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ACTION OF TETANUS TOXIN ON CHOLINERGIC NEUROBLASTOMA×GLIOMA HYBRID CELLS: SELECTIVE BLOCKADE OF Ca SPIKES

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SUMMARY We examined the effect of tetanus toxin on clonal neuroblastoma×glioma hybrid cells, NG108–15, by intracellular microelectrode studies of passive membrane electrical properties and action potentials generated under various conditions. Binding of tetanus toxin to the surface of the cells was demonstrated by indirect immunofluorescent staining but no morphological alteration was observed in tetanus toxin-treated cells under a phase contrast microscope. There is no significant difference between the tetanus toxin-treated and untreated cells in their passive electrical membrane properties, i.e. resting membrane potentials, input resistances, time constants and input capacities. Cells in 120 m*M* Na^+, 2 m*M* Ca^{2+} salt solution showed Na spikes, and cells in high Ca^{2+} (30 m*M*), Na^+-free salt solution showed Ca spikes in response to depolarizing current pulses. While the Na spike was not affected by tetanus toxin, the Ca spike was blocked by the toxin. The minimum dose of tetanus toxin for maximum suppression of the peak potential level of the Ca spike was 250 ng/ml. Addition of tetraethyl ammonium (TEA) to extracellular fluid enhanced the Ca spike in untreated cells. In toxin-treated cells, TEA did not alter the effect of tetanus toxin on the Ca spike. Blockade of the Ca spike by tetanus toxin could be detected even at low extracellular Ca^{2+} concentration (10 m*M*) by adding TEA to the extracellular fluid and adjusting the membrane potential to a steady hyperpolarized level (−80 mV) to ensure optimal and uniform electrical responses. The usefulness of NG108–15 hybrid cells for in vitro investigations on the mechanism of action of tetanus toxin was discussed.

INTRODUCTION

Tetanus toxin has been reported to inhibit the release of neurotransmitters (Bigalke et al., 1978; Bigalke et al., 1981; Collingridge and Davies, 1982; Wendon and Gill, 1982), but

the mechanism of this action is still unknown.

In previous studies, we focused attention on the action of tetanus toxin on the electrical properties of the neuronal membrane. We chose neuroblastoma clone NlE-115 cells (Amano et al., 1972) because their ionic channels have been well characterized by the voltage-clamp technique (Moolenaar and Spector, 1978; 1979). We found that tetanus toxin blocks the Ca spike in NlE-115 cells under high Ca^{2+}, Na^{+}-free conditions (Sugimoto et al., 1983; Higashida et al., 1983). This suggests that it inhibits the release of neurotransmitters by blocking the influx of Ca^{2+}.

For studies on the effect of tetanus toxin on Ca^{2+} influx linked to neurotransmitter release, NlE-115 cells are not suitable because they are unable to release transmitter. Therefore in this study we used neuroblastoma×glioma hybrid cells, clone NG108–15 (Hamprecht, 1977), which have been reported to release acetylcholine in a voltage dependent manner (Nelson et al., 1976; McGee et al., 1978). However, the electrical properties of the membranes of this hybrid cell clone are not so well characterized as those of NlE-115 cells. The purpose of this study was to extend our previous findings to NG108–15 hybrid cells and establish a basis for in vitro investigations on the mechanism of action of tetanus toxin that leads to inhibition of neurotransmitter release.

MATERIALS AND METHODS

1. *Tetanus toxin and rabbit antitoxin*

Clostridium tetani strain Harvard A47, substrain Biken-Ozutsumi, was employed for production of tetanus toxin. Purified tetanus toxin was obtained from bacterial extracts by the method of Matsuda and Yoneda (1975). The toxin preparation formed a single precipitation band against crude horse antitoxin in agar gel immunodiffusion plates and migrated as a single protein band on a polyacrylamide gel plate. The activity of this preparation was 365–390 flocculating units and $0.8–1.6\times10^{7}$ mouse minimum lethal doses per mg protein. Rabbit antitoxin was prepared by intramuscular injection of formalin-treated toxin in incomplete Freund's adjuvant as described by Matsuda and Yoneda (1976).

