DIVALENT CATIONS AND TRANSMITTER RELEASE AT LOW CONCENTRATION OF TETRODOTOXIN

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ABSTRACT Transmitter release from frog motor terminals was studied in the presence of very low concentrations of tetrodotoxin (TTX, 4.10^{-10}–6.10^{-9}g/ml). TTX reversibly reduced the amplitude of the end-plate potential (epp), while leaving the amplitude distribution to follow Poisson's law. The effects of a number of divalent cations were studied in the presence of TTX. It was found that after the addition of TTX there was an increase in the constant of dissociation of calcium and strontium from a hypothetical membrane "release site," while the dissociation constants of magnesium and managanese remained unaltered. It is concluded that the release site is probably intracellular and that a reduced presynaptic spike amplitude, as well as magnesium and manganese ions, decrease the access of calcium and strontium to the site.

INTRODUCTION

The "kinetic model" of transmitter release first suggested by del Castillo and Katz (1954*a*) and further elaborated by Jenkinson (1957) and Dodge and Rahamimoff (1967) has been very useful in describing the interaction of ions in the process of transmitter release. However this model suffered from two major deficiencies: (*a*) It failed to assign a physical location to the "release site" X, (*b*) it failed to consider, explicitly, the role of the presynaptic action potential in transmitter release. Subsequent authors attempted to correct these deficiencies but arrived at conflicting results. Thus, Hubbard et al. (1968), Landau (1969), and Meiri and Rahamimoff (1971) believed X to be extracellular and that membrane depolarization enhances its efficacy in releasing transmitter, whereas Van der Kloot and Kita (1973) and Cooke et al. (1973) believed X to be intracellular and that membrane depolarization enhances the access of Ca^{2+} ions to the site. Another shortcoming of the aforementioned studies was the use of slow changes in presynaptic membrane potential to investigate the effect of presynaptic depolarization on release. With such slow depolarizations, various adaptive mechanisms may set in, which could distort the picture. We therefore chose to investigate this problem by studying the interaction of ions in the release process under the influence of very low concentrations of tetrodotoxin (TTX), which reduce the spike amplitude without abolishing it completely. It can be shown (Results) that if the release site, X, is intracellular, the apparent dissociation constant of the CaX complex (K'_{Ca}), should increase when the spike amplitude diminishes. Our main finding was that low concentrations of TTX do indeed produce an increase K'_{Ca}. This suggests that X is intracellular and that the amplitude of the presynaptic spike regulates the access of Ca^{2+} ions to the release site.

MATERIALS AND METHODS

Preparation and Solutions

The experiments were performed in vitro on the sartorius nerve-muscle preparation of the frog *Rana ridibunda* at room temperature (20°–24°C). The standard bathing Ringer's solution had the following composition (in millimolars): NaCl, 116; KCl, 2–2.5; $CaCl_2$, 0.2–2.5; $MgCl_2$, 0–1; Tris buffer, 5. In some experiments $MnCl_2$ (0.03–0.1 mM) or $SrCl_2$ (1–2.5 mM) were added. Except for the experiments of the type shown in Fig. 2, the divalent ion concentrations were such that no failures of transmission appeared. Experiments in which failures appeared after the addition of TTX were excluded from the study. Muscle contraction was prevented by the addition of tubocurarine (dTC; 1.0–2.0 · 3g 10^{-6} g/ml), or by the process of detubulation with ethylene glycol as described by Sevcik and Narashashi (1972). The pH of the solutions was adjusted to 7.3–7.4 by adding HCl.

When increasing the divalent ion concentration or adding TTX, the existing solution was replaced by washing 100–140 ml of the new solution through a chamber of 10 ml capacity for 1.5–3 min. One such washing was usually adequate to reach a new steady state of quantal release. When lowering the divalent ion concentration or washing TTX our after prolonged exposure, several washings were required. Each experiment was carried out in a single cell. When changes in the resting potential occurred, the end-plate potential (epp) amplitudes were corrected to the initial driving potential, defined as the resting potential minus the reversal potential which was taken as −3 mV (Landau, unpublished observations). Experiments in which large shifts in the resting potential occurred (>10 mV) were rejected.

Stimulation and Recording

The sciatic nerve was stimulated supramaximally using a Grass SD9 stimulator (Grass Instrument Co., Quincy, Mass.). Facilitation was avoided by stimulating at a rate of 0.5 s^{-1} in solutions containing Ca^{2+} and Mg^{2+}, and at a rate of 0.3–0.4 s^{-1} in solutions with Sr^{2+} and Mn^{2+}. Minature end-plate potentials (mepp) and epp were recorded intracellularly with glass micropipettes filled with 3 M KCl. Signals were amplified via a NF1 Bioelectric or WPI electrometer and displayed on a 565 Tektronix oscilloscope screen (amplifier 3A9, Tektronix, Inc., Beaverton, Ore.; frequency range DC to 10 KHz). epp and mepp were recorded on cine film; in addition, the mean of 100–250 epp was calculated by a computer for averaging transients (CAT 400C–TMC) and plotted with an X–Y recorder (H–P 7035B, Hewlett-Packard Co., Palo Alto, Calif.). The mean epp amplitude was corrected for nonlinear summation (Martin, 1955). This correction rarely amounted to 10% of the epp amplitude, and in most cases was <5%. To determine the mean mepp amplitude, 50–60 mepp were measured from film and averaged. The coefficient of variation of the mepp amplitudes varied between 15 and 22%.

