Vol. 135, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1986 Pages 16-24

# INHIBITION OF MACROPHAGE SECRETION BY TETANUS TOXIN IS NOT DIRECTLY LINKED TO CYTOSOLIC CALCIUM HOMEOSTASIS

John L. Ho * and Mark S. Klempner

The Division of Experimental Medicine, Department of Medicine, Tufts University School of Medicine, Boston, MA.

Received January 6, 1986

Tetanus toxin (TT) inhibits secretion of neurotransmitters from neurons and lysozyme from human macrophages (MØ). Because these secretory events are associated with changes in cytosolic free calcium $[Ca^{2+}]_i$, we examined the effect of TT on MØ calcium homeostasis and secretion in response to ionomycin and phorbol myristate acetate (PMA). Using Quin 2 to report $[Ca^{2+}]_i$, basal $[Ca^{2+}]_i$ was similar for control cells (133 nM) and MØ treated with TT (127 nM). In response to ionomycin (50 nM) $[Ca^{2+}]_i$ increased to 548 + 74 nM in control cells and to 357 + 36 nM in TT-treated MØ ($p < 0.02$, $N = 12$). Despite this rise in $[Ca^{2+}]_i$, neither control MØs nor TT-treated MØs secreted the lysosomal enzyme lysozyme in response to this concentration of ionomycin (50 nM). In both control and TT-treated MØ, stimulation with a higher concentration of ionomycin (1000 nM) caused saturation of the quin 2 fluorescence signal. However, lysozyme secretion from TT-MØ was inhibited. In response to the phorbol ester, PMA (3 uM), $[Ca^{2+}]_i$ did not increase in either control MØ or TT-treated MØ. However, secretion of lysozyme from TT-treated MØ was also inhibited in response to this stimulus (70.8% of control, $p < 0.02$, $N = 3$). These data indicate that the ability of TT to inhibit secretion from MØ is not directly linked to alterations of cytosolic calcium homeostasis. © 1986 Academic Press, Inc.

Tetanus toxin is a potent biologic toxin produced by the anaerobic bacterium Clostridium tetani. The clinical manifestations of tetanus can be wholly attributed to intoxication with tetanus holotoxin. When introduced into the systemic circulation the 150 kilodalton (Kd) holotoxin causes the typical syndrome of spastic paralysis (1,2). There is considerable evidence that tetanus holotoxin inhibits neurotransmitter release and preferentially affects presynatic inhibitory motor neurons (3-10). However, the molecular basis for intoxication with tetanus toxin remains undefined. Since neurosecretion and secretion of macrophage lysosomal granular enzymes share several characteristics we previously examined the human macrophage (MØ) as

*To whom reprint request should be addressed.

0006-291X/86 $1.50
*Copyright © 1986 by Academic Press, Inc.*
*All rights of reproduction in any form reserved.*

a model cell to investigate the molecular basis for tetanus intoxication (11). In that study we demonstrated that tetanus toxin inhibited secretion of the lysosomal enzyme, lysozyme, from human MØs. Changes in the intracellular calcium concentration have been shown to participate in stimulus secretion coupling of many cells including neurons and phagocytic cells (12-16). We therefore wondered whether the inhibition of macrophage lysosomal secretion by tetanus toxin might be related to alterations of $[Ca^{2+}]_i$ homeostasis. Using MØ loaded with the calcium sensitive fluorescent probe, Quin 2 acetoxymethylester (17), we report here the effect of TT on MØ $[Ca^{2+}]_i$ homeostasis in both the basal and stimulated state.

## MATERIALS AND METHODS

Materials. Purified Tetanus holotoxin (TT, 2.5 mg of protein per milliliter) was purchased from Calbiochem (Div. Behring, San Diego, CA). The calcium ionophore, ionomycin, phorbol myristate acetate (PMA) and Quin 2 were obtained from Sigma (St. Louis, MO). Quin 2 acetoxymethyl ester (AM) was purchased from Lancaster Synthesis Ltd. (Windham, NH).