2. *Cell culture*

NG108–15 hybrid cells were routinely cultured at 37 C in Dulbecco modification of Eagle's minimum essential medium (DMEM) supplemented with 5% fetal calf serum, 1×10^{-4} *M* hypoxanthine, 1×10^{-6} *M* aminopterine and 1.6×10^{-5} *M* thymidine in a humidified atmosphere of 10% CO_2 in air. For the experiments, NG108–15 hybrid cells were cultured for 2–3 weeks in the presence of 1 m*M* N^6, O^2-dibutyryl-adenosine-3′: 5′-cyclic monophosphate (dibutyryl cAMP) to shift the hybrid cells to a more differentiated state as described by Nelson et al. (1976).

3. *Immunofluorescent staining*

NG108–15 hybrid cells grown on glass coverslips (4×30 mm) were washed with DMEM, and then incubated with tetanus toxin (2 μg/ml) in DMEM at 37 C for 1 h. The cells were stained by sequential incubation with rabbit antitoxin for 1 h and goat anti-rabbit immunoglobulin conjugated with fluorescein for 1 h. After each incubation, the coverslips were dipped successively in 5 beakers containing 100 ml of 40 m*M* N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, containing 120 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* KCl, 1 m*M* $MgCl_2$ and 20 m*M* glucose. After the final incubation and washing, the coverslips were mounted in glycerol-borate buffered saline, pH 8.2 (9: 1 v/v) and examined under a microscope equipped with fluorescence optics (Nikon Optiphoto).

4. *Electrophysiology*

Techniques for intracellular recording of the membrane potentials were essentially as described previously (Sugimoto et al., 1983; Higashida et al., 1983). The hybrid cells were treated with tetanus toxin in DMEM at 37 C for 1 h and then washed with an appropriate buffer solution. For recording the Na spike, cells were bathed in 120 m*M* Na^{+}, 2 m*M* Ca^{2+} salt solution (120 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* KCl, 1 m*M* $MgCl_2$, 20 m*M* glucose). For recording the Ca spike, cells were bathed in high Ca^{2+}, Na^{+}-free salt solution (30 m*M* $CaCl_2$, 5 m*M* KCl, 1 m*M* $MgCl_2$, 20 m*M* glucose). All the solutions were made isotonic (ca. 340 mOsm) with tris (hydroxymethyl) amino methane (Tris)-HCl, pH 7.25. In the solution with TEA, Tris was isoosmotically

replaced by this ion. Cells in an appropriate salt solution were impaled with a single microelectrode (3 *M* KCl; 10–20 MΩ) used for both passing current and recording the voltage. For recording action potentials, a rectangular depolarizing current pulse was applied. For quantitative estimation of the action of toxin on the action potential, the membrane potential was adjusted to a steady hyperpolarized level (−80 mV) and then the peak level of the action potential elicited by 5 nA of depolarizing current pulse was measured.

RESULTS

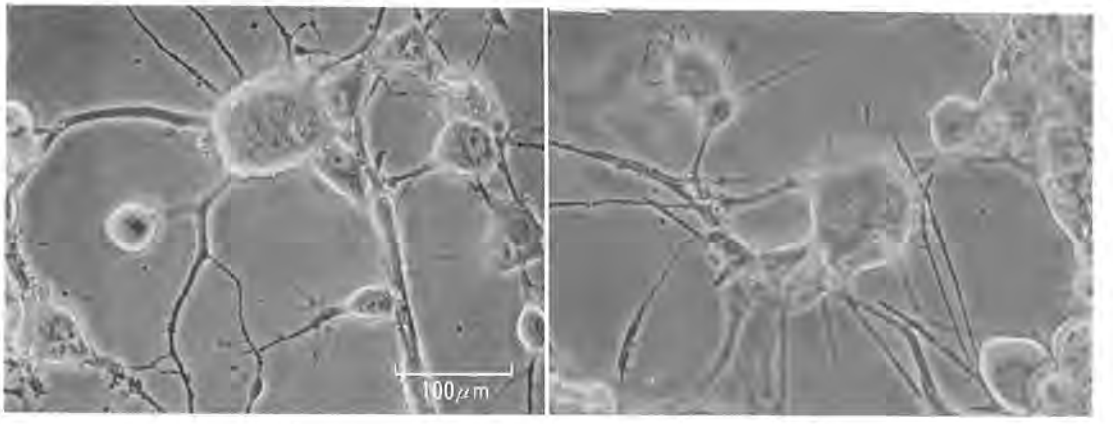
1. *Morphology and immunofluorescent staining of tetanus toxin-treated hybrid cells*

In growth medium, NG108–15 hybrid cells were stelliform with short processes. Addition of dibutyryl cAMP to the medium induced morphological differentiation of the cells. Cells treated with 1 mM dibutyryl cAMP for 2–3 weeks had extended long neurites and become more refractile and larger than those incubated in growth medium: large polynucleated cells (50–150 μm in diameter) with long neurites (up to 500 μm in length) were observed (Fig. 1A). Cells treated with tetanus toxin (2 μg/ml) showed no morphological alteration under a phase contrast microscope (Fig. 1B).

