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PRESYNAPTIC EFFECT OF ERYTHROSIN B AT THE FROG NEUROMUSCULAR JUNCTION: ION AND PHOTON SENSITIVITY

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SUMMARY

1. The anionic dye Erythrosin B increases quantal transmitter release from frog neuromuscular synapses. Experiments were performed to determine the role of ions and light in this presynaptic effect.

2. In calcium-free saline containing 1 mM-EGTA, Erythrosin B increased miniature end-plate potential (m.e.p.p.) frequency at a more rapid rate than in normal saline.

3. The dye's effect was influenced by extracellular calcium ions in a complex manner. Dye-induced release was minimal in Ringer solution containing 0·1 mM-calcium, and higher in calcium concentrations above or below 0·1 mM.

4. Erythrosin B-induced spontaneous release also occurred in saline which contained 1 mM-EDTA and was free of both calcium and magnesium ions.

5. Temporary removal of external sodium ions did not alter the progressive increase in m.e.p.p. frequency produced by the dye.

6. Elevation of the potassium concentration of the external medium (from 2 to 20 mM), which presumably depolarized nerve terminals and increased their calcium permeability, did not change the rate of increase of dye-induced release when preparations were in a reversed (outward) electrochemical gradient for calcium ions.

7. A reduction in light intensity of at least six orders of magnitude reduced the effect of Erythrosin B by 50%, suggesting that photoactivation is not the primary basis for the dye's action.

8. These results indicate that Erythrosin B is not acting solely by altering the ionic permeability of the presynaptic nerve terminal to calcium, magnesium, or sodium ions, or by altering the calcium metabolism of the terminal. The enhanced effect of the dye in calcium-free saline suggests that it may be competing with calcium at a common site, while the enhancement of its effect in elevated external calcium suggests that the dye may also increase the permeability of the nerve terminal to calcium ions.

INTRODUCTION

The anionic dye Erythrosin B alters neuromuscular transmission, by transiently increasing the release of transmitter quanta from the presynaptic nerve terminal and

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subsequently producing 'giant' m.e.p.p.s (Augustine & Levitan, 1982). In this paper we have examined several possible mechanisms which might be responsible for the ability of Erythrosin B to increase transmitter release. As divalent and monovalent cations are thought to directly or indirectly activate the release process (Rahamimoff, Lev-Tov & Meiri, 1980), we have first considered their roles in dye-induced release. Also, because Erythrosin B (Pooler & Valenzeno, 1979) and several other derivatives of fluorescein (Burmistrov & Lyudkovskaya, 1968; Oxford, Pooler & Narahashi, 1977; Baumgold, Matsumoto & Tasaki, 1978) have photodynamic actions on neuronal membranes, we have examined the effect of a reduction in ambient light intensity on the dye's presynaptic action. We report here that the dye's effect cannot be accounted for solely by any known ionic mechanism, and that it is slightly sensitive to large changes in light intensity. The sensitivity of the dye's effect to changes in extracellular calcium ion concentration suggests that the dye may have more than one presynaptic action, perhaps competing for a site normally occupied by calcium in triggering release and also affecting the permeability of the presynaptic nerve terminal. Some aspects of this study have appeared previously (Augustine & Levitan, 1977, 1978, 1980).

METHODS

Exeriments were performed on the isolated cutaneous pectoris neuromuscular junction of the frog (*Rana pipiens*), using techniques described in the previous paper (Augustine & Levitan, 1982). Changes in the frequency of spontaneous miniature end-plate potentials (m.e.p.p.s) were used as an indicator of the effect of Erythrosin B on quantal transmitter release from these junctions. Physiological solutions used are listed in Table 1. Preparations were incubated in these salines for at least 60 min before dye treatment.

To determine the extent to which light was responsible for the effect of Erythrosin B on m.e.p.p. frequency the recording chamber and perfusion system were enclosed in a light-tight box and the fluorescent light fixtures in the laboratory were turned off. Light intensity within the box was measured with a photomultiplier tube (EMI No. 9781A) powered by a high voltage a.c. source (Calibration Standards Corp. Model 120B). In order to compare this intensity with that to which preparations were ordinarily exposed, the box was opened and light levels were again measured. Neutral density filters (Kodak Wratten filter No. 96) were then used to attenuate the light impinging upon the photomultiplier tube by 1 log unit increments, until the photomultiplier tube output was similar to that recorded in dark conditions. Although this method did not provide a measure of absolute light intensities or their spectral composition, we were able to determine that the light within the box in a darkened room was at least 6 log units lower than that to which preparations were normally exposed.

