Effect of tetanus toxin on the accumulation of the permeant lipophilic cation tetraphenylphosphonium by guinea pig brain synaptosomes

(membrane potential/tetanus/neurotransmitters/batrachotoxin/thyrotropin)

SOFIA RAMOS*, EVELYN F. GROLLMAN†, PEDRO S. LAZO†, SHERRY A. DYER†, WILLIAM H. HABIG*, M. CAROLYN HARDEGREE*, H. RONALD KABACK‡, AND LEONARD D. KOHN†

*Bacterial Toxins Branch, Division of Bacterial Products, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland 20205; †Section on Biochemistry of Cell Regulation, Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20205; and ‡Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT Accumulation of the permeant lipophilic cation [^3H]tetraphenylphosphonium (TPP^+) by synaptosome preparations from guinea pig brain cerebral cortex is inhibited 1:10 by medium containing 193 mM K^+ and by veratridine. A further 1:10 to 1:15 decrease in TPP^+ uptake occurs under nitrogen and in the presence of mitochondrial inhibitors such as oligomycin, whereas starvation and succinate supplementation have no effect. These data indicate that, in analogy to intact neurons, there is an electrical potential ($\Delta\psi$, interior negative) of -60 to -80 mV across the synaptosomal membrane that is due primarily to a K^+ diffusion gradient ($\text{K}^+_{\text{in}} \rightarrow \text{K}^+_{\text{out}}$). The data also indicate that mitochondria entrapped within the synaptosome but not free mitochondria make a large contribution to the TPP^+ concentration gradients observed.

Conditions are defined in which tetanus toxin binds specifically and immediately to synaptosomes in media used to measure TPP^+ uptake. Under these conditions tetanus toxin induces dose-dependent changes in TPP^+ uptake that are blocked by antitoxin and not mimicked by biologically inactivated toxin preparations. The effect of tetanus toxin on TPP^+ uptake is not evident in the presence of 193 mM K^+ or veratridine but remains under conditions known to abolish the mitochondrial $\Delta\psi$. Moreover, tetanus toxin has no effect on TPP^+ uptake by isolated synaptosomal mitochondria. The results thus define an *in vitro* action of tetanus toxin on the synaptosomal membrane that can be correlated with biological potency *in vivo* and is consistent with the *in vivo* effects of tetanus toxin on neuronal transmission.

The classic symptomatology of tetanus reflects a syndrome of dysinhibition in which tetanus toxin is thought to abolish neuronal transmission through certain inhibitory pathways in the central nervous system (see ref. 1 for a review). Studies carried out *in vivo* (2-4) suggest that the effect of the toxin is caused by its ability to inhibit the release of inhibitory neurotransmitters such as glycine at specific synaptic termini. However, tetanus toxin also has been shown to block conduction through peripheral cholinergic synapses with a concomitant decrease in acetylcholine release (5-7).

Synaptosome preparations from the central nervous system consist largely of presynaptic nerve termini that maintain a surprising degree of functional integrity. Synaptosome preparations have been shown to fix tetanus toxin (8, 9), to synthesize and accumulate neurotransmitters, and to release neurotransmitters when exposed to depolarizing agents (10-12). Because release of glycine from spinal cord synaptosomes prepared from rats poisoned with tetanus toxin is depressed (12), the following possibilities are suggested: (i) synaptosome

preparations might provide a useful system in which to study the mechanism of action of tetanus toxin; and (ii) inhibition of neurotransmitter release might result from an effect of the toxin on the electrical potential ($\Delta\psi$) across the neuronal membrane. In this regard, synaptosome preparations have been used to study the effect of botulinum toxin on the inhibition of acetylcholine release (13, 14).