RESULTS

In the Presence of TTX There Is a Decrease in epp Quantal Content

Our study is based on an extension of the release-model of Dodge and Rahamimoff (1967). We make the following assumptions: (*a*) the intracellular concentration of calcium near the membrane, $[Ca]_i$, is proportional to the outside bathing calcium concentration, $[Ca]_o$ (cf., Crawford, 1974). The proportionality constant between the two, k_v, is voltage dependent and reflects the ease of access of the Ca^{2+} ions to an intracellular release site, thus

$$[Ca]_i = k_v[Ca]_o . \tag{1}$$

(*b*) Inside the terminal, Ca^{2+} ions bind to an intracellular release site, X, to form a complex CaX, thus

$$[Ca]_i + [X] \underset{}{\overset{K_{Ca}}{\rightleftharpoons}} [CaX] . \tag{2}$$

(*c*) The cooperative activation of four release sites by calcium is required for the release of a quantum of transmitter

$$r = a[\mathrm{CaX}]^4 \tag{3}$$

where r is the rate of release and a an appropriate constant. Conventional treatment of Eqs. 2 and 3 leads to

$$\overline{V} = \overline{V}_{max}\left(\frac{[\mathrm{Ca}]_i/K_{Ca}}{1 + [\mathrm{Ca}]_i/K_{Ca}}\right)^4 \tag{4}$$

where $\overline{V}$ is the average epp amplitude and $\overline{V}_{max}$ is $\overline{V}$ at saturating concentrations of calcium (both corrected for nonlinear summation). If we introduce Eq. 1 into Eq. 4 we obtain

$$\overline{V} = \overline{V}_{max}\left(\frac{[\mathrm{Ca}]_o/(K_{Ca}/k_v)}{1 + [\mathrm{Ca}]_o/(K_{Ca}/k_v)}\right)^4. \tag{5}$$

Eq. 5 has the same form as Eq. 4 except that we have replaced K_{Ca} by an apparent dissociation constant, K_{Ca}/k_v. In the future we shall denote apparent constants by a dash thus, K'_{Ca}. From Eq. 5 we conclude that K'_{Ca} should increase when k_v decreases.

The simplest prediction that our model makes is that when k_v decreases $\overline{V}$ should decrease, provided calcium is present in nonsaturating concentrations. To reduce k_v we decided to employ very low concentrations of TTX, close to the TTX dissociation constant in frog nerve (Ulbricht and Wagner, 1975). In this way we hoped to reduce the amplitude of the nerve action potential without blocking its propagation. The decrease in the spike amplitude should lead to a decreased k_v and hence to a decrease in $\overline{V}$ (Eq. 5). Indeed, our expectation was fulfilled. In the presence of low concentrations of TTX, the epp amplitude diminished in a reversible manner. A typical experiment is shown in Fig. 1: on application of 4.10^{-10} g/ml of TTX the epp amplitude decreased to ~28% of that of control, recovering completely when the TTX was washed out. The effect of TTX was always readily reversible when applied for not longer than 30 min. Thereafter reversibility was more difficult to achieve and required numerous washings (cf., Narahashi et al., 1964; Takata et al., 1966).

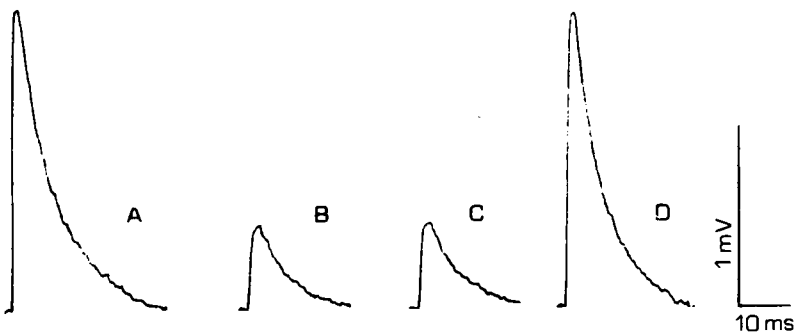

FIGURE 1 The effect of low concentration of tetrodotoxin on the average epp amplitude. *A*, Control epp in Ringer's solution; *B*, epp 7 min after addition of TTX ($4 \cdot 10^{-10}$ g/ml); *C*, 15 min after the addition of TTX; and *D*, 4 min after washout with Ringer's solution. Calibration: Vertical, 1 mV; horizontal, 10 ms. Normal Ringer's solution with Ca_o = 0.5 mM, dTC 10^{-6} g/ml, no Mg^{2+} added.

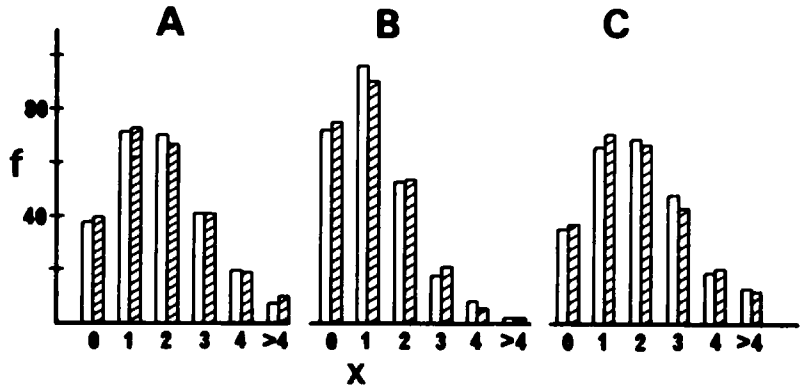

FIGURE 2 The effect of TTX on the statistical properties of transmitter release. Ringer's solution without dTC, $[Ca_o]$ = 0.2 mM, $[Mg_o]$ = 0.9 mM. The amplitude histograms *A*, *B*, and *C* represent each 250 epp amplitudes grouped into classes corresponding to a single quantum (the mean mepp amplitude), two quanta, three quanta, etc., and a group of failures (the column corresponding to $x = 0$). The open columns represent the observed frequency of occurrence, the striped columns represent the expected frequency (Ex) according to the Poisson equation $Ex = e^{-\bar{m}}\bar{m}^x/X!$ where m is given by mean epp/mean mepp (Del Castillo and Katz, 1954*b*). (*A*) Control (Ringer's solution); mean quantal content ($\bar{m}$) = 1.832, χ^2 = 1.347, $P > 0.05$; mean mepp amplitude = 0.181 mV. (*B*) After addition of TTX ($6 \cdot 10^{-10}$ g/ml). $\bar{m}$ = 1.185, χ^2 = 1.72, $P > 0.05$; mean mepp amplitude = 0.184 mV. (*C*) After washout of TTX with control Ringer's solution. $\bar{m}$ = 1.908, χ^2 = 1.22, $P > 0.05$; mean mepp amplitude = 0.185 mV.

