Nature of Increase in Quantal Release by the Thallous Ion at Frog End Plates with and without Nerve Stimulation

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ABSTRACT The monovalent thallous ion (Tl) was evaluated at the frog end plate in vitro with intracellular microelectrodes. Recordings included end plate potentials (EPPs), and miniature end plate potentials (MEPPs). Replacement of extracellular potassium (K) by 2.5 mM Tl (a) caused increases in MEPP and EPP amplitudes, MEPP frequency, and quantal content, and (b) caused complete recovery of the EPP facilitation index at BAPTA-loaded nerve terminals. Tl's effects were reversible and concentration dependent, and persisted for > 3 h. The increase in MEPP frequency and its rate of decline due to Tl washout were more pronounced at 0 calcium (Ca)-2 mM EGTA than at 0.3 mM EGTA, suggesting that Tl's effects were not due to elevation of internal Ca. Unlike heavy metal ions reportedly capable of substituting for Ca, 0.2 mM Tl did not block, but further enhanced, elevated MEPP frequencies, occurring after nerve stimulation or in high K, to greater levels with barium (Ba) than with Ca. 200 nM ω-conotoxin (ω-CTX) blocked Tl's effect, indicating that Tl primarily entered the nerve terminal via Ca channels. A 50% reduction in sodium (Na) did not modify Tl's effect, although removal of K in the presence of 20 μM ouabain and 2.5 mM Tl caused an exaggerated increase in MEPP frequency, which decreased with a 50% reduction in Na. Based on the analysis, Tl neither substituted for Ca nor elevated internal Ca and Na, nor were its effects antagonized by ouabain; Tl increased quantal secretion, possibly by a fusogenic mechanism, after its entry into the nerve terminal.

INTRODUCTION

The monovalent thallous ion (Tl) increases miniature end plate potential (MEPP) frequency at end plates of mouse (Wiegand, Papadopoulos, Csicsaky, and Kramer, 1984). This in vitro effect appears consistent with neurological signs of Tl toxicity, such as paresthesia, convulsions, and perhaps centrally mediated respiratory paralysis. Though it was reported (Wiegand, Lohmann, and Chandra, 1986) that quantal content is inhibited at mouse end plates after prolonged exposure, > 2 h, to 0.5 mM Tl, tissue hypoxia was not ruled out. The reason for this is that a Tl-free control was
not done for a similar period of 2 h or more, and it is known (Czech and Somjen, 1990) that hypoxia is associated with a decrease in transmitter release. But some heavy metal ions, which induce quantal secretion under certain conditions, e.g., lead (Pb), zinc (Zn), and cadmium (Cd) (see Wang and Quastel, 1991), also block quantal secretion. Thus, it was important to determine whether or not TI fit into this group. In addition to chemical similarities between TI and K (Cotton and Wilkinson, 1980), there are also similarities in biological actions. For instance, both ions activate uptake at the Na-K pump (Amdur, Doull, and Klaassen, 1991), though there is a report that TI may inhibit the Na-K ATPase (Ellenhorn and Barceloux, 1988). TI is more permeable than K at Na, K, and end plate channels (Hille, 1992). Thus, it was also important to determine whether TI was a more effective substitute for K in the process of quantal secretion.

METHODS

Intracellular microelectrode recordings were made at the end plate of the sciatic nerve–sartorius muscle in vitro from 2-in. Northern grass frogs (Rana pipiens pipiens). Glass microelectrodes filled with 3 M KCl and with tip resistances of 4–10 MΩ were used. After dissection, the muscle was submerged in saline. After fixation in a 1.5-ml Lucite chamber, the preparation was continuously superfused with saline at 5 ml/min by using a tubing pump (Orion Research Inc., Cambridge, MA). Each experiment was performed at a single end plate of a different sartorius. Records of MEPPs and end plate potentials (EPPs) were obtained with a kymograph camera (Grass Instrument Co., Quincy, MA) and an oscilloscope (World Precision Instruments, Sarasota, FL); membrane potential was recorded in millivolts with a digital meter. Experiments were done at room temperature (20°C). MEPP frequency (in seconds⁻¹) was obtained by standard procedure.

To obtain EPPs, the nerve was stimulated with supramaximal voltages at a duration of 0.05 ms by using a square wave generator (Grass Instrument Co.). At each frequency, 600 shocks were used. EPPs and MEPPs that were obtained at a single end plate were normalized relative to a standard potential of −90 mV to yield corrected EPPs and MEPPs (Katz and Thesleff, 1957). A reversal potential of −15 mV was used. For nonlinear summation, the EPP' was multiplied by a correction factor, (RMP-15)/(RMP-15-EPP'), to yield EPP'' (Martin, 1955). The factor was on average <1.03. The direct method was used to estimate quantal content (m): mean EPP''/mean MEPP'.

For experiments involving frequency-dependent changes in m, 600 nerve shocks were used at each frequency of nerve stimulation from 1 to 8 Hz. The last 100 resultant EPPs at each nerve stimulation frequency were recorded, in addition to MEPPs, and m was obtained with the direct method (above). For EPP facilitation experiments, pulse trains were used at a train rate of 0.1 s⁻¹. Each pulse train was comprised of 20 nerve shocks (pulses) at 40 Hz. The EPP facilitation index (I) was defined as a ratio: the average of the 18th and 19th EPP amplitudes in each pulse train was summed over 19 or 20 pulse trains, and the result was divided by the first EPP amplitude in each pulse train summed over the same pulse trains.

To pretreat with ω-conotoxin (ω-CTX), the preparation was equilibrated for 2 h in 0 Ca, 200 nM ω-CTX-containing saline (Table 1) at pH 7.4. The preparation was then superfused with ω-CTX-free initial control and TI salines (at 0.7 mM Ca), and recordings were made.

A standard procedure for loading BAPTA, a Ca-chelating agent, into the nerve terminal (Kijima and Tanabe, 1988; Tanabe and Kijima, 1989) was used with the following modifications. The sartorius was equilibrated for 3 h with vigorous shaking every 15 min at 30°C in 3 ml of 0 Ca-0.3 mM EGTA saline at pH 7.4, to which was added 0.5 μmol of BAPTA-AM in 15 μl dimethyl sulfoxide (DMSO). The range of dry weights of sartorii was 8.6–18.2 mg. The control, in the contralateral muscle, was equilibrated in 0.5% DMSO + 0 Ca-0.3 mM EGTA,
BAPTA-free saline. Muscles were then returned to BAPTA-free, DMSO-free salines, and the effects of test Tl and K were evaluated on the EPP facilitation index.