Because synaptosomes are too small to be impaled with microelectrodes, efforts to measure $\Delta\psi$ have relied on indirect techniques. Using fluorescent carbocyanine dyes, Blaustein and Goldring (15) have reported values of -55 to -60 mV, whereas Campbell (16) calculated somewhat lower values from the distribution of Na^+ , K^+ , and Cl^- across the synaptosomal membrane. Recent studies have used radiolabeled permeant "lipophilic ions" to measure $\Delta\psi$ in biological systems (17-24). Importantly, recent studies with NG108-15 mouse neuroblastoma-rat glioma hybrid cells (23) and *Escherichia coli* (25) demonstrate that distribution measurements with [^3H]tetraphenylphosphonium $^+$ (TPP^+) yield values for $\Delta\psi$ that are essentially identical to those obtained by using direct intracellular recording techniques under various conditions.

In this communication, accumulation of [^3H] TPP^+ by guinea pig brain synaptosomes is used to monitor $\Delta\psi$. More important, evidence is presented which is consistent with the interpretation that tetanus toxin alters the $\Delta\psi$ across the synaptosomal membrane. The findings document an *in vitro* effect of the toxin that is consonant with its action *in vivo*—i.e., modulation of the electrical potential in neuronal tissue.

MATERIALS AND METHODS

Preparation of Synaptosomes. Synaptosomes were prepared by a described procedure (26) using slices from the top layer of the cerebral cortex of two male guinea pigs (Hartley; 200-300 g). The final washed P2 pellet was suspended in 0.32 M sucrose at 10-15 mg of protein per ml and kept at 4°C until use.

Mitochondrial Preparations. Mitochondria were prepared from synaptosomes as described (27).

Protein Determinations. Protein was assayed colorimetrically (28) on samples treated for 30 min in 0.3 M NaOH by using crystalline bovine serum albumin as a standard.

Uptake of [^3H] TPP^+ . Accumulation of [^3H] TPP^+ was determined by filtration through Millipore cellotrate filters (0.5 μm) (19). Incubations were at 37°C in reaction mixtures containing Krebs-Ringer's phosphate buffer designated "low K^+ medium" (180 mM NaCl/3 mM KCl/2 mM MgSO_4 /10 mM

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Abbreviations: $\Delta\psi$, membrane potential; TPP^+ , tetraphenylphosphonium $^+$; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

NaPi, pH 7.4/10 mM glucose) and 5 μ M [3 H]TPP $^+$ (final volume, 100 μ l). In experiments in which the K $^+$ concentration of the medium was increased, the Na $^+$ concentration was decreased proportionately. Other additions were no greater than 1% of the total assay volume. Reactions were initiated by adding 5 μ l of the synaptosome suspension (50–75 μ g of protein) and terminated by the addition of 2.0 ml of ice-cold 0.8 M NaCl, followed by immediate filtration and washing with an additional 2.0 ml of the same solution. Dilution, filtration, and washing were complete within 10 sec. No differences were observed when ice-cold 0.2–0.8 M NaCl was used to terminate the reactions.

Radioactivity was assayed by liquid scintillation spectrometry after the filters were dissolved in Instabray (Yorktown Research, S. Hackensack, NJ). Corrections for nonspecific adsorption to the filters were made by diluting the reaction mixtures with 0.8 M NaCl prior to addition of synaptosomes, followed by addition of synaptosomes, filtration, and washing. Values obtained in this manner were subtracted from the data presented. Mitochondrial assay medium (170 mM sucrose/60 mM KCl/10 mM NaPi, pH 7.4/2 mM MgSO $_4$ /2 mM sodium succinate) was substituted for Krebs–Ringer's phosphate buffer for studies of [3 H]TPP $^+$ uptake by isolated mitochondria. This medium was aerated at 37°C for 20 min prior to use.