RESULTS

Ionic permeability

To determine whether Erythrosin B acts by modifying the permeability of the presynaptic membrane to specific ions, they were individually removed from the extracellular medium.

Calcium. In calcium-free saline Erythrosin B increased m.e.p.p. frequency (Fig. 1), suggesting that dye-induced release does not depend upon an influx of calcium ions from the external medium. In both normal and calcium-free Ringer solution m.e.p.p. frequency increased roughly exponentially after dye treatment, but the rate of frequency increase, expressed as the rate constant α (Augustine & Levitan, 1982), was about four times greater in calcium-free saline at all dye concentrations tested

TABLE 1 Composition of solutions [in mM]

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Sucrose	Na_2SO_4	K_2SO_4	$CaSO_4$	Tris-HCl (pH 7·2)	Tris-H_2SO_4 (pH 7·2)
Normal A	120	2	1·8	—	—	—	—	—	5	—
Ca-free B	120	2	—	1	—	—	—	—	5	—
Ca-free, high K C	102	20	1	1	—	—	—	—	5	—
Divalent-free D	120	2	—	—	—	—	—	—	5	—
Na-free, Tris E	—	2	1·8	—	—	—	—	—	125	—
Na-free, sucrose F	—	2	1·8	—	220	—	—	—	5	—
Low Cl G	—	2	-	—	—	120	2	10	—	5

Solution B contained less than 5 μM-Ca, as measured on a Perkin–Elmer Model 560 atomic absorption spectrophotometer. EGTA (1 mM) was ordinarily added to this solution to reduce its ionized Ca concentration to less than 10^{-8} M (Portzehl, Caldwell & Ruegg, 1964).

Solution D contained 1 mM-EDTA to reduce the concentration of both Ca and Mg to less than 10^{-6} M. In experiments using solutions C or D, tetrodotoxin (2×10^{-6} g/ml.; Sigma Chemical Co.) was used to prevent muscular action potentials (Ornberg, 1977). When using isotonic sucrose (Birks & Cohen, 1968) as a substitute for Na (F) it was necessary to pre-treat preparations in low Cl saline (G) to prevent muscle depolarization (Hodgkin & Horowicz, 1959).

(Fig. 2). Thus, eliminating external calcium ions increased the sensitivity of the preparation to the dye.

The dye's action on release was considered over a wide range of calcium concentrations (Fig. 3). The dye's effect was lowest in Ringer solution containing 0·1 mM-calcium, with greater rate constants found at lower calcium concentrations. In

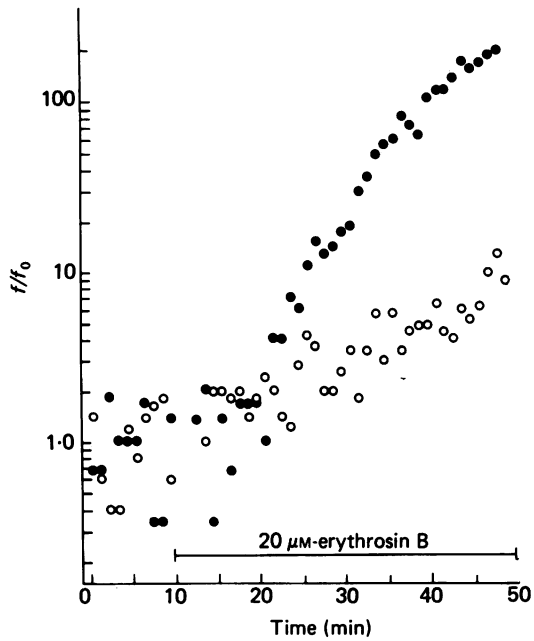

Fig. 1. The m.e.p.p. frequency (f) increases with time relative to its initial level (f_0) after addition of Erythrosin B (20 μM) to normal Ringer solution (○) and calcium-free Ringer solution containing EGTA (●).

addition, the dye's effect was significantly ($0{\cdot}05 > P > 0{\cdot}02$) greater at 20 mM-Ca than in normal saline. Thus the curve relating $\bar{\alpha}$ to external calcium concentration is U-shaped, with a minimum around 0·1 mM-calcium.