Indirect immunofluorescent staining revealed the binding of tetanus toxin to the hybrid cells. When tetanus toxin-treated cells were fixed in cold acetone (−20 C) and then stained with rabbit antitoxin serum and fluorescein conjugated goat anti-rabbit immunoglobulin, they showed intense fluorescence in both their cell bodies and neurites (Fig. 2A). As seen in Fig. 2B, on control staining with non-immunized rabbit serum instead of antitoxin only very weak fluorescence was detected. When the toxin treated-cells were stained without prefixation with acetone, their fluorescence had a honeycomb-like appearance (Fig. 2C). These results indicated that tetanus toxin bound diffusely to the surface of the cells.

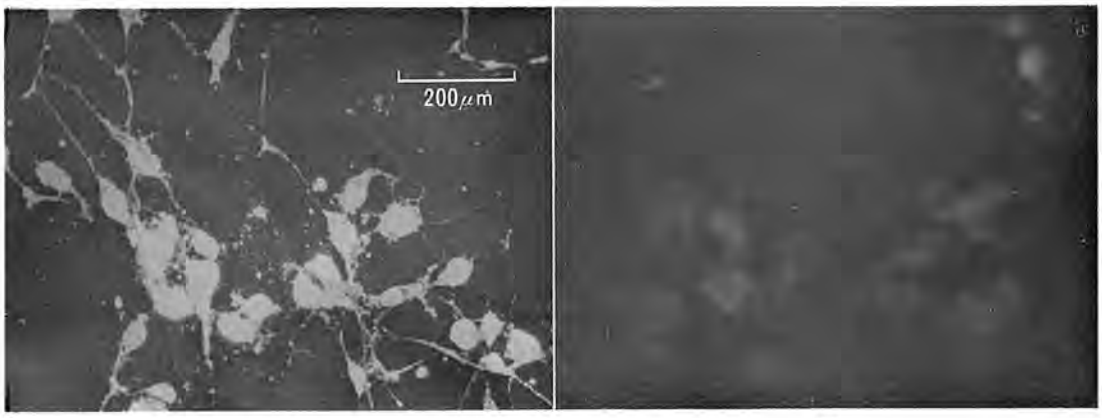
2. *Passive electrical membrane properties of tetanus toxin-treated cells*

When a microelectrode was impaled into a cell, the potential recorded through the electrode fell abruptly and, a few seconds after impaling the electrode, reached a steady level which was negative relative to extracellular fluid. Recording of the membrane potentials from the cells could be continued for several



(A) (B)

FIGURE 1. Phase contrast photomicrographs of NG108–15 hybrid cells. A, untreated cells; B, tetanus toxin-treated cells. NG108–15 hybrid cells were cultured for 20 days in the presence of 1 m*M* dibutyryl cAMP. Cells were washed with DMEM and incubated in DMEM with or without tetanus toxin (2 μg/ml) at 37 C.



(A) (B)

(C)

FIGURE 2. Indirect immunofluorescent staining of NG108–15 hybrid cells. A, cells treated with 2 μg/ml of tetanus toxin in DMEM were prefixed with cold acetone (−20 C) and stained with rabbit antitoxin and fluorescein conjugated goat anti-rabbit Ig. B, similar cells stained with non-immunized rabbit serum instead of rabbit antitoxin. C, surface staining: cells were stained with rabbit antitoxin and fluorescein conjugated goat anti-rabbit Ig without prefixation.

minutes. As shown in Table 1, the resting membrane potentials recorded from tetanus toxin-treated cells and untreated cells were not significantly different.