Determination of Synaptosomal Internal Volume. The internal volume of the synaptosomes was determined as described (29). An average internal volume of 3.6 μ l per mg of synaptosomal protein was calculated from the data; a similar value was obtained when synaptosomes were treated with tetanus toxin (20 μ g per ml) or veratridine (up to 200 μ M) or when they were suspended in Krebs–Ringer's phosphate buffer with K $^+$ replacing Na $^+$. In the presence of 1 μ M batrachotoxin, on the other hand, the internal volume increased to 4.1–4.5 μ l per mg of protein.

Calculation of TPP $^+$ Concentration Gradients and $\Delta\psi$. The internal concentration of TPP $^+$ ([TPP $^+$] $_{in}$) was calculated by using an intrasynaptosomal volume of 3.6 μ l per mg of protein. External TPP $^+$ concentration ([TPP $^+$] $_{out}$) was calculated by subtracting the amount taken up from the concentration of TPP $^+$ originally present in the reaction mixture. In some instances, as much as 60% of the added TPP $^+$ was taken up. Concentration gradients were calculated as ([TPP $^+$] $_{in}$ /[TPP $^+$] $_{out}$); $\Delta\psi$ was calculated as described by Lichtshtein *et al.* (23).

Binding of 125 I-Labeled Tetanus Toxin (125 I-Tetanus Toxin). Synaptosomes were suspended in Krebs–Ringer's phosphate buffer containing 0.5% crystalline bovine serum albumin in a total volume of 100 μ l. Tetanus toxin and 125 I-tetanus toxin were prepared as described (30). To each assay mixture, 6.6 nM 125 I-tetanus toxin was added, the samples were incubated for 30 min at 37°C (unless otherwise noted), and binding was assayed by filtration (30). In some experiments, antitoxin was incubated with 125 I-tetanus toxin for 1 hr at 22°C prior to the binding assay. All assays were performed in duplicate and are reported as specific binding—i.e., after correction for nonspecific binding measured in assays without synaptosomes or in assays containing a 10,000-fold excess of unlabeled toxin.

Materials. [3 H]TPP $^+$ (400 mCi per mmol, 1 Ci = 3.7×10^{10} becquerels) (bromide salt) was synthesized by the Isotope Synthesis Group of Hoffmann–La Roche, under the direction of Arnold Liebman according to unpublished methods. Batrachotoxin was kindly provided by John Daly of the Laboratory of Bioorganic Chemistry (National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, MD 20205). All other materials were of the highest purity available from commercial sources.

RESULTS AND DISCUSSION

TPP $^+$ Accumulation by Synaptosomes. Synaptosome preparations suspended in Krebs–Ringer's phosphate buffer (190 mM Na $^+$ /3 mM K $^+$) took up [3 H]TPP $^+$ rapidly for 3–4 min, achieving a steady-state level of accumulation in about 5 min at which time the apparent internal concentration of the cation was approximately 500 times that of the medium (Fig. 1A). When the protonophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) was added at 20 min, well over 95% of the cation taken up was lost from the synaptosomes within a short time. This indicates that the plateau level represents a steady state rather than conversion of TPP $^+$ into a stable internal product. The capacity of the synaptosome preparations to accumulate TPP $^+$ declined with time, decreasing by about 20% (not shown) within 6 hr.

As opposed to findings with *E. coli* membrane vesicles (unpublished results) and NG108-15 cells (23), the apparent concentration gradient of TPP $^+$ established by guinea pig synaptosomes is relatively sensitive to the external concentration of the lipophilic cation (Fig. 1B). As shown, although a 500-fold concentration gradient was obtained at the steady state with TPP $^+$ concentrations ranging from 0.5 μ M to about 5 μ M, at concentrations higher than 5 μ M significantly less accumulation was observed. At 50 μ M, for instance, a concentration gradient of only about 100-fold was obtained. For this reason, TPP $^+$ was used at concentrations no higher than 5 μ M.