To study interactions, if any, between TIAc, BAPTA (unesterified), and Ca, a Ca-selective electrode (TIPCA; World Precision Instruments, Sarasota, FL) was used. Its response was 30 mV per decade change in Ca concentration in the range 10^{-6}-10^{-1} M. For comparison with TIAc salines, NaAc salines were also used. The electrode’s selectivity coefficient for Na was 10^{-5.5}. A 3 M KCl-filled reference electrode was used, and millivoltage was measured with a pH/ion analyzer (Orion Research Inc., Cambridge, MA). The same meter was used to measure pH.

BAPTA-AM was obtained from Calbiochem Corp. (La Jolla, CA); dextrose and KCl were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other drugs and chemicals (including unesterified BAPTA) were obtained from Sigma Chemical Co. (St Louis, MO). EGTA and BAPTA-AM refer to ethylene-glycol-bis-(β-amino-ethyl)ether-N,N',N'-tetraacetate, and the acetoxymethyl ester of 1,2-bis-(2-amino-phenoxy)ethane-N,N',N'-tetraacetate, respectively. Osmolality was obtained with an osmometer (Advanced Instruments, Inc., Needham Heights, MA). The critical level of significance (P) was 0.05. Paired and unpaired t tests were used to evaluate data. MEPP frequency has a log normal distribution when external Ca is low (Gage and Quastel, 1966); thus, the geometric mean (+SE, -SE) of MEPP frequency (in seconds^{-1}) was obtained. For +SE close to -SE, it was mean ± SE; SE refers to standard error. A semilogarithmic plot was used for log quantal content (or EPP amplitude) versus nerve stimulation frequency (Maeno and Edwards, 1969). Some data were presented as a percentage of control.

**RESULTS**

**Effects of Tl on MEPP Frequency Before and After Nerve Stimulation in Ca Salines**

The initial control and test superfusing salines contained 0.7 mM Ca (Table I). Substitution of 2.5 mM Tl for K caused an increase in MEPP frequency before, 

| Saline* | NaCl | NalSe | KCl | TIAc | CaCl₂ | BaCl₂ | MgCl₂ | EGTA | Dextrose |
|---------|------|-------|-----|------|-------|-------|-------|------|---------|
| Initial control | 95 (114) | — | 2.5 | 0.7 (1) (0.4) | 10 (4) | — | — | — | — |
| Tl substitution | 97 (58) | (59) | (59) | 2.5 (5) | 0.7 | — | 10 | — | — |
| High K | — | 88 | 10 | 0.7 (0.8) | — | 10 | — | — | — |
| Isotonic control | 48 | 50 | 2.5 | — | 0.7 | — | 10 | — | — |
| 0 Ca control | 95 | — | 2.5 | — | — | — | 10 | 0.3 (2) | — |
| Low Na control | 46 | — | 2.5 | — | 0.7 | — | 10 | — | 80 |
| Ba ± Tl | 114 | — | 2.5 | ±0.2 | — | — | 0.4 | 4 | — |
| TTX control | 114 | — | 2.5 | — | 0.4 | — | 2 | — | — |
| Ba, high K, TTX | 96 | 15 | 2.5 | ±0.2 | — | — | 0.4 | 2 | — |
| Pretreatment | 110 | — | 2.5 | — | — | — | 1 | (0.3) | — |

*Parentheses show additional salines; i.e., top row shows saline with 0.4 mM Ca, 4 mM Mg, and 114 mM Na. Isotonic refers to isethionate; tetrodotoxin (TTX) was used at 0.2 μM. Other salines are indicated in the text. Adjustments were made in the NalSe/NaCl ratio to keep KCl product or related ion products constant. Desired pH and constant ionic strengths of salines were achieved by varying the Na₂HPO₄/NaH₂PO₄ ratio and amount of Na phosphate buffer, respectively. Pretreatment salines were only used to incubate muscles; i.e., ω-CTX saline and saline containing 0.3 mM EGTA + 0.5% DMSO ± BAPTA-AM. Osmolality of the TTX control was 221 mosmol/kg. Osmolalities of other salines were similar.
during, and immediately after nerve stimulation (Fig. 1). Unlike Tl, 10 mM K was less effective and did not significantly modify the poststimulus MEPP frequency. Mean MEPP frequency (+SE, -SE) of the control before nerve stimulation was 0.8 s⁻¹ (0.2, 0.1; average of 12 experiments). At 15 min of exposure to 2.5 mM Tl (0 K), and specifically before nerve stimulation, there was a threefold increase in MEPP frequency above the control. The initial rate of decay of MEPP frequency, at 0–2 min after stimulus, was unaffected by Tl. However, MEPP frequency was elevated above the control in the presence of Tl.

The increase in MEPP frequency that normally occurs with nerve stimulation was enhanced by Tl. This latter effect depended on the frequency of nerve stimulation and number of nerve shocks. For instance, in the first minute after 1,200 shocks, 2.5 mM Tl caused a 39-fold increase in MEPP frequency above the control; a threefold increase occurred in the first minute after stimulus without Tl. With 600 shocks at 8 Hz only, the increase was 21-fold; a twofold increase occurred without Tl (Fig. 2).

**Concentration-dependent Effects of Tl in Ca Salines**

Concentrations of Tl substituted for K were (mM): 1.5, 2.5, and 3.5. These Tl salines contained concentrations of NaIse similar to the Ise control after adjustments for the ion product (Table I legend). Means ± SE of the initial control (0.7 mM Ca) for MEPP frequency, MEPP amplitude, EPP amplitude, and quantal content were 0.6 s⁻¹ (0.2, 0.1), 0.3 mV ± 0.02, 0.7 mV ± 0.2, and 3.0 ± 1.1, respectively (averages of 12 experiments each). Similar values were obtained with the Ise control. First, Tl caused significant concentration-dependent increases in MEPP frequency, EPP amplitude, and quantal content (Table II). Second, Tl-induced increases in quantal content were readily reversed upon washout of the cation. Specifically, there was an exponential decline in quantal content with Tl washout (Fig. 3), which was consistent with a first-order process. Assuming that the rate of return of quantal content to control

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**Figure 1.** The ordinate shows effects of 2.5 mM Tl on MEPP frequency (F') before and at 0–12 min (abscissa) after nerve stimulation. 4-Hz stimulation (600 shocks) began at the left arrow, and 8-Hz stimulation (600 shocks) began at the second arrow. Three individual experiments are shown (circles, squares, and triangles). F' during control (filled symbols) and 2.5 mM Tl (open symbols) are shown; open symbols at −4 min represent 15 min of exposure to Tl.
FIGURE 2. MEPP frequency ($F'$) in the first minute after stimulus (ordinate) versus nerve stimulation frequency (abscissa) is shown. $F'$ was normalized to the prestimulation control ($F_0$). Each point is a mean ± SE (three experiments). Effects before TI (closed circles) and with 2.5 mM TI (open circles) are shown. Frequency-dependent enhancement of $F'$ was induced by TI.

equals the rate of removal of TI from the nerve terminal, the half-time of removal of TI was estimated at 16.7 min.