Isolation and culture of macrophages. Mononuclear cells were isolated from heparinized (20 units of heparin/ml) peripheral blood obtained from healthy volunteers using Hypaque-Ficoll density gradient centrifugation (11,18), washed twice with Hanks's balanced salts solution (HBSS) without $Ca^{2+}$ or $Mg^{2+}$ (pH 7.2), and resuspended at a concentration of 4-6 x $10^6$ cells/ml in RPMI-1640 tissue culture medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal calf serum (HyClone Laboratories, Logan, Utah), penicillin (50 units/ml), and streptomycin (50 ug/ml). Adherent monocytes were obtained by differential adherence to glass as previously described (11). Adherent monocytes were then cultured in supplemented medium with or without TT at 1.5 ug/ml (10 nM). After an 18-24 hour incubation, glass vessels were refigerated at 4°C and the cells were harvested by scrapping the culture vessel with a rubber policeman. Control and TT-treated cells from 2-4 vessels were pooled and washed twice with quin buffer containing: NaCl (145 mM), KCl (5 mM), $Na_2HPO_4$ (1 mM), HEPES (5 mM), glucose (5 mM), $MgCl_2$ (1 mM).

Loading of MØ with quin 2 AM. MØs were suspended at 30 to 50 x $10^6$/ml in quin buffer containing $CaCl_2$ (1 mM). Cell suspensions were incubated with quin 2 AM (50 uM) at 37°C for 10 minutes. Quin buffer at three times the initial volume was added and the cell suspensions were incubated for 40-60 minutes. MØs were separated from extracellular Quin 2 AM by centrifugation (150 g) for 10 minutes at 4°C and resuspended in Quin buffer.

Measurement and calculation of $[Ca^{2+}]_i$. The fluorescent response of Quin 2 loaded MØs was measured with a Perkin-Elmer fluorometer (650-10S) using a thermoregulated cell-holder to maintain a constant temperature of 37°C. An excitation wavelength of 339 nm with a slit width of 4 mm was used. MØ at 2 x $10^6$/ml in quin buffer were checked for quin 2 loading by scanning over an emission range of 400 to 500 nm. A peak emission intensity shift from 430 nm to 492 nm confirmed loading with quin 2 AM and the subsequent conversion to quin 2 by cellular esterases. The peak emission was consistantly noted at 492 nm and this wavelength was used to monitor peak fluroescence. For calculation of the intracellular ionized calcium concentration quin 2 loaded MØ at 2 x $10^6$ were suspended in one milliliter of quin buffer with calcium (1 mM) (designated as CaB) or quin buffer

containing EGTA 1mM (LoCaB). The addition of EGTA 1 mM results in a calculated free ionized calcium of < 1 nM (19). Cellular fluorescence stabilized after 4 minutes of continuous recording and was designated as basal fluorescence (F). Maximal fluorescence (Fmax) was recorded when MØ in CaB were disrupted by digitonin (25 uM) and minimal fluorescence (Fmin) was recorded when MØ in LoCaB were disrupted. $[Ca^{2+}]_i$ was calculated using the equation reported by Tsien et al. (17).

To measure changes in $[Ca^{2+}]_i$ in response to different soluble stimuli, basal fluorescence was first recorded for 4 minutes. Stimuli of varying concentrations were then added. Fluorescence was recorded for 2 minutes followed by cell disruption with digitonin (25 uM). For each experiment calibration of fluorescence in CaB was determined using known concentrations of Quin 2. Intracellular Quin 2 was calculated using these calibrations and a cytocrit of 0.15% of th whole suspension (17).

Secretion. Control and TT-treated MØ were suspended at 2 x $10^7$/ml in CaB and LoCaB and stimulated with calcium ionophore, ionomycin or phorbol myristate acetate. After 20 minutes, at 37°C with gentle agitation, the cell free supernatant was obtained by centrifugation at 200 g for 10 minutes at 4°C. Total cellular enzyme content was measured in cell suspensions lysed with 0.2% Triton X-100. Lysozyme, a macrophage lysosomal enzyme (20), was used as an indicator of secretion of granule contents. The percent total enzyme released was calculated by multiplying by 100 the ratio of lysozyme in the cell free supernatant to lysozyme in the 0.2% Triton X-100 lysate. The percent stimulus-induced lysozyme secretion was defined as the percent stimulus-induced lysozyme released minus spontaneous release. Lysozyme was assayed using Micrococcus lysodeikticus as the optical substrate (21). Lactate dehydrogenase (LDH), a cytoplasmic enzyme was used as a marker for cell disruption and was assayed according to the methods of Bergmeyer (22).

Statistical analysis. The two-tailed paired Student's t-test was used.

## RESULTS

Effect of tetanus toxin on basal cytosolic calcium in MØs. In CaB or LoCaB basal $[Ca^{2+}]_i$ of control and TT-treated MØ were not significantly different (C-MØ: 133.8 $\pm$ 7.4 nM and 30 $\pm$ 1.7 nM, TT-MØ: 127 $\pm$ 7.5 nM and 27.2 $\pm$ 0.5 nM), mean $\pm$ SEM of 18-30 separate experiments.