TPP $^+$ Accumulation as a Probe for $\Delta\psi$. With increasing external K $^+$ concentrations, the concentration gradient of TPP $^+$ established at the steady state decreased from 400–450 at an external K $^+$ concentration of 3 mM to about 50 at an external K $^+$ concentration of 193 mM (Fig. 2), indicating that the $\Delta\psi$ across the synaptosomal membrane is probably due primarily to a K $^+$ diffusion gradient as is the case for other neuronal cells (23, 31–33).

When values for membrane potential were calculated from these data and plotted against the log of the external K $^+$ concentration (34), the limiting slope of the plot was -61.5 mV. The minimal value for membrane potential, however, was at -64 mV rather than 0, suggesting that accumulation of TPP $^+$ in this system represents more than simple equilibration of the permeant cation with the $\Delta\psi$ across the synaptosomal mem-

FIG. 1. Uptake of [3 H]TPP $^+$ by guinea pig brain synaptosomes. (A) Synaptosomes (5 μ l containing 50 μ g of protein) suspended in 0.32 M sucrose were diluted into 100 μ l of Krebs–Ringer's phosphate buffer containing [3 H]TPP $^+$ at a final concentration of 5 μ M ($\bullet$). As indicated by the arrow, after 20 min of incubation, CCCP was added to a final concentration of 10 μ M ($\blacktriangle$). Incubations were carried out at 37°C, and at the times indicated samples were assayed and concentration gradients were determined. (B) Experiments were performed as described in A, except that the concentration of [3 H]TPP $^+$ was varied as indicated. $\blacksquare$, [3 H]TPP $^+$ (400 mCi per mmol) at 0.5–5.0 μ M; $\blacktriangle$, 10 μ M [3 H]TPP $^+$ (400 mCi per mmol); $\blacktriangledown$, 50 μ M [3 H]TPP $^+$ (40 mCi per mmol). The bars encompass the individual data points at 0.5–5 μ M.

FIG. 2. Effect of external K^+ concentration on $[^3H]TPP^+$ accumulation. $[^3H]TPP^+$ uptake was measured as described in Fig. 1A with $5 \mu M$ $[^3H]TPP^+$ and concentration gradients were calculated. The K^+ concentration of Krebs-Ringer's phosphate buffer was varied as follows and the Na^+ concentration was varied reciprocally. $\bullet$, External K^+ (3 mM); $\blacktriangle$, 40 mM external K^+ ; $\circ$, 80 mM external K^+ ; $\triangle$, 193 mM external K^+ .

brane—i.e., that TPP^+ accumulation might also occur within internal organelles such as mitochondria. Synaptosomes are enriched with mitochondria, which have a large $\Delta\psi$ (interior negative) (35).

Entrapped mitochondria make a large contribution to TPP^+ accumulation in synaptosome preparations (Fig. 3). When synaptosomes were incubated under conditions that should completely depress the mitochondrial $\Delta\psi$ —i.e., under nitrogen and in the presence of oligomycin—there was a 1:10 decrease in the steady-state level of TPP^+ accumulation from a concentration gradient of about 450–600 to approximately 60. However, there was a further decrease in the concentration gradient from 60 to 6 or 7 when the K^+ concentration of the medium was increased to 193 mM, indicating that the $\Delta\psi$ across the synaptosomal membrane remains when the mitochondria are suppressed. Starvation for 1 hr and succinate supplementation, conditions known to markedly perturb TPP^+ uptake in mitochondrial preparations (unpublished results), had no effect on TPP^+ uptake by synaptosome preparations. The “mitochondrial component” thus reflects the activity of an intrasynaptosomal organelle rather than an extrasynaptosomal contaminant. When $\Delta\psi$ across the synaptosomal membrane was calculated from the difference in TPP^+ uptake in low and high K^+ media and under conditions that abolish the $\Delta\psi$ (interior negative) across the mitochondrial membrane, a value of -83 ± 10 mV was obtained. It must be stressed that in this preparation it is impossible to verify the TPP^+ distribution measurements electrophysiologically.