Magnesium. In the absence of both external magnesium and calcium ions, m.e.p.p. frequency still increased exponentially after treatment with Erythrosin B. Since it was difficult to maintain stable micro-electrode penetration under these conditions a detailed quantitative analysis was not attempted. In six experiments performed with dye concentrations ranging from 10 to 500 μM, rates of frequency increase (α) were obtained in divalent-free medium which were comparable to those found when calcium alone was removed. We therefore conclude that influx of magnesium also is not required for the increase in release produced by the dye.

Sodium. To test the possible role of sodium influx, sodium ions were removed from the bathing medium prior to the addition of Erythrosin B. Prolonged exposure to sodium-deficient medium is of itself known to produce an increase in spontaneous release (Kelly, 1968), perhaps due to an accumulation of calcium ions within the nerve terminal (Baker, 1972; Baker & Crawford, 1975).

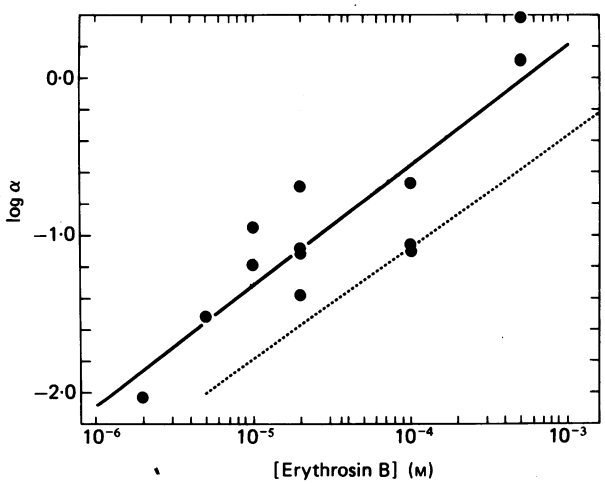

Fig. 2. The rate of increase in m.e.p.p. frequency (α, min^{-1}) was a function of Erythrosin B concentration in calcium-free Ringer. Each point represents a different experiment. The linear regression of these points (continous line), has a slope of 0·76, and correlation coefficient (r^2) of 0·77. The dashed line represents the dye's dose–response curve in normal Ringer solution (taken from Fig. 3 of Augustine & Levitan, 1982).

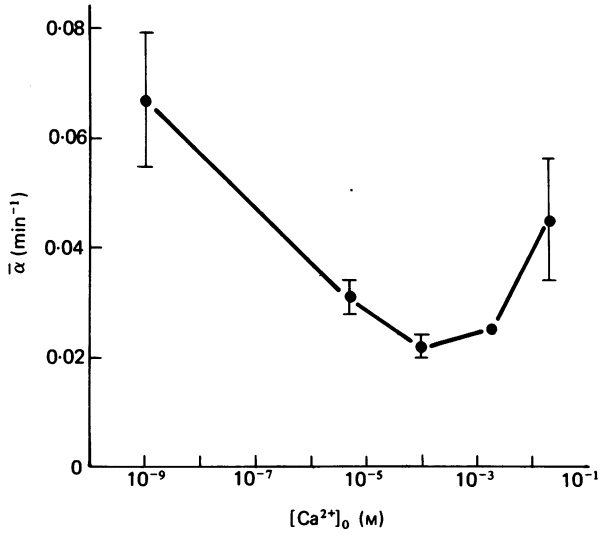

Fig. 3. The mean rate of increase in m.e.p.p. frequency ($\bar{\alpha}$) caused by Erythrosin B (20 μM) is a function of the calcium concentration in the Ringer solution ($[Ca^{2+}]_o$). Calcium-free saline containing 1 mM-EGTA is indicated as 10^{-9} M. Vertical bars indicate ± 1 S.E. and points represent means of three determinations (six for 1·8 mM-Ca).