Effect of TT on Ionomycin-stimulated changes in MØ cytosolic calcium. We next examined the effect of TT on cytosolic calcium homeostasis in response to ionomycin. Ionomycin (10 nM and 50 nM) induced a significantly greater $[Ca^{2+}]_i$ in control-MØ than MØ treated with TT (Figure 1 a,b). Higher concentrations of ionomycin (100 or 1000 nM) caused complete saturation of intracellular Quin 2, N = 5. Intracellular Quin 2 concentration was approximately 0.25 mM. In LoCaB, ionomycin (0.1 uM and 1.0 uM) stimulated a significantly greater increase of $[Ca^{2+}]_i$ in control MØ than TT-treated MØ (Fig. 2).

Dose-dependence of ionomycin induced secretion from MØs. We examined the dose-dependent effect of ionomycin on MØ secretion. In response to

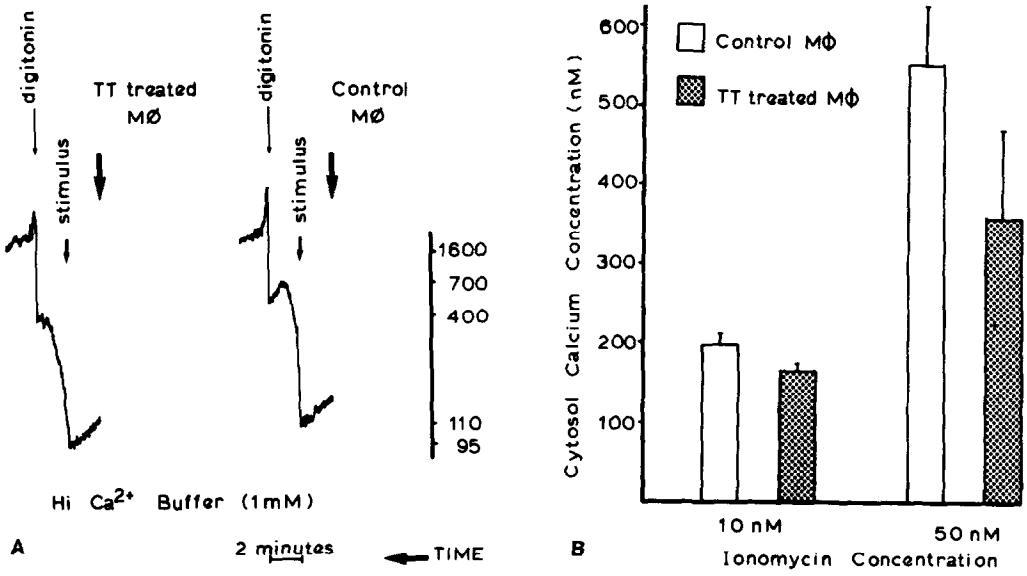



Figure 1. Effect of TT on ionomycin-induced cytosolic calcium in MØs.

MØs were incubated with and without TT (1.5 ug/1m) for 20-24 hours, washed and resuspended in toxin free buffer containing calcium (CaB, 1 mM) or depleted of calcium (LoCaB, EGTA 1 mM; $[Ca^{2+}]$ = 1 nM).

A. In CaB, ionomycin induced a rapid increase of cytosolic free calcium in control and TT-treated MØ.

B. The rise of cytosol free calcium in response to ionomycin was inhibited in TT-treated MØ $p < 0.02$. Results are mean ± SEM of 10-12 separated experiments. Ionomycin at 100 nM or greater caused saturation of intracellular Quin 2 or both control and TT-treated MO.

ionomycin (0.1 uM) control-MØ and MØ treated with TT suspended in CaB` did not secrete lysozyme (Fig.3). However, this concentration of ionomycin caused saturation of intracellular Quin 2 from both control and TT-treated