Much smaller effects were observed with TI on MEPP amplitude and the resting potential of the sartorius endplate. The mean ± SE of the resting potential of the

| Concentration of TI (mM) | N | EPP amplitude (mV ± SE) | MEPP amplitude (mV ± SE) | Mean effect + SE* | MEPP frequency |
|-------------------------|---|------------------------|-------------------------|------------------|----------------|
|                         |   |                        |                         |                  | Before | After |
| 0                       | 12| 0.7 ± 0.2              | 0.3 ± 0.02              | 3.2 ± 1.3        | 0.7 ± 0.2 | --- |
|                         |   |                        |                         |                  | (0.3, 0.2) |      |
| 1.5                     | 4 | 1.6 ± 0.2              | 1.00 ± 0.02             | 1.6 ± 0.2        | 2.5 ± 0.4 | 9.6 ± 0.4 |
|                         |   |                        |                         |                  | (0.4, 0.3) | (2.0, 1.4) |
| 2.5                     | 4 | 1.7 ± 0.2              | 1.00 ± 0.01             | 1.7 ± 0.2        | 3.5 ± 0.4 | 14.8 ± 0.4 |
|                         |   |                        |                         |                  | (0.5, 0.3) | (5.4, 2.3) |
| 3.5                     | 4 | 2.1 ± 0.2              | 1.05 ± 0.01             | 1.9 ± 0.2        | 7.7 ± 0.4 | 28.4 ± 0.4 |
|                         |   |                        |                         |                  | (1.6, 1.1) | (6.3, 4.4) |

*Absolute values of the test control ± SE are shown (first row). TI's effects were obtained as a ratio relative to control, and mean ratios are shown. Mean MEPP frequency (+SE, −SE) is shown before nerve stimulation (before) and in the first minute after 4-Hz stimulation (600 shocks). Effects were recorded at 15–18 min of exposure to TI. All values except MEPP amplitude at 1.5 and 2.5 mM TI and endplate depolarization at 1.5 mM were significantly different from control. Endplate depolarizations (in millivolts) ± SE were 1.4 ± 2.8, 4.0 ± 0.8, and 10.2 ± 0.6 at 1.5, 2.5, and 3.5 mM TI, respectively. $N$ is the number of experiments.
sartorius endplate, obtained with the initial control, was \(-83.3 \text{ mV} \pm 0.7\) (average of 76 experiments). The resting potential for the Ise control was slightly lower at the same sartorius endplate: the mean difference was \(3.6 \text{ mV} \pm 1.6\ SE\) (average of 11 values). Since the resting potential for the Ise control, in each experiment, was recorded 23 min after that obtained with the initial control, the small potential difference was consistent with minor deterioration of the electrode-penetrated sartorius over the time course of the experiments. Thus, it is unlikely that endplate depolarizations caused by 2.5 mM Tl (0 K) contributed significantly to effects observed at this concentration of cation.

![Figure 3](image)

**FIGURE 3.** The ordinate shows quantal content (m) as a function of time (abscissa) before, during, and after Tl in a representative experiment (4 Hz used). Left of first arrow is the Ise control (see text); beginning at the first arrow is 2.5 mM Tl; beginning at the second arrow is the return to the Tl-free control.

Use of Test EGTA Salines without Added Ca

A possible relationship between Ca and the presynaptic effects of Tl was evaluated. The initial control (at 0.7 mM Ca) preceded the use of 0 Ca-EGTA test salines. Test salines contained 2.5 mM Tl and either 0.3 mM EGTA or 2 mM EGTA. Cycles of repetitive nerve stimulation at 4 Hz (600 shocks), followed without interruption by 8 Hz (600) for a total of 1,200 nerve shocks, were used. A 6-min rest interval (no stimulation) followed, during which MEPPs were recorded, before repeating the cycle. MEPP frequency recovered to baseline levels at the time of each recording. The EPP occurred with each nerve shock except during trials with the EGTA containing test saline.
Despite the absence of EPPs, TI caused a dramatic increase in MEPP frequency (Fig. 4, A and B). It could not be attributed to EGTA, as it was not observed with TI-free, 2.5 mM K, 0 Ca-EGTA salines. The TI-induced rise in MEPP frequency was greatest at nerve-stimulated preparations superfused with 0 Ca-2 mM EGTA (Fig. 4 C), indicating that TI's effect was not due to elevations of unbuffered internal Ca. In the absence of nerve stimulation, the increase in MEPP frequency by TI was much smaller, indicative of presynaptic entry of TI with each nerve impulse.

Mean initial control MEPP frequencies (seconds⁻¹ ± SE, before exposure to test salines, were 0.4 ± 0.1 (average of 11 experiments) and 0.5 ± 0.1 (15 experiments) for stimulated and unstimulated preparations, respectively. There was no difference. However, the rate of return of MEPP frequency to control in the TI washout period was dependent on nerve stimulation. The poststimulation decay of MEPP frequency in Fig. 1 was unrelated to the exponential rate of decline in Fig. 4, A and B. In the former, TI was present throughout and sampling of MEPPs began immediately after stimulus, whereas in the latter, TI was washed out by readmission of the control superfusing saline and sampling of MEPPs began at ~ 4 min after nerve stimulation. There was an exponential decline in MEPP frequency with TI washout, which was also consistent with a first-order removal process. The slope of a plot of loge MEPP frequency versus time at 30-60 min of the washout period (in Fig. 4, A-C) was calculated and the elimination half-time (t₁/₂) was determined. Mean t₁/₂'s (in minutes) ± SE were 4.2 ± 0.1 (average of three experiments), 7.6 ± 0.1 (three experiments), and 35.6 ± 10.9 (six experiments) with respect to TI in 0 Ca-2 mM EGTA (nerve-stimulated), 0 Ca-0.3 mM EGTA (nerve-stimulated), and 0 Ca-0.3 mM EGTA (unstimulated), respectively. Thus, exit of TI from the nerve terminal depended on nerve stimulation, and was greater when external Ca was lowered.