To avoid this problem the preparation was exposed to sodium-free solution for a period of time brief enough to avoid significant calcium accumulation. When external sodium was removed for 20 min or less the frequency of m.e.p.p.s on returning to normal sodium concentration was comparable to that observed before sodium deletion (Fig. 4, left). This suggested that either spontaneous release did not

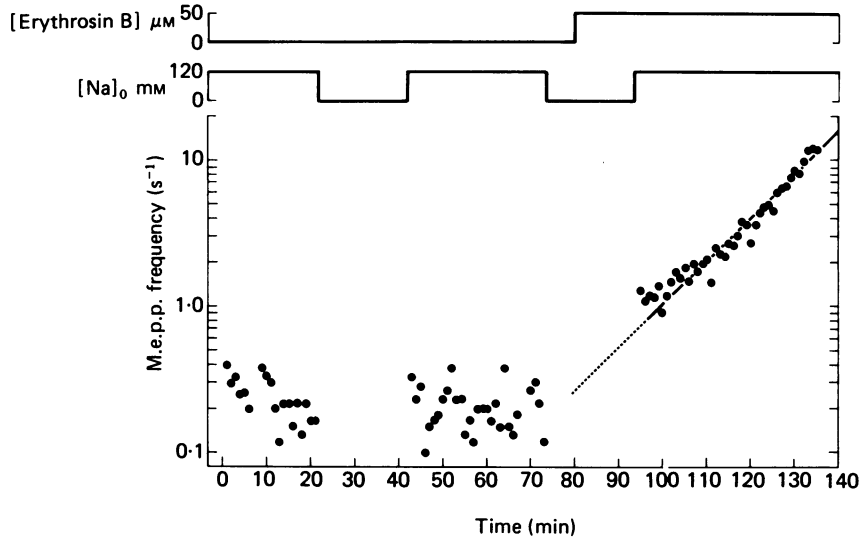

Fig. 4. Effect of temporary removal of sodium ions on m.e.p.p. frequency in the absence and presence of 50 μM-Erythrosin B. At left, sodium ions were removed for 20 min and substituted with Tris. M.e.p.p.s could not be detected during this period as indicated by the first gap in the data points. When sodium was restored little or no change was observed in the frequency of m.e.p.p.s. During a second 20 min period of sodium deletion (right) Erythrosin B was added. At the moment sodium was returned m.e.p.p. frequency was elevated substantially, and continued to increase exponentially afterwards. Points represent average m.e.p.p. frequency determined at 1 min intervals, and the line was calculated for points measured during dye treatment with linear regression analysis.

increase during this brief period, or that recovery from any increase was as rapid as the restoration of external sodium. As the acetylcholine-activated ionic currents responsible for m.e.p.p.s are carried largely by sodium ions (Takeuchi & Takeuchi, 1960; Lewis, 1979) it was not possible to record m.e.p.p.s in sodium-free medium. The m.e.p.p.s did, however, rapidly reappear upon re-introduction of sodium.

Preparations were treated with Erythrosin B during exposure to sodium-free Ringer solution. After 15 min in a sodium-free solution containing dye, the bathing medium was replaced with a second solution containing both sodium and the dye. In each of five experiments using 50–200 μM-Erythrosin B, we found that m.e.p.p. frequency was elevated upon return to normal Ringer solution (Fig. 4). If the effect of Erythrosin B was dependent upon the presence of external sodium, m.e.p.p. frequency would be expected to be *unchanged* upon return to sodium-containing medium, and increase exponentially thereafter. However, if the dye's action was not sodium-dependent, at the instant sodium was restored m.e.p.p. frequency should be

higher than control values, and continue to increase exponentially afterwards. The results obtained suggest that the dye's effect is not dependent upon external sodium.

The Tris ions used in these experiments as a substitute for sodium have been found to have pharmacological actions upon several preparations (Gillespie & McKnight, 1976; Wilson, Clark & Pellmar, 1977), although the effect of Tris on skeletal neuromuscular transmission is reported to be small (Gillespie & McKnight, 1976). To examine whether the above results were due to a secondary effect of Tris ions, we performed similar experiments using sucrose as a substitute for sodium. Results were obtained which were similar to those found using Tris, reinforcing the conclusion that an influx of sodium ions was not necessary for the increase in spontaneous transmitter release produced by Erythrosin B.