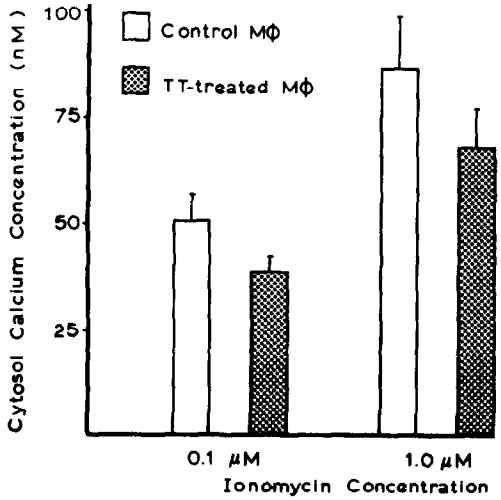
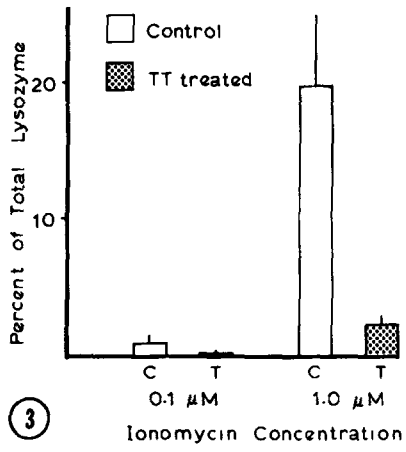



Figure 2. Effect of TT on MØ cytosol free calcium in low calcium buffer.

In LoCaB cytosol free calcium response to ionomycin was inhibited in TT-treated MØs, $p < 0.05$. Results are mean ± SEM of 13-16 separate experiments.



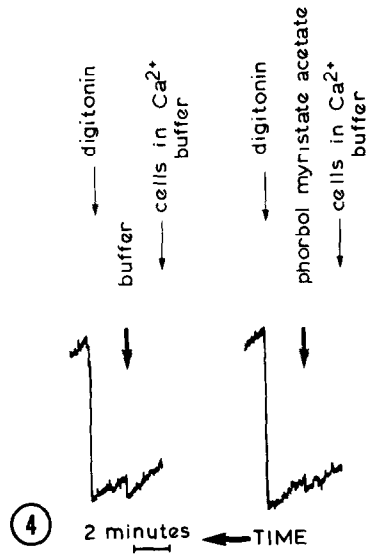




Figure 3. Dose-dependence of ionomycin induced MØ secretion.
C-MØs and TT-treated MØ (1.5 ug/ml) were suspended in CaB and stimulated with ionomycin. Ionomycin at 0.1 uM did not induce secretion even though intracellular quin 2 was saturated. Higher concentration of ionomycin induced secretion from control MØs and inhibited secretion from TT-treated MØs, $p < 0.05$. Spontaneous release of lysozyme from C-MØs and TT-treated MØs was similar, 11.9 ± 1.0% and 13.4 ± 1.4% respectively. LDH release from C-Mφs and TT-treated MØs was similar, 5.8 ± 0.6% and 5.4 ± 0.5% respectively. The results are the mean ± SEM from four separate experiments.

Figure 4. Effect of PMA on MØ cytosol free calcium in MØs.
Similar fluorescence by Quin 2 loaded cells was recorded in response to the addition of PMA (3 uM) or buffer (10 ul). In both TT-treated or control MØs, no detectable increase of cytosolic calcium was observed in response to PMA in CaB or LoCaB, N = 4.

MØs. When stimulated with a higher concentration of ionomycin (1.0 uM), MØs secreted lysozyme which was inhibited by treatment with TT, $p < 0.05$, N = 4. Similar results were observed with Quin 2 loaded MØs.

Effect of TT on PMA-stimulated calcium homeostasis in MØs. We next investigated $[Ca^{2+}]_i$ homeostasis of control and TT-treated MØ in response to PMA. PMA did not induce a detectable $[Ca^{2+}]_i$ elevation in either control or TT-treated MØ. An illustrative recording of PMA stimulated MØ is shown on Figure 4. Compared to the addition of buffer as a control for the effect of stirring the cells, the addition of PMA (3 uM) produced no discernable difference in Quin 2 fluorescence.

Effect of extracellular calcium on PMA-stimulated MØ secretion. In response to PMA (3 uM) control MØs suspended in CaB or LoCaB, secreted a