Use of Ouabain or Low Na, and Effect of TI

Possible roles of the Na-K ATPase or of intraterminal Na in the mechanism of action of TI were evaluated. First, 12-13 recordings of MEPP frequency were made at each endplate at 5-min intervals in the absence of nerve stimulation. Recordings were obtained with the initial control (0.7 mM Ca), and subsequently with and without 20 μM ouabain. Ouabain did not increase MEPP frequency above the control. This result was consistent with the literature (Nishimura, 1986), and different from the effect of ouabain at higher concentrations, in which there is a dramatic increase in MEPP frequency (Haimann, Torri-Tarelli, Fesce, and Ceccarelli, 1985). Consequently, test 0 Ca-0.3 mM EGTA salines with 2.5 mM TI substituted for K were evaluated with and without 20 μM ouabain. In ouabain-free experiments, MEPP frequency decreased after TI washout. In contrast, MEPP frequency with 20 μM ouabain continued to increase even after return to TI-free saline (Fig. 5): in one of five cells, MEPP frequency was too high to be counted manually. MEPP frequency was exaggerated with the combination of 20 μM ouabain + 2.5 mM TI (0 K).

To further evaluate the relationship between ouabain and TI, a nerve stimulation protocol similar to Fig. 4 A was used. The mean initial control MEPP frequency, obtained with 0.7 mM Ca, was 0.4 s⁻¹ (0.2, 0.1; four experiments). At 20 min of exposure to 20 μM ouabain it was 1.0 s⁻¹ (0.8, 0.3). At 20 min of exposure to 2.5 mM TI (0 K), 0 Ca-0.3 mM EGTA (with ouabain), MEPP frequency increased to 150, 82,
FIGURE 4. The ordinate shows MEPP frequency (F') versus time (abscissa) during control saline at -20-0 min and again at 30-60 min. (A) Short bars represent periods of nerve stimulation: 600 shocks each at 4 and 8 Hz. The preparation was rested for 6 min and F' determined. In three experiments, 0 Ca, 0.3 mM EGTA saline + 2.5 mM Ti (filled symbols) was used at 0-30 min (between arrows). In one experiment (open symbols) Ti-free 2.5 mM K EGTA saline was used. (B) 0 Ca, 2 mM EGTA saline + 2.5 mM Ti was used at 0-30 min. The increase in F' by Ti was larger at 2 mM EGTA. (C) MEPP frequency ± SE (error bars) of data in A (circles) and B (diamonds) was normalized to the control at 0 min and compared with those at an unstimulated preparation (triangles, average of six experiments). 0 Ca, 0.3 mM EGTA + 2.5 mM Ti was used at 0-30 min. Ti's effect and its washout were linearly dependent on nerve stimulation and inversely related to the concentration of external Ca.
and 472 times control levels in three experiments (c.f. data in Fig. 4 A). With return to TI-free, ouabain- and Ca-containing saline, MEPP frequency continued to increase for at least 20 min in three of four experiments before subsiding slowly. In some instances EPPs did not recover with return to Ca-containing saline in spite of very high MEPP discharge rates. In other instances, EPPs recovered with dramatic increases in height, and with little change in MEPP height, indicative of a presynaptic increase. In one of these experiments, ouabain-free saline containing 50% reduction in Na (dextrose replacement) was superfused. There was a marked decrease in MEPP frequency (Fig. 6 A), implying that the exaggerated MEPP frequency probably occurred in response to impairment of the Na-K ATPase with elevated intraterminal Na. TI was also evaluated in ouabain-free experiments with 50% reduction in Na;

![Image](image)

**FIGURE 5.** MEPP frequency (ordinate) versus time (abscissa) is shown. In the presence of 20 μM ouabain (open squares), MEPP frequency (F') was normalized to the initial control (F₀, not shown) before exposure to ouabain. In ouabain-free experiments (filled squares) F₀ is at 0 min. 0 Ca, 0.3 mM EGTA salines + 2.5 mM Tl were used at 0–25 min (between arrows). Vertical bars show SE, and each point is a mean of four to five experiments. The effect of ouabain + substitution of Tl for K was not easily reversed.

osmolality was maintained with an osmotic equivalent of dextrose (Table I). The initial mean control MEPP frequency was 0.5 s⁻¹ (0.4, 0.2; three experiments). There were two observations. First, MEPP frequency was significantly reduced with the Na reduction whether or not 1 μM neostigmine was present. After Na reduction in most experiments neostigmine was not required because MEPPs were still much greater than, and distinct from, baseline noise. Second, when 2.5 mM Tl was substituted for K at 0 Ca-0.3 mM EGTA, MEPP frequency increased (Fig. 6 B) to levels similar to that in Fig. 4 A. Thus, in the absence of ouabain, Tl's presynaptic stimulation could not be attributed to either elevated internal Na or interference with the Na-K ATPase. Since Tl's effects were not blocked by ouabain, it is unlikely that significant presynaptic entry of Tl occurred via the Na-K pump.
ω-CTX Pretreatment and Effect of Tl