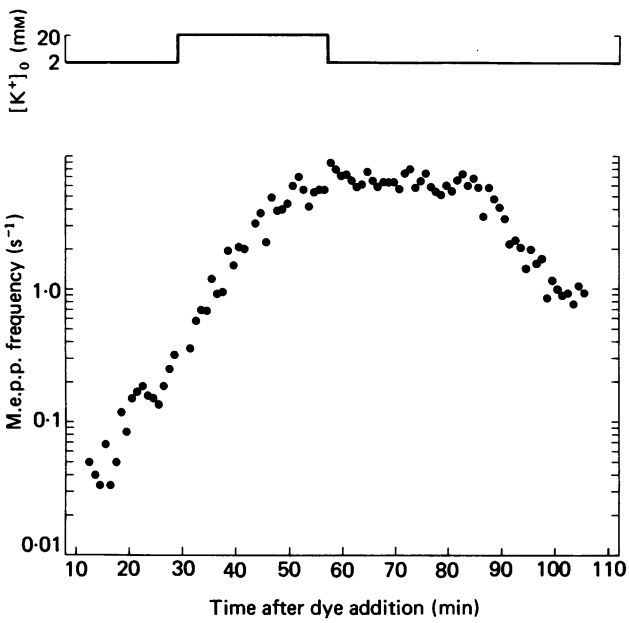

Fig. 5. An examination of the role of intracellular calcium ions in dye-induced release. A preparation was bathed in calcium-free Ringer and treated with 10 μM-Erythrosin B. When the extracellular concentration of potassium ions was increased from 2 to 20 mM, no change was observed in the exponential increase in m.e.p.p. frequency.

Intracellular calcium. Substantial quantities of calcium are stored within the terminal in a sequestered form (Blaustein, Ratzlaff, Kendrik & Schweitzer, 1978), and a number of treatments which increase transmitter release are thought to free this internally bound calcium (Glagoleva, Liberman & Khashayev, 1970; Alnaes & Rahamimoff, 1975). To determine whether Erythrosin B was similarly acting by releasing internally sequestered calcium ions we attempted to establish a condition which would reduce any increase in calcium ions within the nerve terminal induced by the dye.

This was done by promoting an efflux of calcium ions from the nerve terminal through an increase in the calcium permeability of its membrane in the presence of a reversed (i.e. outward) electrochemical gradient for calcium. The calcium permeability of the nerve terminal membrane was increased by depolarization (Katz &

Miledi, 1967; Llinás, Steinberg & Walton, 1981), and a reversed electrochemical gradient for calcium was produced by bathing the preparation in calcium-free Ringer solution. In such conditions a decline in the rate of dye-induced release would be anticipated if an increase in internal calcium were responsible for the dye's effects on release (Shimoni, Alnaes & Rahamimoff, 1977; Erulkar, Rahamimoff & Rotshenker, 1978). In fact, no decline in the rate of increase of m.e.p.p. frequency produced by Erythrosin B was observed when an increase in external potassium (from 2 to 20 mM) was used to depolarize the presynaptic terminal (Liley, 1956; Takeuchi & Takeuchi, 1961; Blaustein, 1975) in calcium-free medium (Fig. 5). This suggests that Erythrosin B does not enhance release by freeing calcium from internal stores.

In these experiments, despite the continued presence of the dye, m.e.p.p. frequency stopped increasing upon returning extracellular potassium concentration to normal. This was followed by a decline in m.e.p.p. frequency to values below those measured before dye addition. This was consistently observed in experiments using various dye concentrations and various periods of exposure to elevated external potassium. The reason for this observation is unknown, but it could possibly reflect an acceleration of the decline in dye-induced release which normally occurs after prolonged exposure (see Augustine & Levitan, 1982).

Photosensitization

The previous experiments suggests that Erythrosin B can alter release via a mechanism that is not exclusively due to a flux of any of the ions tested. Since several other dyes have been found to alter axon excitability (Oxford *et al.* 1977; Baumgold *et al.* 1978), and to increase transmitter release (Burmistrov & Lyudkovskaya, 1968) only upon exposure to light, we attempted to determine whether such a photodynamic action might also be involved in the presynaptic effect of Erythrosin B.