The electrical properties of the cell membrane were determined by passing electrical currents through intracellularly impaled microelectrodes and recording the voltage changes in the membrane potential elicited by the currents. An example of hyperpolarizing membrane potentials elicited by outward steps of the current in tetanus toxin-treated cell is shown in Fig. 3A. The membrane input resistance, calculated from the slope of the voltage-current relation (Fig. 3B), was 15 MΩ. Figure 3C shows an analysis of the time course of the voltage-transient elicited by 3 nA of hyperpolarizing current pulse for determining the membrane capacity. The membrane time constant was 21.5 msec, so the membrane input capacity of this cell was 1.4 nF. The membrane input resistances, membrane time constants and membrane input capacities of tetanus toxin-treated cells and untreated cells are shown in Table 2.

The results indicate that tetanus toxin does not affect the passive electrical membrane properties of the hybrid cells.

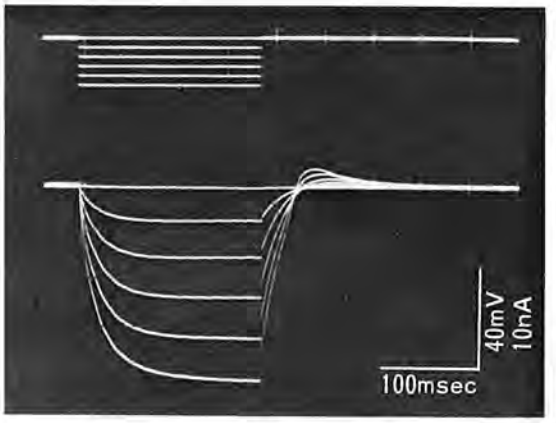
3. *Effect of tetanus toxin on the Na spike in the hybrid cells*

In 120 m*M* Na^+, 2 m*M* Ca^{2+} salt solution, action potentials with sharp spikes were elicited in the cells by depolarizing current pulses. An example of the graded responses of the membrane potential to depolarizing current stimulations (0–5 nA) is shown in Fig. 4A.

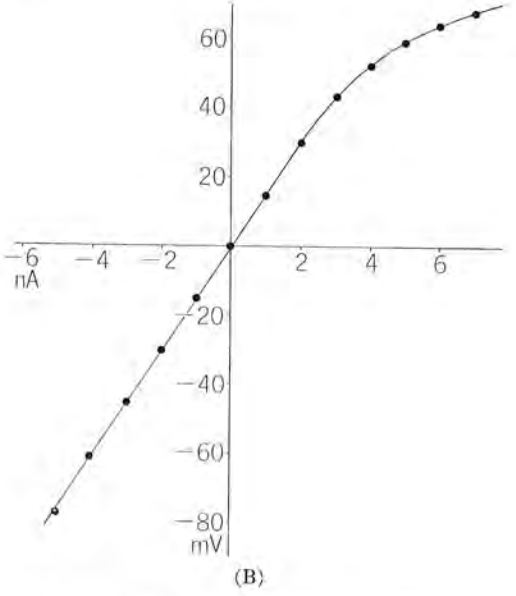
TABLE 1. *Resting membrane potentials of tetanus toxin-treated and untreated NG108–15 cells*[a]

Treatment	Number of cells	Resting membrane[b] potential (mV)	Probability of significant difference
None	52	37.9±1.7	
			0.818
Tetanus toxin (2 μg/ml)	67	38.9±1.4	

[a] Cells were bathed in 120 m*M* Na^+, 2 m*M* Ca^{2+} solution.
[b] Values are means±S.E.M.



(A)



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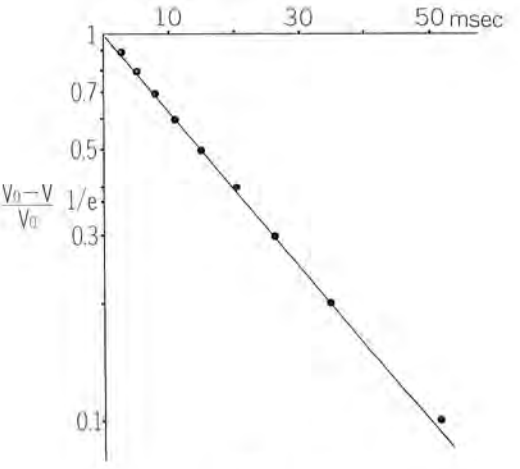


(C)