The reduction in epp amplitude was the result of an inhibition of transmitter release, because the mean quantal content of the epp decreased on TTX application, while the amplitude of the mepp did not change (Fig. 2). Both in the presence and in the absence of TTX, the epp amplitudes were distributed in Poisson fashion (confirmed by χ^2 test; Fig. 2). This indicates that the decrease in epp quantal content was not due to a failure of the action potential to propagate into the nerve terminal, but rather to a decrease in the probability of release of a quantum by the action potential. This decrease in release probability was not due to a direct effect of TTX on the release apparatus or on the coupling between the presynaptic depolarization and release, as TTX concentrations much higher than those used in the present study do not affect the ability of nerve terminal depolarization to release acetylcholine (Katz and Miledi, 1967). The likeliest explanation for the effect of TTX is that the nerve action potential was decreased in amplitude (Takata et al., 1966) without being abolished completely. This should reduce the entrance of calcium into the ending and the epp amplitude should decrease according to our Eq. 5 (cf., Katz and Miledi, 1970).

In the Presence of TTX, There Is an Increase in the Dissociation Constant of Calcium from X

Another prediction arising from Eq. 5 is that the addition of TTX (leading to a decrease in k_v) should result in an *increase* in the apparent dissociation constant for calcium, defined as K_{Ca}/k_v. This prediction was tested by studying the calcium kinetics before and after the addition of TTX. In most experiments 0.5 mM Mg^{2+} was added to the Ringer's solution to avoid large changes in the excitability of the nerve (Frankenhaeuser, 1957). However, we have evidence that the apparent dissociation constant of Mg from X, K'_{Mg}, does not change

TABLE I
THE PARAMETERS OF EQ. 1 AND THE R VALUES
(EQS. 7 AND 9) ESTIMATED FROM THE VARIOUS EXPERIMENTS

	Experiments			
	Ca^{2+}	Mn^{2+}	Mg^{2+}	Sr^{2+}
No. of Exp.	4	7	3	4
K'_{Ca}*	0.62 ± 0.18‡	0.54§	0.50 ± 0.09	(0.52)‖
K'_{Ca} {TTX}	0.70 ± 0.20§	0.82§	0.64 ± 0.10	0.67 ± 0.05
K'_{Mg}	—		4.0 ± 1.8	—
K'_{Mn}	—	0.126§	—	—
K'_{Sr}	—	—	—	(1.5)‡‡
K'_{Sr} {TTX}	—	—	—	2.09 ± 0.47
b	—	—	—	0.37 ± 0.07 §§
R	1.19 ± 0.11¶	0.77 ± 0.04**	0.91 ± 0.02	1.107 ± 0.035

*All dissociation constants in millimolars; used are the apparent constants derived from the experiments; b and R are dimensionless. All values are mean ± 1 SD unless otherwise stated.

‡These values were corrected for the presence of 0.5 mM Mg^{2+} by dividing the value derived from the Lineweaver-Burke analysis by the factor $(1 + [Mg]/K_{Mg})$ see Eq. 1. K_{Mg} was taken to be 4 mM, according to the results of the Mg^{2+} experiments.

§The values were obtained from the results of four experiments in which the $\bar{V}$ values were very similar and were averaged before the calculation of the dissociation constants.

¶The result of the two experiments in which the low concentrations of Ca^{2+} were used, were included when calculating this value.

‖Taken as an average of the values in the previous two columns.

**This value was obtained from all seven experiments with 0.1 mM Mn^{2+}.

‡‡Taken from Meiri and Rahamimoff (1971).

§§Meiri and Rahamimoff (1971) give b values in the range 0.3–0.5.

following TTX application (cf., Fig. 6). In that case the Mg term will be included in the apparent Ca dissociation constant, thus

$$K'_{(Ca)} = (K_{Ca}/k_v)\,(1 + [Mg]_o/K_{Mg}) \tag{6}$$

from which K_{Ca}/k_v can easily be extracted (Table I). In one out of four experiments no Mg^{2+} at all was added, yet the results were essentially the same. One of the experiments will be presented in detail (Fig. 3): the epp amplitude was measured at five different concentrations of bathing Ca^{2+}, from 0.4 to 1.3 mM. TTX (8.10^{-10} g/ml) was then added and the epp amplitude re-examined at the same concentrations of Ca^{2+}. Finally the Lineweaver-Burke plots of $1/\sqrt[4]{\bar{V}}$ against $1/[Ca]_0$ for both series of experiments (in TTX and in control) were constructed, and regression lines drawn by the least-squares method (Fig. 3 *A*). The two lines have different slopes but a common intercept with the Y-axis. This implies that while K'_{Ca} increases after TTX treatment, the $\bar{V}_{max}$ remained unchanged (54.7 mV in control and 56.4 mV in TTX). The increase of the apparent Ca dissociation constant was from 0.54 to 0.65 mM, a change of 20%. A similar result was obtained in three more experiments. $\bar{V}_{max}$ did not change in either of the experiments and was assumed to be constant in the analysis of the rest of the data.