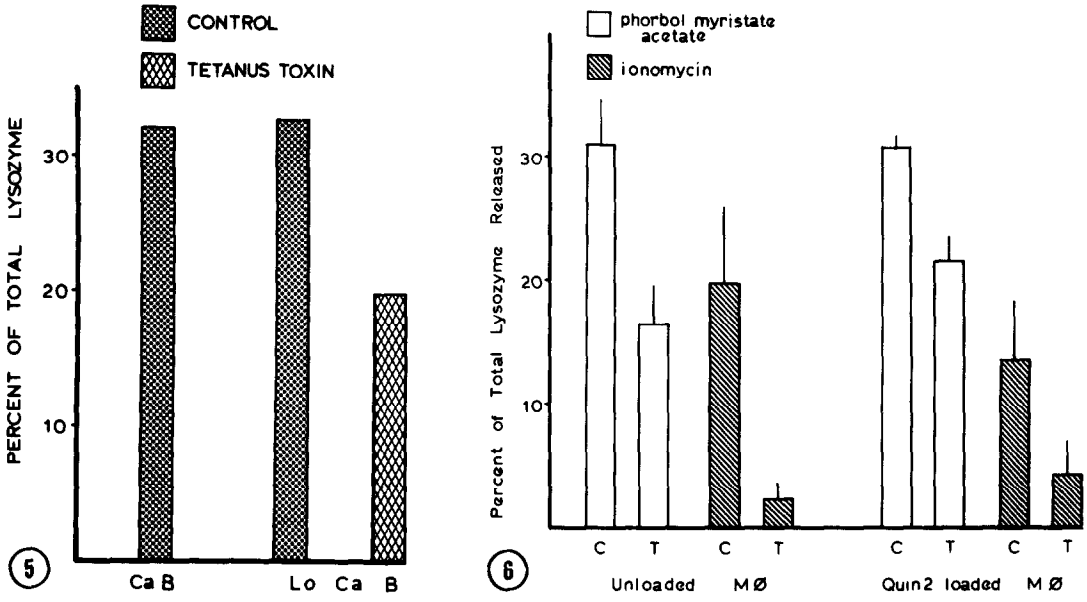



Figure 5. Effect of extracellular calcium on PMA-stimulated MØ secretion.

PMA (3 uM) - induced secretion was not affected by depletion of extracellular calcium. Moreover, in response to PMA, secretion from MØ treated with TT was inhibited ($p < 0.02$). The results are mean ± SEM of 3 experiments. Spontaneous release of lysozyme was similar in CaB or LoCaB from control and TT-treated MOs (C-MØ: 12.9 ± 0.2%, 11.1 ± 0.9%; TT-MØ: 12.0 ± 0.2%). LDH release in calcium containing or depleted medium from C-MØs and TT-MØ were similar (C-MO: 8.0 ± 0.7%, 9.2 ± 1.3%; TT-MO: 11.9 ± 2.4%).

Figure 6. Effect of Quin 2 loading on MØ secretion.

MØs were incubated overnight with and without TT (1.5 ug/ml), resuspended in toxin-free buffer and loaded with Quin 2AM (50 uM). Split cell samples unloaded with Quin 2 demonstrated similar percent of total lysozyme secretion in response to PMA (3 uM) and ionomycin (1 uM). In addition, Quin 2 loading did not alter the inhibition of MØ secretion by TT. The percent of stimulus-induced lysozyme secretion was defined as the percent stimulus-induced lysozyme release minus spontaneous release. The spontaneous release of lysozyme from control and TT-treated MØ without and with Quin 2 incorporation were similar (C-MØ: 11.9% ± 1.0%, 5.4% ± 0.6%; TT-MØ: 13.4% ± 1.4%, 4.1 ± 2.1%; respectively without and with Quin 2). LDH from control and TT-treated MØ without and with Quin 2 loading were respectively, C-MØ: 5.8% ± 0.6%, 4.9% ± 0.5%; TT-MØ: 5.4% ± 0.5%, 3.9% ± 1.0%. Results are the mean ± SEM of three separate experiments.

similar percentage of lysozyme (Fig. 5). However, secretion of lysozyme from TT-treated MØs was inhibited in response to this stimulus in CaB or LoCaB (70.8% and 70.2% of control). Thus, the inhibition of MØ secretion by TT in response to PMA (3 uM) was independent of extracellular $[Ca^{2+}]_i$.

Secretion of Quin 2 loaded MØ. PMA (30 or 3 uM) and ionomycin (1 uM) induced comparable percent of lysozyme secretion from MØ not loaded and MØ loaded with Quin 2 (Fig. 6). Moreover, TT-treated MØ that had been loaded

with Quin 2 continued to demonstrate inhibition of lysozyme secretion in comparison to control MØ (Fig. 6).