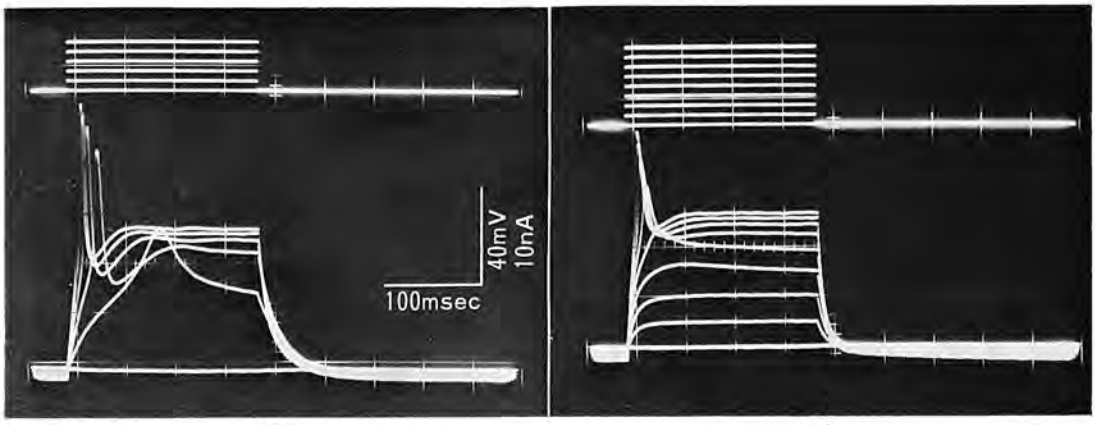
FIGURE 3. Determination of passive electrical membrane properties. A, oscilloscope recordings obtained with an intracellular microelectrode. A cell was stimulated with hyperpolarizing current pulses (upper traces) and the membrane potentials elicited (lower traces) were displayed on an oscilloscope. The resting membrane potential of the cell was −42 mV. B, voltage-current relation. Electrotonic potentials were measured at the end of the current pulse. C, a semilogarithmic plot of the hyperpolarizing voltage transient obtained from a trace of the membrane potential elicited by a current pulse of 3 nA in (A). V_0 represents the voltage change at the end of the current pulse, V is the voltage change at various times after the onset of the current pulse. The abscissa indicates the time after the onset of the current pulse.

Sharp spikes in the action potentials reached a peak within 35 msec after initiation of the depolarizing pulses. The action potential elicited by 5 nA of depolarizing pulse overshot the zero membrane potential by about 15 mV (Fig. 4A). The sharp spikes elicited in 120 m*M*

TABLE 2. *Passive electrical membrane properties[a] of tetanus toxin-treated and untreated NG108–15 cells*

	Untreated cells	Tetanus toxin treated cells	P[b]
Input resistance (MΩ)	24.2±4.9	28.2±5.4	0.582
Time constant (msec)	19.3±1.8	20.1±1.7	0.749
Input capacity (nF)	1.2±0.2	1.28±0.2	0.749
Number of cells	16	15	

[a] Values are means±S.E.M.
[b] Probability of significant difference.



(A) (B)

FIGURE 4. Intracellular recording of Na spikes in tetanus toxin-treated and untreated NG108–15 hybrid cells bathed in 120 m*M* Na^+, 2 m*M* Ca^{2+} solution. A, untreated cell; B, tetanus toxin (2 μg/ml)-treated cell. In both A and B, upper traces show stimulation currents and lower traces show membrane potentials elicited by the currents.

TABLE 3. *Peak potential levels of the Na spike in tetanus toxin-treated and untreated NG108–15 hybrid cells*

Treatment	Number of cells	Peak potential[a] level (mV)	Probability of significant difference
None	25	+14.6±1.6	0.267
Tetanus toxin (2 μg/ml)	30	+12.2±1.4	

[a] Valus are means±S.E.M.