Since Tl's presynaptic stimulation was masked by external Ca (Fig. 4, A–C), ω-CTX was used to further evaluate the nature of the presynaptic entry of Tl. After incubation of sartorii with 200 nM ω-CTX, tissues were removed and continuously superfused with ω-CTX-free Ca-containing salines: first with the initial control (at 0.7 mM Ca), then with 2.5 mM Tl (0 K), followed by return to control (Tl washout). The nerve was stimulated with the same protocol as in Fig. 4, A and B. No EPPs occurred at nerve-stimulated preparations pretreated with ω-CTX, implying that Ca entry was effectively blocked. The mean control MEPP frequency (s⁻¹) ± SE, after pretreatment

\[ F' \text{(s}^{-1}) \]

versus time (abscissa) is shown. Short bars represent nerve stimulation as in Fig. 4, A and B. Left arrow indicates exposure to 2.5 mM Tl (0 K) with 0 Ca, 0.3 mM EGTA; Tl washout began at right arrow. (A) An individual experiment is shown. The ouabain-free initial control F' was 0.2 s⁻¹. 20 μM ouabain was present at −10–40 min (filled circles). Tl superfusion began at 0 min. Compare the increase in F' by Tl to Fig. 4 A. F' did not subside with Tl washout after 20 min. Superfusion with ouabain-free, low Ca, low Na saline (see text) began at 40 min (open circles). (B) MEPP frequency (F') was normalized to the initial control (F₀). Each point is an average of three experiments; vertical bars show SE. F₀ was 0.5 s⁻¹ (0.4, 0.2). With dextrose substituted for 50% NaCl (filled circles), F' was one-third F₀. Relative to F' at 0 min, Tl caused an 18-fold increase in F' at 30 min; cf. Fig. 4 A.
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with ω-CTX, was 0.6 ± 0.1 (average of three experiments). There was no significant difference from controls in untreated, ω-CTX-free preparations. However, there was a 38-fold increase in MEPP frequency at 20 min of exposure to Tl in untreated, ω-CTX-free experiments. In contrast, there was a significantly smaller increase, only fourfold, with Tl in ω-CTX-pretreated experiments (Fig. 7). The estimated elimination half-times (in minutes) for Tl removal were 5.4 ± 0.8 and 33.7 ± 18.7 at untreated and CTX-treated preparations, respectively. Consequently, the stimulation dependence of Tl's presynaptic effect as well as its washout primarily resulted from an ω-CTX-sensitive process. Tl not only entered but apparently also exited the nerve terminal via voltage-gated Ca channels.

Substitution of Ba for Ca, and Effect of Tl

With Ba, the elevated MEPP frequency with high K is inhibited (more than for Ca) by heavy metal ions capable of substituting for Ca (Wang and Quastel, 1991). To determine if Tl fits into this group of ions, 15 mM K (high K) ± Ba substitution was used at TTX-treated endplates (Table I). MEPP frequency rose from a mean TTX control value of 0.1 s⁻¹ ± 0.1 to a value of 50.2 s⁻¹ (15.7, 10.2) with Ba at high K (average of three experiments). To achieve similar rises in MEPP frequency with Ca at high K, 1 mM Ca and 1 mM Mg were used. MEPP frequency rose from a mean TTX control value of 0.3 s⁻¹ (0.4, 0.1) to a value of 23.1 s⁻¹ (38.2, 9.9) with 1 mM Ca at high K (three experiments). With 0.2 mM Tl, MEPP frequency rose further with either Ca or Ba substitution (Fig. 8); there was no inhibition. Tl caused significant increases in MEPP frequency to 1.6 ± 0.1 and 1.3 ± 0.1 times levels achieved at high K with Ba and Ca, respectively (average of three experiments each). Unlike the heavy metals (alluded to above), Tl probably did not substitute for Ca.

FIGURE 7. The ordinate for both panels shows MEPP frequency (F') during exposure to control saline at -10--0 min (abscissa), and again at 20--40 min. At 0--20 min (between arrows) the test saline contained 2.5 mM Tl. Short bars represent periods of nerve stimulation as in Fig. 4, A and B. F' in three untreated, ω-CTX-free experiments is shown at left (filled symbols). Another three experiments pretreated with 200 nM ω-CTX (open symbols) are shown at right. ω-CTX blocked the Tl-induced increase in F'.

With Ba, the elevated MEPP frequency with high K is inhibited (more than for Ca) by heavy metal ions capable of substituting for Ca (Wang and Quastel, 1991). To determine if Tl fits into this group of ions, 15 mM K (high K) ± Ba substitution was used at TTX-treated endplates (Table I). MEPP frequency rose from a mean TTX control value of 0.1 s⁻¹ ± 0.1 to a value of 50.2 s⁻¹ (15.7, 10.2) with Ba at high K (average of three experiments). To achieve similar rises in MEPP frequency with Ca at high K, 1 mM Ca and 1 mM Mg were used. MEPP frequency rose from a mean TTX control value of 0.3 s⁻¹ (0.4, 0.1) to a value of 23.1 s⁻¹ (38.2, 9.9) with 1 mM Ca at high K (three experiments). With 0.2 mM Tl, MEPP frequency rose further with either Ca or Ba substitution (Fig. 8); there was no inhibition. Tl caused significant increases in MEPP frequency to 1.6 ± 0.1 and 1.3 ± 0.1 times levels achieved at high K with Ba and Ca, respectively (average of three experiments each). Unlike the heavy metals (alluded to above), Tl probably did not substitute for Ca.
As an alternative to high K for causing nerve terminal depolarizations, the effect of Ba (0 Ca) ± 0.2 mM Tl was also evaluated in nerve-stimulated experiments. An initial prestimulation control MEPP frequency was obtained at 0.4 mM Ca. With 0.4 mM Ba (0 Ca) salines, 2–4 mM Mg was used to prevent muscle contraction. Nerve stimulation was initiated in the presence of Ba at 4 Hz (600 shocks), followed without interruption by 8 Hz (600 shocks). Stimulation cycles were repeated after 10-min rest intervals (no stimulation). For comparison, nerve stimulation was also used with the Ba-free, 0.4 mM Ca control saline. 2 min before the start of the second stimulation cycle, Tl was included in the superfusing saline. When 2.5 mM Tl was substituted for K, the discharge of MEPP quanta was so high that MEPPs could not be discriminated from each other. In another experiment, the control MEPP frequency was 1.1 s⁻¹. The superfusing saline was then changed to 0.4 mM Ba (0 Ca), 2 mM Mg; 2 min later nerve stimulation was initiated. At 4 s and 4 min after stimulus, MEPP frequencies were 17.9 and 1.4 s⁻¹, respectively. The 4-min poststimulus MEPP frequency with Ba was similar to the prestimulus Ca control. 2 min before the start of the second cycle of nerve stimulation, 0.2 mM Tl was included in the superfusing 0.4 mM Ba, 2.5 mM K saline; MEPP frequencies rose further to 188.3 and 28.0 s⁻¹ at 4 s and 4 min after nerve stimulation, respectively. Similar changes occurred with 4 mM Mg-containing

![Figure 8. MEPP frequency (F') (ordinate) versus time (abscissa) is shown in three experiments with TTX. The TTX control (open symbols) is shown. At left arrow, K was increased to 15 mM with 0.4 mM Ba (0 Ca), 2 mM Mg (filled circles, solid lines, one experiment), or with 1 mM Ca, 1 mM Mg (filled triangles, broken lines, two experiments). At the middle arrow, 0.2 mM Tl was included; at right arrow, Tl-free saline was superfused. Tl did not block, but invariably enhanced, F' at high K with Ba, more than with Ca.](image-url)
salines (Table III). At 4 s poststimulus, Ba (not Ca) caused the greater MEPP frequency. In parallel, TI’s presynaptic stimulation was greater in the presence of Ba. TI blocked neither Ba- nor Ca-dependent quantal secretion. No EPPs were present with Ba, and despite a decrease in the resting potential, MEPPs were clearly visible above the baseline noise.