The average K'_{Ca} and K'_{Ca}[TTX] values for all experiments are shown in Table I (column 1). Although the scatter of the results was relatively large, K'_{Ca}[TTX] was larger than K'_{Ca} in each

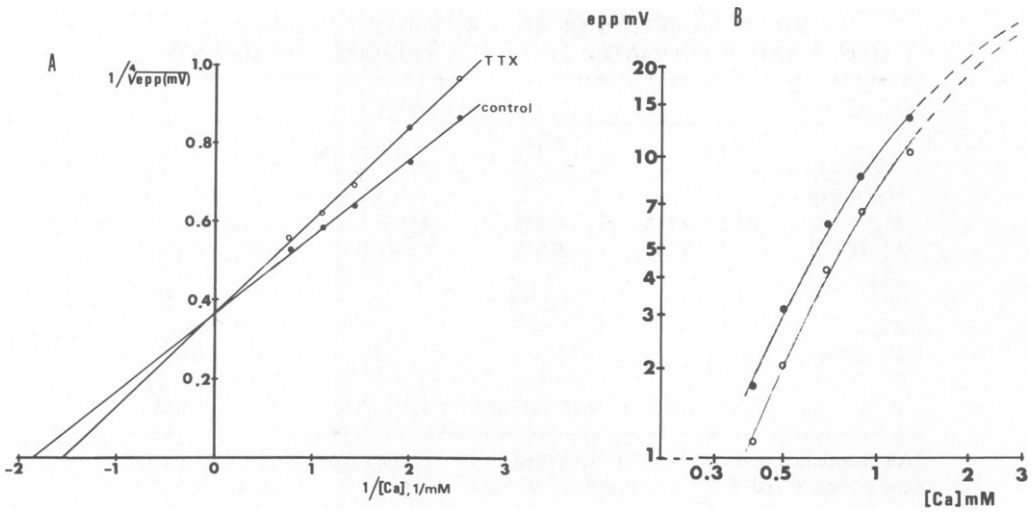

FIGURE 3 The effect of Ca^{2+} ions on transmitter release in the presence of TTX. Measurements were performed in the presence of Ringer's solution containing 0.5 mM Mg^{2+}. (*A*) The relationship between the fourth root of mean epp amplitude (ordinate) and $[Ca_o]$ (abscissa) on double reciprocal coordinates (Lineweaver-Burke plot). ●, without TTX, ○, with TTX ($8 \cdot 10^{-10}$ g/ml). (*B*) The relationship between the mean epp amplitude (ordinate) and $[Ca^o]$ (abscissa) on logarithmic coordinates. The same experiment as in Fig. 3 *A*. ●, without TTX; ○, with TTX ($8 \cdot 10^{-10}$ g/ml). The lines were drawn according to Eq. 5 with V_{max} = 54.7 mV, $\bar{V}_{max}$ {TTX} = 56.4 mV, K'_{Ca} = 0.54 mM, K'_{Ca} {TTX} = 0.65 mM; these values were taken from the Lineweaver-Burke analysis (Fig. 3 *A*). The dotted lines are extrapolations.

of the four experiments. The percentage increase in K'_{Ca}[TTX] was 12.3±6.6% (SD), and this was significantly larger than zero ($P<0.05$, t test). The increase in apparent K_{Ca} was smaller in this set of experiments than it was in the other experiments shown in Table I, probably because lower TTX concentrations were employed here. The range was 4–8 · 10^{-10} vs. 1–6 · 10^{-9} g/ml in the other experiments of this paper.

The results were also plotted on double logarithmic coordinates ($\bar{V}$ against $[Ca]_0$), and curves fitted using Eq. 5 and the apparent K_{Ca} and $\bar{V}_{max}$ values obtained from the Lineweaver-Burke analysis (Fig. 3 *B*). The fit was clearly good.

As can be seen from Fig. 3 *B*, the initial slope of the log $\bar{V}$ − log $[Ca]_0$ curves did not change after TTX application. In two addition experiments at low $[Ca]_0$ (0.3–0.45 and 0.3–0.6 mM) this phenomenon was studied further by examining the effect on $\bar{V}$ of four to six Ca^{2+} concentrations both before and after adding TTX. The initial slopes of the log $\bar{V}$ − log $[Ca]_0$ curves before and after TTX addition were the same within 10% in both experiments, and were in the range of 2.5–3, which is in accord with the data of Dodge and Rahamimoff (1967) for this range of divalent ion concentrations. The constancy of the slope is in accordance with our model which assumes a constant cooperative number, n, for calcium action (Eq. 3). However, our findings do not exclude models where n is variable, because the range of presynaptic depolarizations examined was probably not very large.

We conclude that the decrease in epp quantal content and amplitude, following a reduction in presynaptic spike amplitude, is due to an increase in the apparent K_{Ca}. This is exactly what

is predicted by our model (Eq. 5), assuming that TTX reduced k_v, i.e., the inflow of Ca^{2+} into the terminal, by reducing the amplitude of the nerve action potential.

The Blocking Effect of Manganese Is Enhanced in the Presence of TTX

To further elucidate the effect of reducing the spike amplitude on transmitter release, the inhibitory action of Mn^{2+} was examined. According to previous work, this ion competes with calcium for the release site, X (Meiri and Rahamimoff, 1972; Landau and Nachshen, 1975). The classic equation for release which includes the effects of competing, inhibitory ions is the following (Dodge and Rahamimoff, 1967):

$$\bar{V} = \bar{V}_{max}\left(\frac{[Ca]_o/K_{Ca}}{1 + [Ca]_o/K_{Ca} + [I]_o/K_i}\right)^4 \tag{7}$$

where $\bar{V}$ and $\bar{V}_{max}$, $[Ca]_o$ and K_{Ca} have the same meaning as in Eq. 5 and $[I]_o$ is the bathing concentration of some inhibitory ion, I, and K_I the dissociation constant of I from X. We have already seen that according to our model, K_{Ca} is replaced by the apparent dissociation constant K_{Ca}/k_v, which increases after treatment with TTX. What will be the fate of apparent K_I in TTX? To elucidate this problem, we performed a number of experiments where the effect of adding a given concentration of Mn^{2+} was compared before and after treatment with TTX.