Experiments were performed in conditions where the light impinging upon the preparation was reduced by at least six orders of magnitude. In such dark conditions, Erythrosin B produced an exponential increase in spontaneous release which occurred at a somewhat slower rate than that observed in normal laboratory lighting. This difference was quantified by comparing the mean rate of increase ($\bar{\alpha}$) obtained with 20 μM-dye in normal ambient light with that obtained in darkness. Reducing the light intensity significantly ($P < 0.05$) reduced the mean rate of increase in m.e.p.p. frequency ($\bar{\alpha}$) from 0.025 ± 0.001 min^{-1} to 0.013 ± 0.01 min^{-1} (mean $\pm$ S.E. of mean) in four experiments conducted at each light intensity. Thus the dye still affected release in the virtual absence of light, although large increases in light intensity seemed to enhance its effect.

DISCUSSION

In an attempt to determine the basis for the ability of Erythrosin B to increase transmitter release, it was found that removal of external calcium, magnesium or sodium ions did not reduce the dye's effect and, in the case of calcium ions, removal enhanced the rate of dye-induced release. Dye-induced release did vary as a function of external calcium, increasing with concentrations greater or less than 0·1 mM. Depolarization of terminals in a reversed electrochemical gradient for calcium ions did not reduce Erythrosin B's presynaptic effect, and the dye's effect was partially attenuated by a large reduction in light intensity. These results suggest that

dye-induced release is not mediated solely by any ion thought to play a role in release and is not primarily due to a photodynamic action of the dye.

The complex relationship between extracellular calcium and dye-induced release suggests that Erythrosin B has more than one presynaptic action. The enhancement of the dye's effect by high external calcium concentrations would be expected if the dye were increasing the permeability of the presynaptic membrane to calcium ions. Recent experiments on artificial lipid bilayer membranes reveal that Erythrosin B produces an increase in membrane conductance to calcium ions (Colombini & Wu, 1981). The dye cannot increase release exclusively by increasing the calcium permeability of the presynaptic membrane because dye-induced release occurred in calcium-free saline. It will be necessary to apply Erythrosin B to a 'giant' synapse preparation, where it is possible to measure presynaptic calcium permeability (Llinás *et al.* 1981; Charlton, Smith & Zucker, 1982) and intraterminal calcium concentrations (Llinás & Nicholson, 1975; Miledi & Parker, 1981; Charlton *et al.* 1982), to determine whether such a mechanism partially underlies the dye's action on transmitter release.

The enhancement of the dye's effect as calcium is reduced below 0·1 mM suggests an antagonism between Erythrosin B and calcium ions. Reducing the extracellular concentration of calcium ions could influence the dye's action in at least two ways. The binding of calcium-transporting 'ionophores' to cells apparently is enhanced by pre-incubation in calcium-free saline (Selinger, Eimerl & Schramm, 1974). Calcium deletion perhaps could similarly facilitate the association of dye with the presynaptic membrane. Calcium removal could somehow allow Erythrosin B to occupy a presynaptic site which calcium normally occupies when triggering release, so that the dye and calcium would be competing for a common site. The observation that the dose–response curves for the presynaptic effect of the dye in normal and calcium-free Ringer are roughly parallel (Fig. 2) is an indication of competitive inhibition (Dodge & Rahamimoff, 1967) and therefore is consistent with such a possibility.

The fact that Erythrosin B increases release in divalent cation-free solution suggests other possible mechanisms. Black widow spider venom, dinitrophenol and ouabain also increase release from neuromuscular junctions in calcium free saline containing magnesium, but their effects are eliminated by removal of magnesium (Ornberg, 1977). It has been suggested that the effect of black widow spider venom depends upon external magnesium because the venom increases the permeability of the nerve terminals to cations (Finkelstein, Rubin & Tzeng, 1976; Gorio & Mauro, 1979; Misler & Hurlbut, 1979), and that magnesium, upon entry, can trigger release (Blioch, Glagoleva, Liberman & Nenashev, 1968; Hurlbut, Longenecker & Mauro, 1971; Kita & Van der Kloot, 1976; Lev-Tov & Rahamimoff, 1980; Kharasch, Mellow & Silinsky, 1981). Although the effect of dinitrophenol and ouabain on the magnesium permability of nerve terminals is unknown , a different mechanism has been proposed for the magnesium dependence of their effects. Ornberg (1977) has suggested that magnesium ions may be required as a normal cofactor for release. Based on this interpretation, the persistence of the dye's effect in divalent cation-free conditions would indicate that the dye is substituting for magnesium and/or bypassing the magnesium-dependent step in the transmitter release process. Hyperosmotic solutions also increase release in the absence of external divalent cations (Ornberg, 1977; Shimoni, *et al.* 1977; Misler & Hurlbut, 1979).