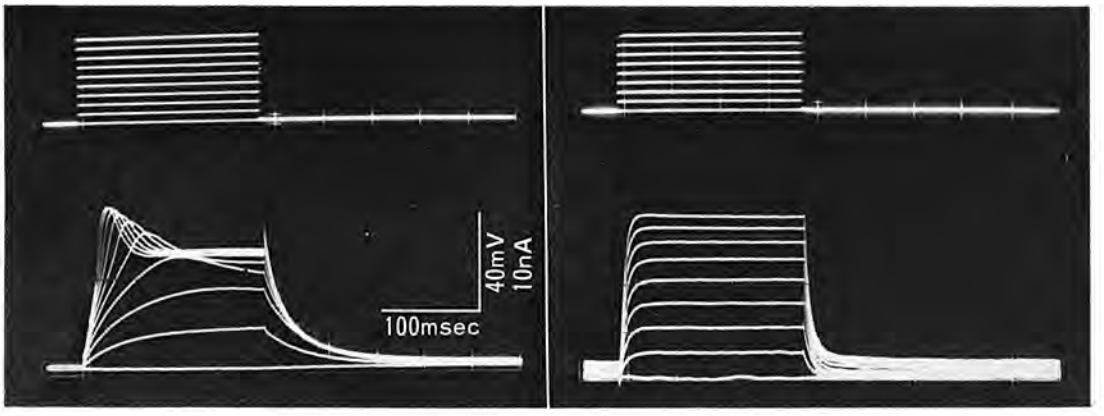
Na^+, 2 m*M* Ca^{2+} salt solution were abolished by adding 3 μ*M* tetrodotoxin, a Na^+ channel blocker, to the extracellular fluid. So the spikes were identified as Na spikes.

In cells treated with tetanus toxin (2 μg/ml) also, the Na spikes were elicited by depolarizing current pulses (Fig. 4B). For quantitative comparison of the Na spikes in tetanus toxin-treated cells with those in untreated cells, the peak potential levels of the spikes elicited by

a depolarizing current of 5 nA were measured. As shown in Table 3, there was no significant difference between the peak potential levels of tetanus toxin-treated and untreated cells. These results indicate that the mechanism generating the Na spike in response to current stimulation of the cells was not affected by tetanus toxin.

4. *Effect of tetanus toxin on action potentials elicited in high Ca^{2+}, Na^{+}-free solution*

Even in high Ca^{2+} (30 m*M*), Na^{+}-free solution, the cells showed active resoponses of membrane potential to depolarizing current pulses, though the responses appeared quite different from those elicited in 120 m*M* Na^{+}, 2 m*M* Ca^{2+} salt solution. Figure 5A shows the graded responses elicited in high Ca^{2+}, Na^{+}-free solution. Slow spikes in the active responses were elicited at 4 nA or more and reached a peak potential more than 28 msec after the beginning of stimulation. The slow spike elicited by 9 nA of current overshot the zero membrane potential by about 4.5 mV. These slow spikes elicited in high Ca^{2+}, Na^{+}-free solution were resistant to tetrodotoxin (3 μM) and were abolished by Co^{2+} (10 m*M*) or Mn^{2+} (10 m*M*). The peak potential levels of the slow spikes depended on the extracellular Ca^{2+} concentration (data not shown). There-



(A) (B)

FIGURE 5. Responses of membrane potential to current stimulation in tetanus toxin-treated and untreated NG108–15 hybrid cells in high Ca^{2+}, Na^{+}-free solution. A, untreated cell; B, tetanus toxin (2 μg/ml)-treated cell. Cells with or without treatment with tetanus toxin were washed and bathed in 30 m*M* Ca^{2+}, Na^{+}-free solution. Recordings were from a microelectrode impaled intracellularly. In A and B, upper traces show stimulation currents and lower traces show membrane potentials elicited by the currents. Note that slow spikes (Ca spikes) were elicited by current stimulation in the untreated cell (A) but not in the tetanus toxin-treated cell (B).

TABLE 4. *Peak potential levels of the Ca spike elicited in high Ca^{2+}, Na^{+}-free solution in tetanus toxin-treated and untreated NG108–15 hybrid cells.*

Treatment	Number of cells	Peak potential[a] level (mV)	Probability of significant difference
None	25	+4.2±1.5	
			$\ll 0.002$
Tetanus toxin (2 μg/ml)	32	−15.8±1.5	

[a] Values are means±S.E.M.

fore, the slow spikes were identified as Ca spikes according to the criterion described by Hagiwara and Byerly (1981).

In contrast to the Na spikes, the Ca spikes in the cells were blocked by treatment with tetanus toxin (2 μg/ml). As shown in Fig. 5B, the responses of the membrane potential of tetanus toxin-treated cells to current stimulation were passive and Ca spikes were not generated even when the stimulation intensity was increased to 8 nA. Table 4 shows that the peak potential levels of the responses in the membrane potential of the cells to current stimulation were greatly suppressed by treatment with tetanus toxin (2 μg/ml).

