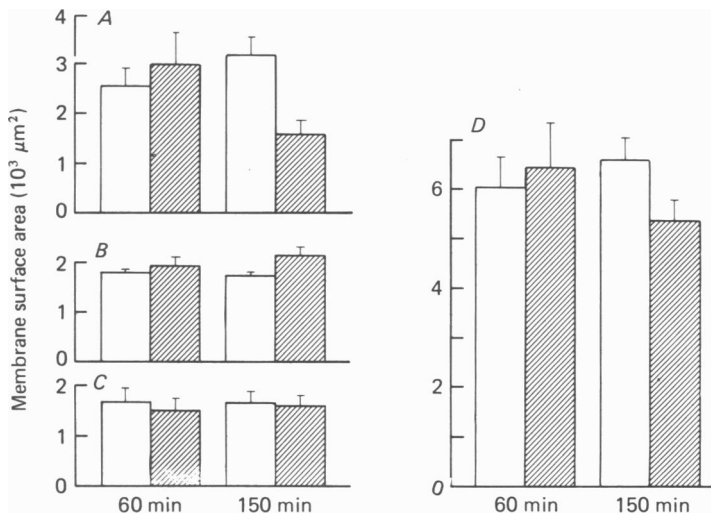

Fig. 9. Distribution of membrane in synaptic vesicles (A), presynaptic plasma membrane (B), and cisternae and smooth endoplasmic reticulum (C) in normal and dye-treated terminals. Preparations were exposed to either normal Ringer solution (open bars) or Ringer solution containing 100 μM-Erythrosin B (shaded bars) for 60 or 150 min. The total amount of synaptic membrane (D) changed little in these various conditions, although the amount of vesicular membrane decreased after exposure to the dye for 150 min. Error bars indicate S.E. of the mean, determined independently for each graph.

of the surface area of synaptic vesicles, presynaptic plasma membrane, and other membrane-bound organelles, exclusive of mitochondria (Fig. 9), revealed that the dye did not cause a redistribution of membrane within these several presynaptic compartments. Coated vesicles, usually frequent in terminals undergoing vigorous transmitter secretion (Heuser & Miledi, 1971; Heuser & Reese, 1973; Gennaro, Nastuk & Rutherford, 1978), were rare in control terminals and were not observed in dye-treated terminals (Pl. 1).

Appearance after decline of release. After 150 min in normal Ringer solution, junctions were indistinguishable from those incubated for only 60 min (Pl. 2A). However, distinct structural changes occurred in nerve terminals exposed to 100 μM-Erythrosin B for 150 min. The most pronounced effect was a reduction in the number of synaptic vesicles (Pl. 2B, C). Sections of dye-treated terminals contained 46.4 ± 8.5 vesicles per section (mean $\pm$ S.E. of mean, $n = 22$), compared to 97.2 ± 10.7 vesicles per section ($n = 23$) measured in untreated terminals. This difference was statistically

significant ($P < 0.01$). Mean synaptic vesicle diameters were similar, 55.1 ± 2.6 nm in control terminals and 55.7 ± 3.0 nm in terminals treated with Erythrosin B ($0.20 > P > 0.10$).

This decrease in the number of synaptic vesicles was reflected in the distribution of membrane within the terminal, with the amount of vesicular membrane decreasing by about half (Fig. 9). The decrease in vesicular membrane appeared to be partially offset by an increase in the area of plasma membrane, which also appeared to be invaginated (Pl. 2C).

Occasionally the terminal appeared to be divided into several compartments separated by Schwann cell processes. In other cases terminals appeared virtually devoid of organelles, except for poorly defined, membranous elaborations (Pl. 2C). All these images could be a consequence of extreme infolding of the plasma membrane of dye-treated terminals. The inability to accurately quantify the area of invaginated membrane may account for the significant ($0.05 > P > 0.02$) decrease in the total membrane area of dye-treated terminals (Fig. 9).