In all experiments with Mn^{2+}, no Mg^{2+} was present in the Ringer's solution. A typical experiment is shown in Fig. 4. Here, before applying TTX, the addition of 0.1 mM Mn^{2+} ($[Ca]_o$ = 1 mM) reduced the average epp amplitude, $\bar{V}$, to 53.2% of that of control. (Fig. 4, traces 1 and 2); when Mn^{2+} was washed out, $\bar{V}$ recovered completely (Fig. 4, trace 3). Next, TTX (4.10^{-9} g/ml) was applied, whereupon $\bar{V}$ again decreased (Fig. 4, traces 4 and 4*a*). The effect of addition of Mn^{2+} at the same concentration as before, was now more pronounced, $\bar{V}$ being 39.5% of the value obtained in TTX alone (i.e., before adding Mn^{2+}) (Fig. 4, trace 5).

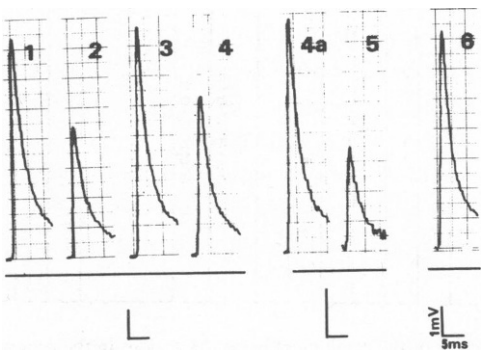

FIGURE 4 The effect of TTX on the inhibition by Mn^{2+} ions of transmitter release. Each trace represents the mean of 100 epp. No Mg^{2+} added to Ringer's solution. Trace 1: in control medium. Trace 2: after the addition of Mn^{2+} (0.1 mM). Trace 3: after washout with control Ringer's solution. Trace 4: in Ringer's solution containing TTX ($4\cdot10^{-9}$ g/ml). Trace 4*a*: same as trace 4, but at a higher amplification at the *X-Y* plotter (note change in vertical calibraton). Trace 5: Mn^{2+} (0.1 mM) added in the presence of TTX. Amplification as in trace 4*a*. Trace 6: Mn^{2+} and TTX washed out. Return to initial amplification. Vertical calibration: 1 mV; horizontal calibration: 5 ms.

Thus, the blocking effect of Mn^{2+} on transmitter release is enhanced in the presence of TTX.

To provide a quantitative measure of the effect of TTX treatment on the blocking of release by Mn^{2+}, a master ratio, R, was defined as follows:

$$R = \frac{(\bar{V}\{\text{Mn, TTX}\}/\bar{V}\{\text{TTX}\})}{(\bar{V}\{\text{Mn}\}/\bar{V}\,\text{control})} \tag{8}$$

where $\bar{V}$ is the mean epp amplitude, and {Mn} and {TXX} denote the presence of Mn^{2+} ions and TTX, respectively.

This ratio will be equal to 1 if the blocking effect of Mn^{2+} is the same both in the presence and in the absence of TTX, and will be <1 if the block is stronger in the presence of TTX. In the experiment shown in Fig. 4, R = 0.395/0.532 = 0.74. This ratio provides a tool for comparison and statistical analysis of experiments performed at different mean epp amplitudes and ion concentrations. Seven experiments similar to that shown in Fig. 4 ($[Ca]_o$ = 1–1.2 mM, [Mn] = 0.1 mM) are summarized in Fig. 5 *A*. All the ratios were less than unity, and the scatter of the results was small (mean R = 0.77±0.04 (±SD), significantly <1 at the 0.001 level).

R can also be defined in terms of the ion concentrations and dissociation constants. To achieve this we can simply substitute Eq. 7 into Eq. 8. Note, however, that before doing this we should replace all the dissociation constants in Eq. 7 with apparent dissociation constants,

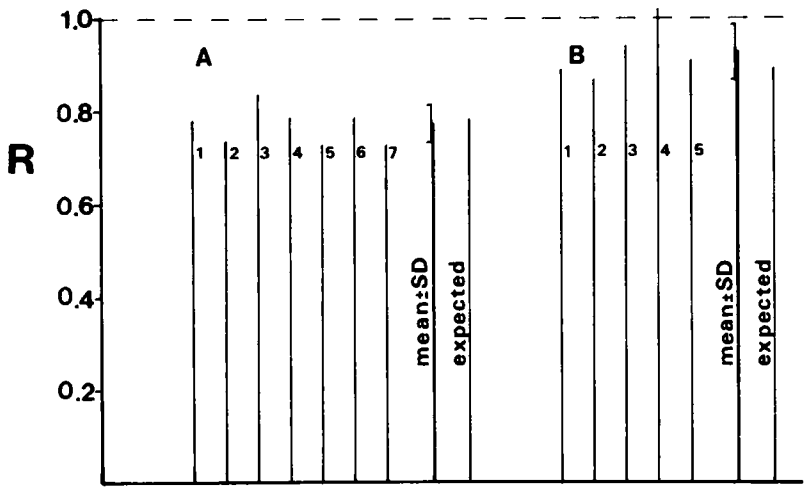

FIGURE 5 The enhancement of the blocking effect of Mn^{2+} ions in the presence of TTX: summary. (*A*) Each column (numbered 1–7) represents the value of R calculated from Eq. 8, of one out of seven experiments of the type shown in Fig. 4. $[Ca_o]$ in these experiments was 1–1.2 mM, $[Mg_o]$ was 0, and $[Mn_o]$ was 0.1 mM. The last bar but one represents the mean R ±1 SD. The last bar represents the mean expected R, calculated using the Eq. 9 with K_{Ca} = 0.54 mM, $K_{Ca}\{\text{TTX}\}$ = 0.82 mM, K_{Mn} = 0.126 mM and $\bar{V}_{max}$ unchanged in the presence of TTX. (*B*) As for Fig. 5 *A*, represents the results of five experiments with $[Ca_o]$ = 0.3–0.5 mM, $[Mn_o]$ = 0.03–0.05 mM, $[Mg_o]$ = 0. The bars 1 to 5 represent the value of R for each of the five experiments. The last bar but one is the mean R ±1 SD, and the last bar is the mean expected R value calculated using Eq. 9 and the constants derived from the experiments of Fig. 5 *A*.