5. *Relation between toxin dose and peak potential level of Ca spikes in the hybrid cells*

As shown in Fig. 6, the peak potentials of the Ca spikes elicited by depolarizing current stimulation (5 nA) in high Ca^{2+}, Na^{+}-free salt solution varied inversely with the dose of tetanus toxin. A dose of 25 ng/ml caused no suppression of the peak potentials of the Ca spikes, but suppression was detectable at a dose of 45 ng/ml and reached a maximum at more than 250 ng/ml of toxin. The ED_{50} for suppression of Ca spikes in the hybrid cells was 92 ng/ml.

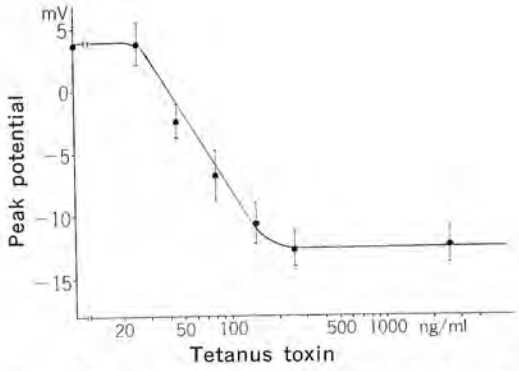
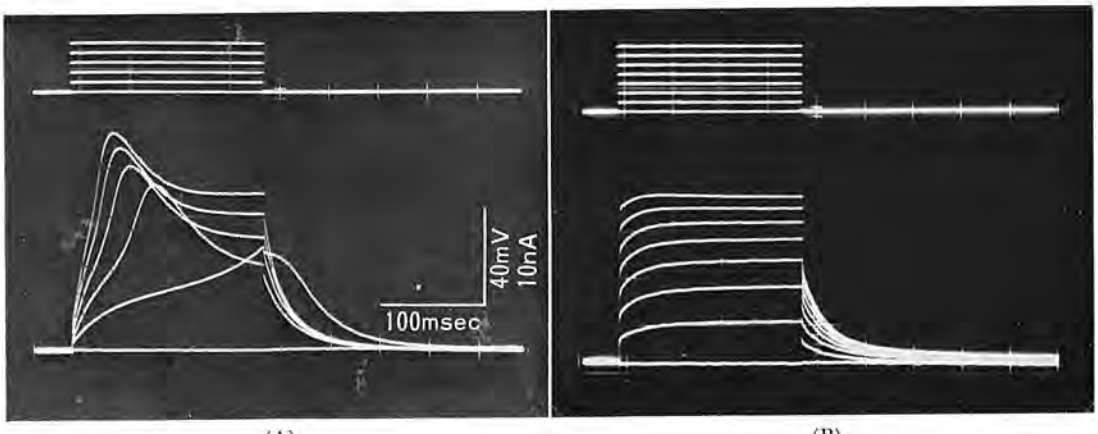


FIGURE 6. Toxin dose-response relationship in suppression of the Ca spike by tetanus toxin in NG108–15 hybrid cells. Cells were treated with various concentrations of tetanus toxin in DMEM for 1 h at 37 C. Then, the medium was replaced by 30 m*M* Ca^{2+}, Na^{+}-free solution for intracellular recording of the Ca spike. Peak levels of the response to a stimulation current of 5 nA were read on an oscilloscope. Symbols and bars show mean values and standard errors for 20 cells.



(A) (B)

FIGURE 7. Responses to current stimulation of the membrane potential of tetanus toxin-treated and untreated NG108–15 hybrid cells bathed in 10 m*M* Ca^{2+}, Na^{+}-free solution containing 15 m*M* TEA. A, untreated cell; B, tetanus toxin-treated cell. In A and B, upper traces show the stimulation current, and lower traces the membrane potentials elicited by the current. Note that the untreated cell showed a more marked Ca spike in solution cotaining TEA than in 30 m*M* Ca^{2+}, Na^{+}-free solution (Fig. 5A) and that the tetanus toxin-treated cell showed no active response even in medium containing TEA.