Although the dye's effect does not appear to depend upon entry of sodium from the extracellular medium, it is possible that the concentration of sodium within the nerve terminal is increasing by some other means. Increasing the concentration of sodium within nerve terminals increases transmitter release (Charlton & Atwood, 1977) and this effect may be mediated via a subsequent increase in intraterminal calcium concentration (Rahamimoff *et al.* 1980). If this is true, then the conclusion that the dye does not increase intracellular calcium (see below) further diminishes the likelihood of a role for sodium in the dye's effect.

Since and elevation of external potassium ions in calcium-free Ringer solution did not reduce Erythrosin B-induced release, the dye is apparently not acting by elevating intracellular calcium. Although many agents are thought to increase the concentration of calcium ions within nerve terminals, this experiment has been attempted only with terminals exposed to hyperosmotic sucrose (Shimoni *et al.* 1977) and lithium (Fig. 19 of Crawford, 1975). In both cases depolarization of the terminal with 20 mM-potassium in calcium-free media produced a *decline* in m.e.p.p. frequency. This observation is consistent with the interpretation that an increased intracellular calcium concentration is responsible for the observed effects of these agents. The fact that we did not observe a similar decline in m.e.p.p. frequency under the same experimental conditions suggests that Erythrosin B does not cause an appreciable increase in intracellular calcium, although more direct measurements are needed to strengthen this conclusion.

The reduction in the dye's effect with very large reductions in light intensity suggests that it has, at least in part, a photodynamic basis. This is consistent with the results of Pooler & Valenzeno (1979), who reported that Erythrosin B decreases the excitability of lobster axons when they are exposed to light of sunlight intensity. We cannot be sure that a photodynamic effect is entirely responsible for the dye's action, because in the virtual absence of light the dye still had an effect. Perhaps even at this greatly reduced intensity illumination triggered a photoactivated effect. Alternatively, the action of the dye may have a photosensitive and a photoinsensitive component.

Whether photoactivated or not, the molecular mechanism underlying the action of Erythrosin B remains uncertain. The activity of xanthene dyes, including Erythrosin B, has been found in other systems to be strongly correlated with their lipid solubility, suggesting that the dyes interact with the hydrophobic regions of all membranes (Levitan, 1977; Carroll & Levitan, 1978). This may also be true at the neuromuscular junction. Erythrosin B affects the activity of isolated protein molecules, including several enzymes (Wassarman & Lentz, 1971; Jacobsberg, Kantrowitz & Lipscomb, 1975; Somerville & Quiocho, 1977). It could thus be altering the properties of a calcium-activated protein which is responsible for release, such as actin or myosin (Berl, Puszkin & Nicklas, 1973), calmodulin (De Lorenzo, Freedman, Yohe & Maurer, 1979), synexin (Creutz, Pazoles & Pollard, 1978), or others (Kelly, Deutsch, Carlson & Wagner, 1979). Biochemical experimentation will clearly be necessary to better understand the action of Erythrosin B at the molecular level.

In summary, the ability of Erythrosin B to increase transmitter release does not depend solely upon any known ionic mechanism and may partially rely on some photodynamic property of the dye. Its effect could involve enhancing calcium entry

into nerve terminals as well as interacting with a site which calcium occupies during normal transmitter secretion. Because Erythrosin B stimulates transmitter release, with an apparently calcium-independent component, it could be a useful addition to the presynaptic neurotoxins previously identified. Although the specific presynaptic mechanisms of the dye are not clear, its actions are probably understood as well as those of most other presynaptic neurotoxins. These other agents have proved valuable (Kelly *et al.* 1979; Howard & Gunderson, 1980; Ceccarelli & Hurlbut, 1980), although knowledge of their specific sites of action shouls permit more definite statements to be made about the molecular basis of transmitter release. This task may prove simpler for a substance with well-defined physical properties, such as Erythrosin B.

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