K', which may contain the factor k_v (cf., Eq. 5). We then obtain:

$$R = \left[\frac{(1 + [Ca]_0/K'_{Ca}\{TTX\})(1 + [Ca]_0/K'_{Ca} + [Mn]_0/K'_{Mn})}{(1 + [Ca]_0/K'_{Ca})(1 + [Ca]_0/K'_{Ca}\{TTX\} + [Mn]_0/K'_{Mn}\{TTX\})}\right]^4. \quad (9)$$

We already know that TTX treatment causes K'_{Ca} to increase, i.e., $K'_{Ca}\{TTX\} > K'_{Ca}$. Concerning K'_{Mn}, two limiting cases may exist: (*a*) On TTX addition K'_{Mn} increases to the same extent as does K'_{Ca}. In this case Eq. 9 predicts that R should always be greater than unity. This is clearly in disagreement with the results of Fig. 5 *A*. (*b*) K'_{Mn} remains unaltered in TTX. In this case, R values should be always <1, as indeed was found experimentally. (Fig. 5 *A*). Intermediate cases (i.e., K'_{Mn} increases in TTX, but to a lesser extent than K'_{Ca}) are not excluded, but will not be discussed further for the sake of simplicity.

From the $\bar{V}$ values obtained in experiments with Mn^{2+} (Fig. 4, traces 1, 2, 4*a*, 5), and taking $\bar{V}_{max}$ and K'_{Mn} as constant, we could calculate K'_{Ca}, $K'_{Ca}\{TTX\}$ and K'_{Mn}, using Eq. 7, after having replaced the dissociation constants in that equation with our apparent dissociation constants, (K'). Note that K'_{Ca} is clearly increased in the presence of TTX (Table I). After determining the constants we computed R from Eq. 9, and this value (shown in Fig. 5 *A* as "expected") was very close to the mean R obtained experimentally.

Another point of interest arising from the analysis of Eq. 9 is that in the presence of lower Ca^{2+} concentrations, Mn^{2+} should produce an R value closer to unity, because the calcium terms in the equation would have less weight. The value of R thus obtained should be exactly predictable from the dissociation constants derived from the high-calcium experiments. We therefore repeated the experiments using lower concentrations of Ca^{2+} ($[Ca]_o$ = 0.3–0.5 mM, $[Mn]_o$ = 0.03–0.05 mM, $[Mg]_0$ = 0). The individual R values from five experiments and the mean R ±1 SD are shown in Fig. 5 *B*, and indeed they are greater (0.93 ± 0.06, mean ±1 SD) than the R values obtained in the high-calcium experiments (cf., Fig. 5 *A* and *B*). The difference in R was significant at the 0.001 level (t test). The expected R value (0.88), was computed using Eq. 9, employing the parameters derived from the high-calcium experiments. The fit allowed no free parameters, yet it was satisfactory.

Two conclusions may be drawn from the experiments with Mn^{2+}. First, the value of K'_{Ca} increases in the presence of TTX. This supports the conclusion arrived at in the previous section. Second, K'_{Mn} does not change to the same extent as does K'_{Ca} on addition of TTX, and probably does not change at all.

The Effect of Magnesium Ions Is Similar to That of Manganese

Similar experiments to those described for Mn^{2+} were conducted with Mg^{2+}. These experiments were technically difficult, because in effective concentrations of Mg^{2+}, the conduction of the nerve impulse tended to fail in terminals treated with TTX. Nevertheless, we successfully carried out three experiments, the results of which are shown in Fig. 6. The finding that R (defined as in the previous section) for Mg^{2+} is smaller than unity again indicates that the major effect of TTX is to increase K'_{Ca}. An increase in K'_{Mg} similar to the increase in K'_{Ca} is excluded, because then R should be greater than unity (Eq. 9).

If one again assumes that $\bar{V}_{max}$ and K'_{Mg} are unaltered by TTX, then one can compute from each experiment the values of K'_{Mg}, K'_{Ca}, $K'_{Ca}\{TTX\}$. These were computed as in the previous

section, and the values obtained are recorded in Table I. It should be pointed out that although the variability in the individual K'_{Ca} and K'_{Ca}[TTX] values was large, K'_{Ca} increased in every experiment, the mean percent of increase being 30.7±6.8% (±1 SD), which is significantly greater than zero ($P < 0.01$, t test). Inserting these computed average values and the actual ion concentrations into Eq. 9, we obtained the expected value of R for each experiment, the mean value of R obtained in this way being shown in Fig. 6. Clearly, it fits closely the experimentally observed average. The experiments with Mg^{2+} again demonstrate that in TTX it is K'_{Ca} that increases, whereas K'_{Mg} probably does not change.

What could explain the finding that K'_{Mg} and K'_{Mn} are unchanged in TTX whereas K'_{Ca} increases? According to our model the increase in K'_{Ca} indicates a decrease in k_v, i.e., the accessibility of Ca^{2+} ions to the intracellular release-site, X (cf., Eqs. 1, 5). The constancy of K'_{Mg} and K'_{Mn} may indicate that the channels subserving these ions are not affected by changes in the presynaptic spike amplitude. However, a simpler explanation could be that these ions inhibit release by competing with Ca^{2+} for an extracellular site, Y, which may be located at the outer surface of the Ca^{2+} channel. Clearly, the access of Mg^{2+} or Mn^{2+} to an extracellular site should not be affected by the transmembrane potential difference. A simple two-site