The ability of TPP^+ to be concentrated secondarily within internal organelles by comparison to the fluorescent probes used by Blaustein and Goldring (15) may be an advantage in future studies. Differences in the comparative values determined by using the two techniques not only may be a good indication of a functioning internal compartment but also may give a quantitative indication of the ability of that compartment to magnify perturbations affecting the plasma membrane. In essence, TPP^+ could then be a good measure of an amplification mechanism important for regulation of cell function.

[§] The $\Delta\psi$ across the mitochondrial membrane is generated by proton extrusion via the respiratory chain or ATP hydrolysis catalyzed by proton-translocating Ca^{2+} , Mg^{2+} -ATPase. Thus, by omitting oxygen and inhibiting ATPase activity with oligomycin, the ability of the mitochondria to generate $\Delta\psi$ should be completely abolished (36).

FIG. 3. Effect of anaerobiosis and oligomycin on $[^3H]TPP^+$ accumulation. $[^3H]TPP^+$ uptake was measured as described in Fig. 1A with $5 \mu M$ $[^3H]TPP^+$ and concentration gradients were calculated. The external K^+ concentration was either 3 mM ($\bullet$ and $\circ$) or 193 mM ($\triangle$). $\circ$ and $\triangle$, Incubations were carried out under nitrogen, and the reaction mixtures contained oligomycin at a final concentration of $50 \mu g/ml$.

Effect of Veratridine and Batrachotoxin on TPP^+ Accumulation. TPP^+ accumulation was diminished to a level of approximately that observed in high K^+ medium when veratridine (and to a lesser extent batrachotoxin) was added to the synaptosomes, a finding that is consistent with the known ability of the alkaloid to depolarize neuronal cells by increasing electrogenic Na^+ influx (see ref. 37 for a review). As shown in Fig. 4A, when synaptosomes were allowed to accumulate TPP^+ to a steady state for 4–5 min and veratridine was then added, rapid loss of TPP^+ occurred, and within 2 min the level of accumulation approximated that observed when the synaptosomes were incubated in high K^+ medium (see Fig. 2); batrachotoxin exhibited a similar but less dramatic effect (Fig. 4A). The slower onset of depolarization by batrachotoxin when compared to

FIG. 4. Effect of veratridine, batrachotoxin, or tetrodotoxin on $[^3H]TPP^+$ accumulation. $[^3H]TPP^+$ uptake was assayed as described in Fig. 1A with $5 \mu M$ $[^3H]TPP^+$. (A) Four minutes after initiation of the reaction, veratridine (in ethanol) ($\blacktriangle$) or batrachotoxin (in methanol) ($\circ$) was added to final concentrations of $200 \mu M$ (1% ethanol) or $1 \mu M$ (1% methanol), respectively. Incubations were continued for given times, and the samples were assayed. $\bullet$, Control. (B) $[^3H]TPP^+$ uptake was assayed without further additions [control ($\bullet$)] and in the presence of tetrodotoxin at a final concentration of $17 \mu M$ ($\circ$), veratridine at a final concentration of $20 \mu M$ ($\blacktriangle$), and tetrodotoxin and veratridine at final concentrations of $17 \mu M$ and $20 \mu M$, respectively ($\blacktriangle$). Ethanol or methanol at final concentrations of 1% had no significant effect on $[^3H]TPP^+$ accumulation.

FIG. 5. Binding of ^{125}I -tetanus toxin to synaptosomes. Binding assays were performed in 100 μl of Krebs-Ringer's phosphate buffer containing 6.6 nM ^{125}I -tetanus toxin. (A) Binding as a function of synaptosomal protein; incubations were carried out at 37°C for 30 min. (B) Binding as a function of time; incubations were carried out at 37°C and contained 50 μg of synaptosomal protein. (C) Specificity of binding. Upper curve: in the presence of unlabeled tetanus toxoid, diphtheria toxin, insulin, prolactin, ACTH, growth hormone, or thyrotropin. Preparations were the same as those used in refs. 30 and 39. Amounts up to 1000-fold molar excess were added for 30 min at 37°C prior to addition of radiolabeled tetanus toxin. Lower curve: unlabeled tetanus toxin alone. Assays were performed for 30 min but otherwise were the same as in B. (D) Inhibition of ^{125}I -tetanus toxin binding by tetanus antitoxin. Binding was assayed as described in C. Antitoxin was dialyzed for 48 hr at 0°C against Krebs-Ringer's phosphate buffer without glucose prior to use.