TABLE 5. *Effect of TEA on peak potential level of the Ca spike elicited in 10 mM Ca^{2+}, Na^{+}-free solution in tetanus toxin-treated and untreated NG108–15 hybrid cells*

TEA (15 m*M*)	Peak potential level[a]		Difference of means	Probability of significant difference
	Untreated cells	Tetanus toxin-treated cells		
Absent	-4.8 ± 1.9 (20)[b]	-16.2 ± 1.9 (20)[b]	11.4	$\ll0.002$
Present	6.8 ± 1.5 (32)[b]	-16.8 ± 1.5 (35)[b]	23.6	$\ll0.0002$

[a] Values are means±S.E.M.

[b] Values in parentheses are numbers of cells examined.

6. *Effect of externally added TEA on the effect of tetanus toxin on Ca spikes in the hybrid cells*

In the presence of 15 m*M* of TEA, a voltage-dependent K^{+} channel blocker, the Ca spikes recorded in 10 m*M* Ca^{2+}, Na^{+}-free salt solution became more marked (Fig. 7A). Under these conditions the Ca spikes elicited by 5 nA of depolarizing current overshot the zero membrane potential by about 7 mV, whereas the mean peak potential recorded in 10 m*M* Ca^{2+}, Na^{+}-free solution without TEA was about −5 mV. Blockade of the Ca spikes by tetanus toxin was not influenced by externally added TEA (Fig. 7B). As shown in Table 5, addition of TEA to the extracellular fluid markedly increased the difference between peak potentials of tetanus toxin-treated and untreated cells.

DISCUSSION

In previous work (Sugimoto et al., 1983; Higashida et al., 1983), we used neuroblastoma clone NlE-115 cells and found that tetanus toxin blocks the Ca spike under high Ca^{2+}, Na^{+}-free conditions. In the present experiments, we extended our previous findings to NG108–15 neuroblastoma×glioma hybrid cells, which release the neurotransmitter acetylcholine (Nelson et al., 1976; McGee et al., 1978). Under refined conditions, we confirmed the selective blockade of the Ca spike by tetanus toxin in the hybrid cells.

In this study, we used cells that were sufficiently differentiated to generate a definite Na spike and adjusted membrane potentials to a steady hyperpolarized level to ensure optimal and uniform electrical responses to depolarizing pulses. Under these conditions, all the control cells responded to stimulation, indicating that the conditions were better than those in previous investigations. Moreover, by adding TEA to the extracellular fluid, we could detect blockade of the Ca spike by tetanus toxin at lower extracellular Ca^{2+} concentrations than those in previous studies.

Experiments on passive electrical membrane properties indicated that tetanus toxin does not injure the cell membrane. The fact that the Na spikes were generated in tetanus toxin-treated cells as well as in untreated cells suggests that the voltage-dependent Na^{+} channels in tetanus toxin-treated cells operate normally. Externally added TEA, a volgate-dependent K^{+} channel blocker, did not influence blockade of the Ca spike by tetanus toxin, while it enhanced the Ca spike in untreated cells, possibly by reducing the counter-current of K^{+} (Katz and Miledi, 1969; Hagiwara and Byerly, 1981). This suggests that tetanus toxin does not affect the TEA-sensitive K^{+} current. Thus the present results suggest that tetanus toxin selectively blocks the voltage-dependent inward Ca^{2+} current in NG108–15 hybrid cells.

The minimum dose of tetanus toxin for blocking the Ca spike in NG108–15 hybrid cells was 250 ng/ml. Though this dose is apparently larger than the dose (ca. 100 pg) required for killing mice by toxin injection, it is similar to, or less than, the doses so far reported to inhibit neurotransmitter release from

particulate brain or brain slices in vitro (Bigalke et al., 1981; Collingridge and Davies, 1982).

NG108–15 hybrid cells have been reported to release acetylcholine in response to various stimulations, such as high extracellular K^+ (McGee et al., 1978) and electrical stimulation (Nelson et al., 1976). Wendon and Gill (1982) using NG108–15 cells reported that tetanus toxin (5 μg/ml) inhibited the release of acetylcholine evoked by high extracellular K^+. NG108–15 hybrid cells were also reported to form functional synapses with striated muscle cells in culture (Nelson et al., 1976). These findings, together with the present results indicate that NG108–15 hybrid cells are a suitable in vitro system for electrophysiological studies on the effect of tetanus toxin on neurotransmitter release in synaptic transmission.

We are examining the action of tetanus toxin on the Ca^{2+} current linked to the release of acetylcholine at synapses formed in vitro by these hybrid cells and striated muscle cells in culture.

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