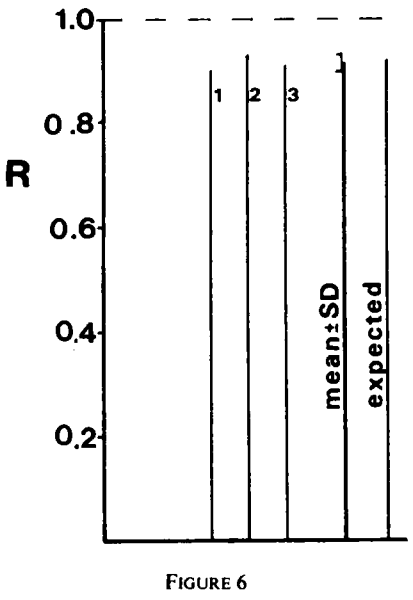

FIGURE 6

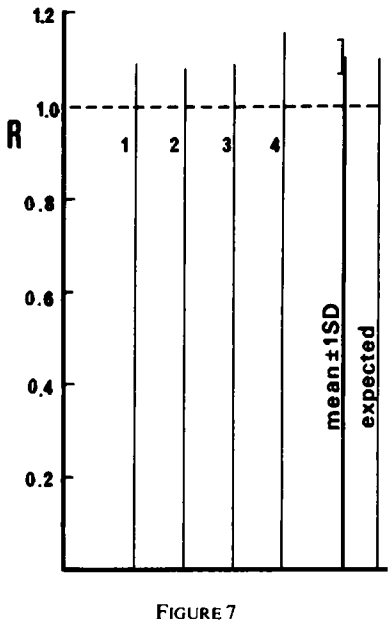

FIGURE 7

FIGURE 6 The enhancement of the blocking effect of Mg^{2+} ions in the presence of TTX. Each bar represents the value of R (Eq. 8) for a single experiment. The mean R ±1 SD and the mean expected R (Eq. 9) are also shown. The expected R values were computed using the constants; K'_{Ca} = 0.5 mM, K'_{Ca}[TTX] = 0.64 mM, K_{Mg} = 4 mM. $\bar{V}_{max}$ K_{Mg} were assumed to be unaltered on addition of TTX. [Mg_o] varied between 1 and 2.5 mM and [Ca_o] varied between 0.7 and 1 mM. dTC was present in all experiments.

FIGURE 7 The effect of TTX on the blockage by strontium ions of transmitter release. The mode of presentation is the same as in Figs. 5 and 6. [Ca_o] was 1.2–1.4 mM, [Sr_o] was 1–2.5 mM, [Mg_o] = 0. dTC was always present. The expected mean R value was computed using the constants K_{Ca} = 0.52 mM, K_{Ca}[TTX] = 0.67 mM, K_{Sr} = 1.5 mM, K_{Sr}[TTX] = 2.09 mM, b = 0.37. Eq. 7 was used with a $b[Sr_o]/K_{Sr}$ term added in the numerator (Meiri and Rahamimoff, 1971).

model can be derived as follows: Assume that extracellular Ca^{2+} ions form a complex, CaY, with the channel site, and that $[Ca]_i = k_v[CaY]$. ($[Ca]_i$ and k_v have the same meaning as in Eq. 1). The intracellular Ca^{2+} ions form the complex CaX and release is proportional to $[CaX]^4$. Mg^{2+} and Mn^{2+} compete with Ca^{2+} only for Y. Under these assumptions, $[Ca]_i$ is given by

$$[Ca]_i = k_v[Y]_t \cdot \frac{[Ca]_o/K_o}{1 + [Ca]_o/K_o + [I]_o/K_I} \tag{10}$$

where $[Y]_t$ is the total concentration of the sites Y, K_o—the CaY dissociation constant, $[I]_o$ is the extracellular concentration of the ion competing with Ca^{2+} for Y, and K_I is the IY dissociation constant. The release is proportional to $[CaX]^4$, and this yields the following equation:

$$\bar{V} = \left(a[X]_t \cdot \frac{[Ca]_o/(K_iK_o/k_v[Y]_t)}{1 + [Ca]_o/(K_iK_o/k_v[Y]_t) + [Ca]_o/K_o + [I]_o/K_I}\right)^4 \tag{11}$$

where a is a voltage-independent coefficient, $[X]_t$—the total concentration of sites, X, and K_i is the CaX dissociation constant. In this equation, the maximum epp amplitude, $\bar{V}_{max}$, will be voltage depedent:

$$\bar{V}_{max} = \left(\frac{a[X]_t}{1 + K/(k_v[Y]_t)}\right)^4. \tag{12}$$

From the finding that $\bar{V}_{max}$ does not change following TTX application (Fig. 3), we may conclude that under our experimental conditions $K_i/(k_v[Y]_t) << 1$. In that case we may neglect the $[Ca]_o/K_o$ term in the denominator of Eq. 11 and obtain an equation identical to Eq. 7, where $\bar{V}_{max} = (a[X]_t)^4$, and $K'_{Ca} = K_iK_o/k_v[Y]_t$.

This equation predicts that as k_v decreases, following a reduction in spike amplitude, K'_{Ca} should increase, whereas K_I should remain unchanged, as can indeed be concluded from the results shown in Figs. 5 and 6.

The Inhibitory Action of Strontium Is Decreased in the Presence of TTX

Another ion of interest is Sr^{2+}. This ion can substitute for Ca^{2+} in the process of transmitter release (Miledi, 1966; Meiri and Rahamimoff, 1971), and in four experiments yielded values of R greater than unity (Fig. 7). The mean R value was 1.107 ± 0.035, (± 1 SD, $n = 4$), and this was significantly >1 (t test, $P < 0.05$).