veratridine has been reported in other systems (38). Veratridine exhibited a similar effect when the synaptosomes were incubated anaerobically with oligomycin (not shown). Moreover, addition of tetrodotoxin to the synaptosomes prior to veratridine partially blocked the effect of the alkaloid, although tetrodotoxin itself had no effect on TPP^+ accumulation (Fig. 4B).

Interaction of Tetanus Toxin with Synaptosomes. Brain synaptosomes and brain plasma membrane preparations bind ^{125}I -labeled tetanus toxin (Fig. 5 and ref. 39, respectively). When synaptosomes were incubated in Krebs-Ringer's phosphate buffer at 37°C with 1 μg of ^{125}I -labeled tetanus toxin per ml (6.6 nM), binding of toxin increased markedly from 1 μg to about 50 μg of synaptosomal protein and remained constant at higher synaptosomal concentrations (Fig. 5A). At high synaptosomal protein concentrations, binding was complete in about 10 sec and remained constant for at least 30 min (Fig. 5B).

Binding was specific (Fig. 5C), and preincubation of ^{125}I -toxin with tetanus antitoxin for 1 hr at 22°C blocked toxin binding (Fig. 5D). At 5 μg of antitoxin, the toxin was no longer neurotoxic to mice (data not shown). Binding was inhibited by gangliosides that are able to inhibit neurotoxicity, analogous to reported data (30). Analogous results were seen in high K^+ medium.

Effect of Tetanus Toxin on TPP^+ Accumulation. Tetanus toxin caused an increase in the steady-state level of TPP^+ accumulation by synaptosomes in low (3 mM) K^+ media (Fig. 6A; $t = 4$ min). This effect was dose dependent (i.e., 0.7 and 2 μg of tetanus toxin per ml caused increases of 0.5 and 0.9 nmol of TPP^+ per mg of synaptosomal protein, respectively) and was blocked by antitoxin. When 2 μg of toxin per ml was added at 0 time, moreover, uptake of TPP^+ exceeded that of untreated synaptosomes by 15–20% over the entire time-course of the

FIG. 6. Effect of tetanus toxin on $[\text{H}]\text{TPP}^+$ accumulation. (A) Effect of toxin under control conditions—i.e., Krebs-Ringer's phosphate buffer (low K^+), and 56 μg of synaptosomal protein ($\bullet$). As indicated by the arrow, tetanus toxin or tetanus toxin pretreated with antitoxin was added at 4 min at the following final concentrations: tetanus toxin, 0.7 μg per ml (Δ); tetanus toxin, 2 μg per ml ($\blacktriangle$); tetanus toxin, 2.7 μg per ml, preincubated for 1 hr at 22°C with 2 units of antitoxin ($\square$). (B) Effect of tetanus toxin when synaptosomes were incubated under nitrogen and in the presence of oligomycin (50 μg per ml) ($\circ$). At the time indicated by the arrow, tetanus toxin was added to the reaction mixtures at a final concentration of 2 μg per ml (∇). (C) Effect of tetanus toxin on mitochondria ($\square$). TPP^+ uptake was assayed as described in A in mitochondrial assay buffer. At the time indicated by the arrow, tetanus toxin was added at a final concentration of 20 μg per ml (Δ). $\square$, Control. (D) Effect of tetanus toxin on TPP^+ uptake in Krebs-Ringer's phosphate buffer (pH 7.0) containing 0.01 mM CaCl_2 and 40 μg of synaptosomal protein ($\circ$). As indicated by the arrow, tetanus toxin or toxoid was added at 3.5 min at the following final concentrations: tetanus toxin, 0.22 μg per ml (Δ); tetanus toxin, 2.2 μg per ml ($\blacktriangle$); tetanus toxin, 22 μg per ml ($\blacktriangledown$); tetanus toxoid, 14.5 flocculation units per ml ($\square$). Tetanus toxoid (1900 flocculation units/mg of protein) was from Connaught Laboratories (Toronto, ON, Canada).