**Frequency-dependent Changes in Quantal Content by TI and K**

Effects of TI and K were evaluated beginning 15–20 min after inflow with salines containing these cations. 10 mM K depolarized a representative sartorius endplate by 20 mV. There was a corresponding decrease in the uncorrected MEPP amplitude.

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**Table III**

| Mean prestimulus control MEPP frequency | Cycle 1 | Cycle 2 |
|----------------------------------------|---------|---------|
| Mean poststimulus MEPP frequency | s⁻¹ | s⁻¹ | Ca | Ba | TI | Cycle1 |
| **N** | | | mM | mM | mM | |
| 1.2 | 3 | 6.1 | 7.0 | 0.9 | 0.4 | 1.0 ± 0.1 |
| (0.9,0.4) | (26.1,3.1) | (0.7,0.3) | (32.7,3.6) | (0.8,0.3) |
| 0.4 | 3 | 3.7 | 20.7 | 2.1 | 0.4 | 0.2 | 4.9 ± 1.1 |
| (0.1,0.1) | (7.5,1.6) | (0.1,0.1) | (37.5,9.1) | (1.1,0.6) |
| 1.7 | 3 | 4.5 | 6.6 | 3.1 | 0.4 | 0.2 | 1.6 ± 0.1 |
| (1.5,0.6) | (5.4,1.8) | (2.1,0.7) | (6.7,2.4) | (3.0,1.1) |

*N* is the number of experiments; parentheses show (+SE, −SE). The prestimulus control was obtained with 0.4 mM Ca (0 Ba), 4 mM Mg. Each cycle represents 4 s and 4 min after nerve stimulation (see text); 0.4 mM Ba (0 Ca) was present for both cycles (first two rows); 0.2 mM TI was present during cycle 2 only (last two rows); 0.4 mM Ca was present for both cycles (last row only). In each the ratio cycle2/cycle1 (TIs effect) was obtained; mean value ± SE is shown (right). Ba caused the greater increase in MEPP frequency (i.e., a mean sevenfold change relative to control); with Ca, it was only threefold. In parallel, TI caused significant mean fivefold and twofold increases in MEPP frequency (i.e., cycle2/cycle1) with Ba substitution and with Ca, respectively.

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from 0.36 mV (the 0.7 mM Ca initial control) to 0.26 mV (with 10 mM K). These were the measured values before the correction was made for membrane potential differences (see Methods). At the same endplate, the uncorrected EPP amplitude went from 1.0 mV (control) to 1.6 mV (10 mM K). Thus, K caused an unambiguous increase in quantal content. Frequency-dependent effects of 10 mM K on quantal content are shown (Fig. 9A). In each recording period, stimulus frequency was increased in increments at 1–8 Hz (600 shocks each) during sustained nerve stimulation for 20.5 min. At 5 mM (0 K), TI was more effective because it caused the greater increase in quantal content at all stimulus frequencies. Frequency-dependent
changes were also observed when 2.5 mM TI was substituted for K (Fig. 9 B), supporting the idea that TI entered the nerve terminal with each nerve impulse.

**Temporal Sequence of TI's Presynaptic Actions**

TI-induced increases in MEPP amplitude were more readily apparent at 15 min of exposure to the cation. At this time of exposure, 2.5 mM TI (0 K) caused a small but

![Figure 9](image)

**Figure 9.** The ordinate shows quantal content (m); the abscissa shows nerve stimulation frequency. Each panel shows the results of an individual experiment. Open circles show the control. (A) Left and middle panels show effects of 10 mM K (triangles); at right are effects of 5 mM TI (closed circles). K increased m at 1–4 Hz, but TI was more effective. (B) All three panels show effects of 2.5 mM TI (filled circles). Compare frequency-dependent changes with those of Figs. 2 and 4, A and B.

significant increase in the mean MEPP amplitude to 104% ± 1.2 SE of the control (average of five experiments). More importantly, the effect of TI increased with time. For instance, at a single, representative endplate, MEPP amplitude went from 0.35 mV at 15 min of exposure to 0.46 mV at 80 min; the TI-free control amplitude was
Temporal shifts in Tl's effects on EPP amplitude and quantal content were larger. Increases reached a maximum at 80 min during 2-Hz nerve stimulation (Fig. 10, A and B). The observed changes were consistent with presynaptic entry of Tl.

The EPP Facilitation Index in the Presence of Test K and Tl

Since presynaptic entry of Tl apparently occurred via Ca channels, it was important to determine if the entry or perhaps a downstream action of Tl was acid sensitive.

Because EPP facilitation is a Ca-dependent process, it was used as the means for evaluation. The mean ± SE of the control EPP facilitation index at pH 7.4 and 0.7 mM Ca was 2.3 ± 0.1 (average of 15 experiments). Recordings at pH 6.4 were made beginning at 15 min of inflow with pH 6.4 salines. At pH 6.4 the EPP facilitation index was 2.7 ± 0.2 (average of 10 experiments). There was no pH-dependent

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**FIGURE 10.** The evolution of synaptic effects (ordinate) induced by 2.5 mM Tl is shown as a function of nerve stimulation frequency (abscissa) and time. (A) EPP amplitudes (ordinate) at a representative endplate are shown for control (open circles) and after exposure to Tl for 20.5 min (filled circles), 80 min (triangles), and 152 min (squares). (B) Each panel shows quantal content (m, ordinate) in an individual experiment during control (open circles) and after exposure to Tl for 20.5 min (filled circles), 80 min (triangles), 134 min (squares), and 182 min (diamonds).
difference in the EPP facilitation index, even though there was a significant decrease in the mean EPP at pH 6.4 to 65.3% ± 11.0 of the control (three experiments). No significant change in membrane potential at the sartorius endplate was observed with the pH change. Furthermore, enhancement of the EPP facilitation index by 0.8 mM Ca (relative to 0.7 mM Ca) was unaffected at pH 6.4. Thus, Ca entry and its enhancement of EPP facilitation was not affected at pH 6.4.