A R value greater than unity indicates that K'_{Sr} probably increases on the addition of TTX. However, it should be remembered that Sr^{2+} can also bring about transmitter release, a fact which necessitates the inclusion of the term $b[Sr]/K'_{Sr}$ in the numerator of Eq. 7. This term implies that Sr^{2+} ions can induce acetylcholine release, but less effectively than Ca^{2+}, thus $b < 1$ (Meiri and Rahamimoff, 1971). Because of this, the experiments recorded in Fig. 7 do not provide enough information to determine all the unknown parameters. However, if we take K'_{Ca} (control) to be 0.52 mM, (the average of the values obtained from the Mn^{2+} and Mg^{2+} experiments), and K'_{Sr} to be 1.5 mM (from Meiri and Rahamimoff, 1971), and if we assume that $\bar{V}_{max}$ does not change in TTX, then we can compute K'_{Ca}[TTX], K'_{Sr} [TTX], and b for each of the five experiments (Table I). It can be seen that the K'_{Ca} and K'_{Sr} values were 29 ± 9 and

$39 \pm 12\%$ higher, respectively, in the presence of TTX. For both K'_{Ca} and K'_{Sr}, the increase was significant at the 0.01% level (t test). Although K'_{Sr} was chosen somewhat arbitrarily, this choice was not critical, because K'_{Ca} and $K'_{Ca}\{TTX\}$ were calculated from the epp amplitudes obtained in the absence of strontium and were thus independent of the value chosen for K'_{Sr}. Secondly, the percentage increase of K'_{Sr} after the addition of TTX was very similar, whether K'_{Sr} was chosen to be 1, 1.5, or 2 mM.

In view of the ability of Sr^{2+} ions to effect the release of transmitter, it is reasonable to assume that Sr^{2+} ions compete with Ca^{2+} for both the extracellular site Y and the intracellular site X. It would therefore be expected that the effect of TTX addition on K'_{Sr} would be similar to its effect on K'_{Ca}. A quantitative model, similar to that of Eq. 11, can easily be derived for the case of Sr^{2+} , but we have at present not enough information to warrant a detailed exposition of such a model.

DISCUSSION

Our results demonstrate that the effect of divalent cations on evoked transmitter release is modified in the presence of low concentrations of TTX. Thus, on addition of TTX, Mn^{2+} and Mg^{2+} become more effective while Sr^{2+} becomes less effective in inhibiting transmitter release (see the R values, in Table I). Also, Ca^{2+} increases release more in TTX than it does in its absence. The latter assertion can be demonstrated by computing a R value for Ca^{2+} according to the equation:

$$R = \frac{(\bar{V}\{Ca_2,TTX\}/\bar{V}\{Ca_1,TTX\})}{(\bar{V}\{Ca_2\}/\bar{V}\{Ca_1\})} \tag{13}$$

where $\bar{V}\{Ca_2\}$ and $\bar{V}\{Ca_1\}$ are the average epp values in two different calcium concentrations ($Ca_2 > Ca_1$), and the suffix {TTX} denotes the presence of TTX. For the six calcium experiments mentioned in the Results, R was 1.187 ± 0.107 (± 1 SD) and this was significantly greater than unity (t test $P < 0.01$).

The results of the present study could arise in one of two ways: (*a*) the divalent ions, in the presence of TTX, modify the presynaptic spike. (*b*) the release mechanism becomes altered in the presence of TTX. The first explanation rests on the well-known positive correlation between the spike amplitude and transmitter release (Katz and Miledi, 1970). Basing the explanation of the results of our study on the first possibility, one would have to assume that, in the presence of TTX, the spike amplitude is further reduced by Mg^{2+} and Mn^{2+} but is somewhat increased by Ca^{2+} and Sr^{2+}. However, this assumption is unreasonable in view of the similarity of action of all divalent cations on the sodium channels subserving the action potential in frog nerve (Hille et al., 1975). We therefore reject this explanation of our results and base our interpretation on the second possibility.

If we analyze the data in terms of a model in which Ca^{2+} ions combine with a site X in order to release transmitter, the major conclusion is that K'_{Ca}, the apparent Ca^{2+} dissociation constant from X, is increased in the presence of TTX (Table I). Three interpretations are possible. First, TTX itself is a competitive inhibitor of Ca^{2+} at the site X. This possibility can be excluded, because even at very high TTX concentrations, up to 10^{-6} g/ml, direct depolarization of the terminals evokes the same transmitter release as before TTX application (Katz and Miledi, 1967). Second, TTX may lower the affinity of the site X for Ca^{2+}, directly

or through the reduction of the spike amplitude. This, too, seems to us unlikely, because in this case the affinity of X for Mn^{2+} and Mg^{2+} would probably also be affected.

A third explanation, which appears likely to us, is that in the presence of TTX, the access of Ca^{2+} to an intracellular release site is reduced. As shown earlier, this could indeed increase K'_{Ca} (see Eq. 5). Sr^{2+} ions probably have the same action as Ca^{2+} and therefore the increase of K'_{Sr} in TTX is not surprising. Thus, the central concept of our study is that of an intracellular release site for which Ca^{2+} and Sr^{2+}, but probably not Mn^{2+} and Mg^{2+}, will normally compete. This concept is in line with earlier suggestions of Cooke et al. (1973) and Van der Kloot and Kita (1973).

In contrast to K'_{Ca} and K'_{Sr}, K'_{Mg} and K'_{Mn} fail to increase upon TTX application. This indicates that these ions prevent the influx of Ca^{2+} ions into the nerve terminals, as has already been demonstrated by Katz and Miledi (1969). This may occur by neutralization of negative charged, fixed at the outer surface of the membrane (Muller and Finkelstein, 1974; Landau and Nachshen, 1975; Misler and Hurlbut, 1980). This in turn would cause a reduction of $[Ca]_o$ near the surface of the membrane, in a manner independent of the spike amplitude. The quantitative effect of such screening is equal to that of introducing the term $[I]_o/K_I$ in Eq. 7 (Landau and Nachshen, 1975). Surface charge theory also allows for the neutralization of surface charges by binding rather than by screening (Landau and Nachshen, 1975; Misler and Hurlbut, 1980). A second explanation for the extracellular action of Mg^{2+} and Mn^{2+} would be that these ions compete with Ca^{2+} for a specific binding site, perhaps at the entrance of the presynaptic calcium channel (Llinás, et al., 1976). Although we employed the idea of the specific site for our model, the surface charge explanation is equally effective, and cannot be excluded at present.

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