Although the surface area of cisternal membrane was essentially unchanged (Fig. 9), in some sections the volume contained within individual cisternae appeared to increase (Pl. 2B). Mitochondria appeared even more swollen than those from junctions treated for 60 min with Erythrosin B. The mean area of mitochondria in terminals exposed to Erythrosin B for 150 min was $16.7 \times 10^{-2} \pm 3.8 \times 10^{-2} \mu m^2$ (fourteen mitochondrial profiles in twenty-two sections), more than five times greater than the mean of $3.0 \times 10^{-2} \pm 0.1 \times 10^{-2} \mu m^2$ in control terminals (101 mitochondrial profiles in twenty-three sections); $P < 0.01$ for this difference). As in preparations treated with Erythrosin B for 60 min, coated vesicles were not observed after 150 min exposure to the dye.

DISCUSSION

These experiments examined the effects of the dye Erythrosin B on the physiology and morphology of frog neuromuscular synapses. This dye initially increased both m.e.p.p. frequency and e.p.p. quantal content. These increases were followed by a decline in m.e.p.p. frequency and the abolition of e.p.p.s. After this decline in release, spontaneous potentials with a wide range of amplitudes appeared. While the dye was enhancing transmitter release presynaptic mitochondria were swollen. Prolonged dye treatment decreased the mean number of synaptic vesicles contained within presynaptic terminals, caused mitochondria to swell further, and frequently produced elaborate infolding of the presynaptic membrane. Some of these actions of Erythrosin B can be understood by considering the relationship between the dye's physiological and morphological effects.

The kinetics of the increase in transmitter release caused by Erythrosin B provides a possible clue to its mode of action. The exponential increases in dye-induced release with time are similar to the dye's effects on artificial membranes (Colombini & Wu, 1981). Although the ability of Erythrosin B to enter cells is not known, other fluorescein analogoues may be able to cross cell membranes (Oxford, Pooler & Narahashi, 1977); thus the dye's kinetics could reflect entry of the dye into the presynaptic membrane or into the interior of the terminal. The presynaptic effect of the dye seems to involve irreversible binding because dye-induced release is not

reversible under the conditions tested. Re-application of dye after a brief initial exposure (as in Fig. 4) appears to sum with bound dye to produce an additive increment in release.

The total number of quanta which Erythrosin B releases (approximately 4×10^5) is similar to previous estimates of the total number of releasable quanta in a frog nerve terminal (reviewed in Ceccarelli & Hurlbut, 1980), so that the decline in Erythrosin B-induced release is probably due to a depletion of releasable quanta. This is consistent with the reduction of synaptic vesicles observed in dye-treated nerve terminals. E.p.p. failure occurs while m.e.p.p. frequency is still elevated and may be due to other causes, such as blockade of presynaptic action potential propagation (Krnjević & Miledi, 1958).

Depletion of synaptic vesicles suggests that Erythrosin B is impeding the subsequent recovery of vesicular membrane from the plasmalemma (Ceccarelli & Hurlbut, 1980). Inhibition of membrane retrieval could also produce the invaginations observed after prolonged dye exposure. Because coated vesicles are thought to normally be resonsible for retrieval of vesicular membrane (Heuser & Reese, 1973; Gennaro *et al.* 1978) the absence of coated vesicles in dye-treated synapses suggests that Erythrosin B may be affecting membrane recycling by interfering with coated vesicle formation. If true, this could make the dye useful as a pharmacological probe of the recycling process.