When 2.5 mM Tl was substituted for K, there was a significant increase in EPP amplitude. This effect was readily reversed upon washout of Tl. 10 mM test K increased EPP amplitude, as well as the EPP facilitation index. In contrast to effects of 0.8 mM Ca (above), the EPP facilitation index and EPP amplitudes occurring with either 10 mM K or 2.5 mM Tl (0 K) were reduced at pH 6.4 (Fig. 11), indicating that Tl's entry via Ca channels could not have been reduced at pH 6.4 since the entry of Ca was unaffected.

Effect of BAPTA Loading of the Nerve Terminal

Tl's effects were also evaluated after the nerve terminal was loaded with the Ca-chelating agent, BAPTA. After incubation in 0.5% DMSO ± BAPTA-AM, muscles were removed and continuously superfused with BAPTA-free, DMSO-free 1 mM Ca salines: first with the initial control, then with 2.5 mM Tl substituted for K. Since BAPTA decreased EPP amplitudes, 1 mM Ca (rather than 0.7 mM) was used. In BAPTA-free preparations, the mean EPP amplitude (in millivolts) ± SE, before exposure to Tl, was 6.4 ± 4.6 (average of four experiments). In BAPTA-loaded
preparations, it was $1.0 \pm 0.2$ (six experiments). The EPP facilitation index was significantly reduced in BAPTA-loaded preparations. However, at 10 min of exposure to 2.5 mM Tl, the EPP facilitation index recovered completely (Table IV). This effect was associated with a fivefold increase in EPP amplitude.

To determine whether Tl could displace Ca from BAPTA, Ca ionic activity was measured in four cell-free salines (i.e., 0.1 M TlAc, 0.2 M TlAc, 0.1 M NaAc, and 0.2 M NaAc) in which 1 mM Ca with or without 1 mM BAPTA (unesterified) was added to each. First, the measured Ca ionic activity was the same in TlAc and NaAc salines. Second, Ca activity was the same at 0.1 and 0.2 M TlAc (as well as at 0.1 and 0.2 M NaAc). Finally, in an individual trial, measured Ca ionic activity at 0.2 M TlAc was 0.45 mM without BAPTA and 0.11 mM in the presence of 1 mM BAPTA. In another trial, Ca activity at 0.2 M NaAc was 0.56 mM without BAPTA and 0.14 mM with BAPTA. Thus, with Tl or Na in excess of Ca at 100-fold or more, there was no interference with the ability of BAPTA to bind Ca.

**TABLE IV**

| Pretreatment       | Mean EPP facilitation ± SE | Test EPP amplitude normalized to reference |
|--------------------|-----------------------------|---------------------------------------------|
|                    | 2.5 mM K (reference)        | 2.5 mM Tl (test)                            |
| BAPTA-free         | 4                           | 3.1 ± 0.1                                   | 5.6 ± 0.8 | 1.6 ± 0.3 |
|                    | 6                           | 1.6 ± 0.2                                   | 3.3 ± 0.2 | 5.3 ± 1.4 |

*N is the number of experiments. Both reference and test conditions were evaluated at a single endplate in each preparation. All were incubated (pretreatment) for 3 h in 0.5% DMSO, 0 Ca-EGTA saline with BAPTA (BAPTA-loaded) or without BAPTA (BAPTA-free). EPP amplitude and facilitation were then determined in the presence of either K (reference) or at 10 min of exposure to Tl (test). As shown, though EPP facilitation was reduced at the BAPTA-loaded reference nerve terminal, it recovered completely with Tl and was associated with a fivefold increase in EPP amplitude. Reference EPP amplitudes are listed in the text.

**DISCUSSION**

Tl's enhancement of MEPP frequency, quantal content, and the EPP facilitation index apparently involved the same fundamental presynaptic mechanism even though onset and washout of Tl's presynaptic effect appeared to be faster for MEPP frequency than for quantal content. But the results show that presynaptic entry was the deciding factor in Tl's actions. For instance, the rise in MEPP frequency by Tl, and its rate of exponential decline upon Tl washout, were linearly related to both the frequency of nerve stimulation and the number of nerve shocks.

Analyses of Figs. 4 C and 7 together indicate that ω-CTX-sensitive presynaptic entry of Tl apparently occurred via voltage-sensitive N-type Ca channels at the nerve terminal. ω-CTX selectively blocks N-type Ca channels at the nerve terminal of the frog neuromuscular junction (Cohen, Jones, and Angelides, 1991), such that quantal
secretion is reduced due to decreased presynaptic entry of Ca. Tl's effects, in spite of being masked by external Ca (Fig. 4, A–C), were inhibited by pretreatment with ω-CTX. The novel finding was that Tl apparently entered and left the nerve terminal primarily via Ca channels.

By itself, 20 μM ouabain had negligible effects on MEPP frequency (Fig. 5), consistent with an earlier report (Nishimura, 1986). But substitution of Tl for K resulted in an exaggerated increase in MEPP frequency. This effect was reduced with 50% reduction in Na (Fig. 6 A), implying that elevated internal Na was involved. Intraterminal Na leads to elevations in quantal secretion (Rahamimoff, Lev-Tov, and Meiri, 1980). Na was probably elevated due to inhibition of the presynaptic Na-K ATPase by ouabain. Since ouabain binds primarily to the phosphorylated ATPase (E₂P form) (Glynn, 1985), and extracellular Tl or other K congeners catalyze hydrolysis of this form of ATPase (Glynn and Richards, 1984), then antagonism might be expected between Tl and ouabain. But such antagonism was inconsistent with observations. Perhaps inhibition of the Na-K ATPase by ouabain occurred upon removal of extracellular K. 20 μM ouabain was present before K was removed without any major change in MEPP frequency; the exaggerated effect occurred upon K removal (with Tl substitution). Since Tl readily entered the nerve terminal via voltage-gated Ca channels, intraterminal Tl could have enhanced ouabain's toxicity. But it is also likely that removal of K from the extracellular face of the Na-K ATPase precipitated the binding of ouabain. In the absence of ouabain, Tl's presynaptic stimulation was not due to Na (Fig. 6 B), implying that Tl neither elevated intraterminal Na nor inhibited the Na-K ATPase.