assay. Although not shown, ouabain did not block the increase in TPP^+ accumulation induced by tetanus toxin. This is in contrast to recent studies with cultured cell lines (40) in which an increase in intracellular Na^+ can lead to hyperpolarization secondary to activation of the Na^+, K^+ -ATPase. Because tetanus toxin increased TPP^+ uptake in synaptosomes incubated under nitrogen with oligomycin (Fig. 6B) but had no effect on TPP^+ uptake in isolated mitochondria (Fig. 6C), the effect of tetanus toxin seen in Fig. 6A reflects a perturbation of the $\Delta\psi$ across the synaptosomal membrane specifically.

These data (Fig. 6 A, B, and C) are notable in several other respects. First, tetanus toxin enhanced TPP^+ uptake to greater than 100% over control uptake in Fig. 6B by comparison to only 15–20% in Fig. 6A—i.e., the effect of the toxin on TPP^+ accumulation was relatively enhanced when the mitochondrial TPP^+ uptake process was suppressed. Second, the uptake of TPP^+ by isolated mitochondria (Fig. 6C) based on protein was low compared to synaptosomes (Fig. 6A), again suggesting that free mitochondria in synaptosomal preparations are not a significant problem in these studies. Last, as seen in Fig. 6B, tetanus toxin does not cause a “stable” enhancement of TPP^+ uptake. The transient increase in TPP^+ uptake appears to be the beginning of a biphasic process that ultimately leads to a decrease in TPP^+ uptake. Under different conditions (lower membrane protein concentrations, 0.01 mM external Ca^{2+} , and pH 7.0), tetanus toxin caused a dose-dependent decrease in TPP^+ uptake (Fig. 6D). Again, this effect was inhibited by antitoxin (data not shown), was not mimicked by tetanus toxoid (Fig. 6D), and was eliminated if the tetanus toxin was heated to 100°C for 5 min, a condition that destroys its bioactivity.

Although not shown, the toxin did not stimulate or decrease TPP^+ accumulation when the synaptosomes were suspended in high K^+ medium or when experiments were performed in the presence of veratridine (200 μM).

In summary, addition of tetanus toxin to synaptosomes under conditions in which the toxin is specifically bound leads to dose-dependent increases or decreases in TPP^+ accumulation which are blocked by antitoxin and are not evidenced with tetanus toxoid or when the toxin is inactivated by boiling. The phenomenon is not altered when the mitochondria are suppressed by anaerobiosis and oligomycin; in contradistinction, the effect of tetanus toxin is relatively enhanced because the mitochondrial background is depressed. These effects are not observed when the synaptosomes are incubated in high K^+ (i.e., under conditions that abolish $\Delta\psi$), although toxin binding is unaffected.

Tetanus toxin-induced hyperpolarization or depolarization is related to the conditions utilized. Thus, current evidence indicates that the different effects are dependent on synaptosomal protein concentration, pH, and external Ca^{2+} concentration. The change in conditions may induce responses by different subsets of synaptosomes (unpublished results). Nevertheless, these results clearly imply that binding of tetanus toxin under these experimental conditions leads to alterations in the electrical potential across the synaptosomal membrane that is consistent with its *in vivo* activity and correlates with the bioactivity of the preparation.

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