The occurrence of spontaneous potentials of unusual amplitudes suggests that long-term dye treatment has other presynaptic effects. The expansion of the amplitude distribution of spontaneous potentials to include both larger and smaller events rules out an explanation based exclusively on post-synaptic alterations. Other treatments have been shown to produce 'giant' m.e.p.p.s which resemble those produced by Erythrosin B (Katz & Miledi, 1969; Heuser, 1974; Pécot-Dechavassine, 1976; Smith, Clark & Kuster, 1977; Fritz, Atwood & Jahromi, 1980). Dye-induced 'giant' m.e.p.p.s occur in the presence of tetrodotoxin (Fig. 6) and in calcium-free Ringer solution (G. J. Augustine & H. Levitan, unpublished) and are therefore not due to spontaneous action potentials in the nerve terminal. Dilated cisternae are also observed after prolonged dye treatment and could be responsible for the 'giant' m.e.p.p.s (Heuser, 1974; Pécot-Dechavassine & Couteaux, 1975; Fritz *et al.* 1980).

Dye-induced small m.e.p.p.s may have a different origin. Erythrosin B inhibits choline uptake in mammalian synaptosomes (Logan & Swanson, 1979); such an action would be expected to decrease m.e.p.p. amplitude (Elmqvist & Quastel, 1965). Alternatively, it is possible that Erythrosin B is releasing a distinct population of small m.e.p.p.s (Kriebel & Gross, 1974; Magleby & Miller, 1981). Our observations do not permit distinction between these two possibilities.

Dye-induced swelling of mitochondria could mean that they are affected directly or indirectly by Erythrosin B. Swelling of presynaptic mitochondria is commonly observed when nerve terminals vigorously release transmitter (Jones & Kwanbun-bumpen, 1970; Heuser & Reese, 1973; Smith *et al.* 1977; Gennaro *et al.* 1978; Fritz *et al.* 1980) and could reflect uptake of calcium ions entering during dye exposure (Augustine & Levitan, 1982). Mitochondria can also swell in conditions which do not directly involve calcium ions (Hunter, Haworth & Southard, 1976) so that dye-induced changes in mitochondrial morphology could be due to causes other than calcium

uptake or release. Similarly, dye-induced cisternal swelling could be due to calcium uptake (Henkart, Reese & Brinley, 1978; McGraw, Somlyo & Blaustein, 1980; Ornberg & Reese, 1980), or other causes (Miller & Heuser, 1979).

The actions of Erythrosin B in increasing transmitter release are qualitatively similar to those of several other pharmacological agents, such as lanthanum (Heuser & Miledi, 1971), spider venom (Longenecker *et al.* 1970; Fritz *et al.* 1980), lithium (Crawford, 1975), cation-selective ionophores (Kita & Van der Kloot, 1976), ouabain (Baker & Crawford, 1975) and other metabolic inhibitors (Alnaes & Rahamimoff, 1975). Although the ability of many of these agents to deplete synaptic vesicles and produce 'giant' and small m.e.p.p.s is not known, such effects could be a general feature of treatments which trigger massive transmitter secretion. The following paper (Augustine & Levitan, 1982) considers the means by which the dye increases transmitter release, and demonstrates that it differs from other agents in several respects. Its properties may make it useful as a novel pharmacological tool in the study of the transmitter release process.