Based on the observations, potential Ca-related actions must be considered. First, the finding that EPPs did not reappear with Tl (at 0 Ca) does not rule out the possibility that Tl could have acted as a Ca substitute. However, this possibility is unlikely because heavy metal ions that are potential substitutes for Ca (i.e., Pb, Cd, and Zn) block voltage-dependent stimulation of MEPP frequency by Ba with greater potency relative to Ca (Wang and Quastel, 1991). Tl did not fit into this group because the cation, as low as 0.2 mM, invariably enhanced voltage-dependent MEPP frequencies occurring with Ba more than with Ca. Since Ba persists longer at quantal release sites than Ca (Quastel and Saint, 1988), it is apparent that Tl's effects are further enhanced by conditions associated with Ca-related triggering of quantal secretion (i.e., with Ba or internal Na). Second, Tl is univalent, whereas the group mentioned above is divalent. Third, increased EGTA (from 0.3 to 2 mM) in 0 Ca salines during nerve stimulation should have caused a reduction in Tl's presynaptic stimulation (a) if Tl was significantly bound to EGTA, (b) if Tl acted by enhancing Ca influx, and/or (c) if Tl acted by releasing internal stores of Ca. Presynaptic stimulation by ryanodine, an agent that releases internal stores of Ca, becomes negligible at very low external Ca (Nishimura, Tsubaki, Yagasaki, and Ito, 1990), perhaps because of a reversed electrochemical plasmalemmal gradient for Ca (Erulkar, Rahamimoff, and Rotshenker, 1978). But Tl's presynaptic stimulation was not reduced when external Ca was negligible. Rather, it was enhanced by nerve stimulation at the higher concentration of EGTA (Fig. 4, A–C).

Two additional findings make it unlikely that Tl could have released internal stores of Ca. First, if Tl acted to elevate internal Ca, there should have been antagonism
between Ti and Ba because Ca antagonizes Ba-induced increases in MEPP frequency (Silinsky, 1978). But 0.2 mM Ti enhanced presynaptic stimulation by Ba (Table III, Fig. 8). The second additional finding is that in spite of decreased EPP facilitation due to chelation of internal Ca by BAPTA, substitution of Ti for K restored the EPP facilitation index (Table IV). Ti did not displace Ca from BAPTA. Together, the above reasons make it unlikely that Ti's effects resulted from elevation of internal Ca.

EPP facilitation, i.e., the fast developing and decaying increase in quantal release that occurred, in this instance, with a pulse train at 40 Hz and 500 ms duration, was probably due in part to residual Ca (Katz and Miledi, 1968). But it is unlikely that the increase in the EPP facilitation index observed at elevated K resulted solely from Ca influx. The reason for this is that the EPP facilitation index at elevated extracellular K, rather than that at elevated extracellular Ca, was reduced at pH 6.4 (relative to pH 7.4; see Fig. 11). Like elevated K, but unlike elevated Ca, the EPP facilitation index in the presence of Ti was also reduced at pH 6.4, perhaps indicative of related, acid-sensitive mechanisms for Ti and K. Since restoration of the EPP facilitation index with Ti at BAPTA-treated nerve terminals (Table IV) apparently resulted in part from enhanced entry of Ti with each successive nerve impulse in the pulse train, the acid sensitivity of Ti's entry was considered. But enhancement of the EPP facilitation index by raised extracellular Ca apparently involved Ca entry via Ca channels, and it was unaffected at pH 6.4. Thus, it is likewise improbable that Ti's permeability at the same Ca channel was reduced. Consequently, protons apparently reduced Ti's (and perhaps K's) presynaptic stimulation at some point downstream from the \( \omega \)-CTX site at the Ca channel.

The finding that Ti entered the nerve terminal primarily via voltage-sensitive Ca channels is not inconsistent with a potential intraterminal K-like action of Ti. The idea that Ti and K, the former being much more potent, may have fundamentally similar underlying mechanisms is because of similarities (a) in their chemistry (Cotton and Wilkinson, 1980), (b) in their enhancement of quantal content (Fig. 9, A and B), and its pH dependence (Fig. 11), and (c) in the ability of Ca to mask presynaptic effects of Ti (Fig. 4 C) and K (Cooke and Quastel, 1973). There are earlier reports (Liley and North, 1953; Takeuchi and Takeuchi, 1961), which suggest that K increases nerve-evoked transmitter release. The present findings support as well as extend these earlier findings.

As to a specific mechanism, first, it was shown (Ceccarelli, Fesce, Grohovaz, and Haimann, 1988) that external K induces fusion between presynaptic vesicles and the nerve terminal membrane, suggestive of either K-induced exocytosis of transmitter or K-mediated endocytosis (or retrieval) of vesicle membrane. Cell-mediated endocytosis is blocked when internal K stores are depleted, and recovers with reconstitution by either K or rubidium (Larkin, Brown, Goldstein, and Anderson, 1983). Second, like Ti (Table III and Legend), MEPP frequency and quantal content are both increased by DMSO in spite of Ba substitution for Ca (McLarnon, Saint, and Quastel, 1986). Like K, DMSO has been implicated in fusogenic membrane events (Geron and Meiri, 1985). MEPP frequency, associated with a fusogenic K-like action of Ti, would be enhanced in the presence of Ba or with conditions resulting in elevated internal Na.

In summary, substitution of 2.5 mM Ti for K resulted in frequency-dependent
increases in quantal content and MEPP frequency, and caused complete recovery of the EPP facilitation index at the BAPTA-loaded nerve terminal. TI-induced increases in MEPP frequency, MEPP amplitude, EPP amplitude, and quantal content were reversible and concentration dependent. TI, as low as 0.2 mM, did not block, but enhanced voltage-dependent changes in MEPP frequency with Ba more than with Ca substitution. The increase in MEPP frequency by TI and washout of this effect were enhanced by nerve stimulation at 0 Ca-EGTA-treated preparations. The greater the concentration of EGTA, the greater the increase. Pretreatment with 200 nM ω-CTX reduced TI's effect. The combination of 20 μM ouabain + 2.5 mM TI (0 K) caused an exaggerated increase in MEPP frequency, which was reduced by a 50% reduction in Na. 50% reduction in Na, by itself, did not affect TI's presynaptic stimulation.

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