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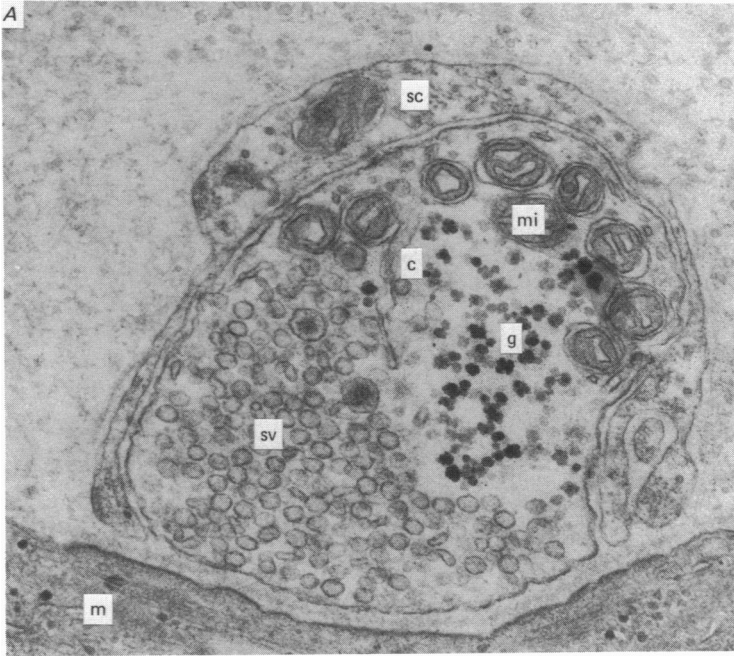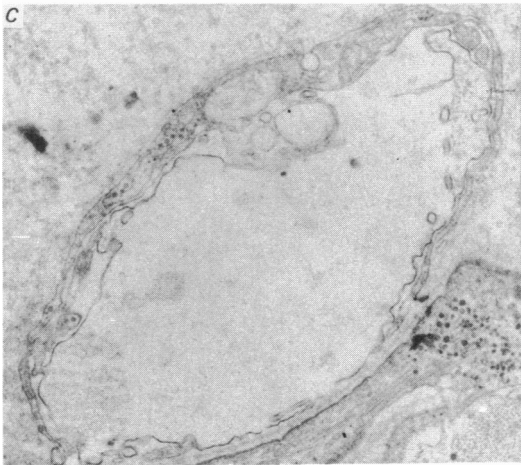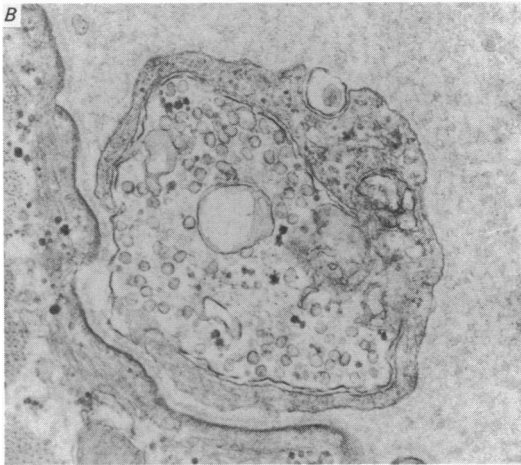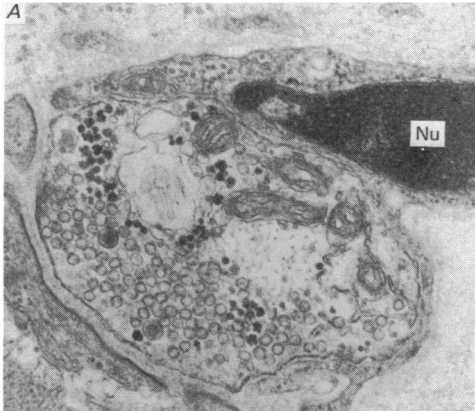
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EXPLANATION OF PLATES

PLATE 1

Morphology of frog motor nerve terminals exposed for 60 min to either normal Ringer solution (*A*) or solution containing 100 μM-Erythrosin B (*B*). Both contain synaptic vesicles (SV), cisternae (c), and mitochondria (mi), which are swollen in *B*. *A* also contains glycogen (g). Both are surrounded by a thin Schwann cell (sc), and rest upon a post-synaptic muscle fibre (m). Calibration bar represents 1μm for these and subsequent micrographs.

PLATE 2

Morphology of terminals exposed to either normal Ringer solution (*A*) or 100 μM-Erythrosin B (*B*, *C*) for 150 min. Terminals in Ringer solution (*A*) appear normal, but those in dye have a reduced number of synaptic vesicles, swollen mitochondria, and an increased number of dilated cisternae. The Schwann cell in *A* contains a nucleus (Nu). Terminal in *C* has very few synaptic vesicles, and an abundance of poorly-defined membranous profiles.