## DISCUSSION

Tetanus toxin inhibits secretion of neurotransmitters from neurons (3-10) and secretion of the lysosomal enzyme, lysozyme, from human MØs (11). Several events that couple cell stimulation to secretion are similar in neurons and pnagocytic cells. These include ligand-receptor interactions, phospholipid metabolism and the generation of cyclic nucleotides (12-14,23). Calcium homeostasis is also thought to play a role in stimulus secretion coupling in both systems. All cells investigated thus far maintain a cytosolic free $[Ca^{2+}]$ that is approximately 10,000-fold lower than the extracellular environment. In response to a variety of stimuli, $[Ca^{2+}]_i$ increases prior to subsequent cell response (eg., secretion). In this report we investigated the effect of TT on MØ calcium homeostasis.

Using Quin 2 as a probe to examine intracellular calcium nomeostasis, basal $[Ca^{2+}]_i$ in calcium containing buffer (1 mM) was approximately 130 nM and was comparable to that reported in numan monocytes by Scully et al (25). TT did not alter resting $[Ca^{2+}]_i$ of human MØs. In response to ionomycin, a calcium dependent stimulus, $[Ca^{2+}]_i$ increased in a dose-dependent manner. TT inhibited the rise in $[Ca^{2+}]_i$ in response to a low concentration of ionomycin. However, ionomycin at 0.1 uM or higher, caused a similar rise in $[Ca^{2+}]_i$ from control MØs and MØs-treated with TT. Moreover, we observed a dissociation of the $[Ca^{2+}]_i$ response and MØ secretion since ionomycin (0.1 uM) induced a $[Ca^{2+}]_i$ response that saturated the intracellular Quin 2 elicited no secretion. The dissociation between maximum $[Ca^{2+}]_i$ response and cell function has also been observed by Korcnak et al (26) in human neutorphils, another phagocytic cell.

The observation that TT did not alter resting $[Ca^{2+}]_i$ is consistent with that reported by Sugimoto et al (27). These autnors noted similar

resting membrane potential in untreated and TT-treated neuroblastoma cells. The major contribution to the resting membrane potential was due to the transmembrane $Ca^{2+}$ gradient because these cells were suspended in $Na^{+}$ free buffer, the resting membrane potential was resistant to tetrototoxin, a voltage dependent $Na^{+}$ channel blocker and was abolished by $Ca^{2+}$ antagonists, $Co^{2+}$ and $Mn^{2+}$ (10 mM) (27).

Our observation that TT alters $[Ca^{2+}]_i$ homeostasis in response to low concentrations of ionomycin but not in response to higner concentrations ($\geq$0.1 uM) of this ionopnore cannot be compared to the findings of Sugimoto et al. These autnors suspended control and TT-treated NIE-105 cells in varying concentrations of calcium and measured, by micropuncture technique, their action potential in response to a constant electrical pulse (1 nA). In experiments using high extracellular calcium (7.2 mM to 80 mM) but not lower or physiological calcium concentrations, Sugimoto et al (27) found a significantly lower peak Ca spike potential from TT-treated neuroblastoma cells than control cells. Moreover, neurosecretion was not assayed.

Neither control MØs no TT-treated MØs demonstrated measurable alteration of $[Ca^{2+}]_i$ in response to PMA. However, this stimulus elicited secretion from control-MUs which was inhibited in TT-treated MØs. The lack of a $[Ca^{2+}]_i$ increase in response to PMA has been demonstrated in other systems (28,29). These results suggest that TT inhibits MØ secretion at a step distal to the rise of cytosolic calcium observed following some stimuli. The ability of the toxin to inhibit PMA induced secretion raises the possibility that protein kinase C activity, the likely receptor for phorbol esters, is interfered with in tetanus intoxicaton.

ACKNOWLEDGMENT

This work was supported in part by grants AI-22145 and AI-16732 from the National Institute of Allergy and Infectious Disease, U.S. Army contract DAMD17-85C-5280 and Gastroenterology Research on Absorptive and Secretory Processes, Public Health Service grant P30AM39428-01.

## REFERENCES


1. Holmes, M.J., and Ryan, W.L. (1970) Infect. Immun. 3,133-40.
2. Matsuda, M. and Yoneda, M. (1975) Infect. Immun. 12,1147-53.
3. Mellanby, J., and Green, J. (1981) Neuroscience 6,281-300.
4. Curtis, D.R., Felix, D., Game, C.J.A., and McCulloch, R.M. (1973) Brain Res. 51,358-62.
5. Osborne, R.H., Bradford, H.F. (1973) Nature; New Biology 244,157-8.
6. Collingridge, G.L., Collins, G.G.S., Davies, J., James, T.A., Neal M.J., and Tongroach, P. (1980) J. Neurochem. 34,540-7.
7. Bigalke, H., Heller, I., Bizzini, B., and Habermann, E. (1981) Naunyn Schmiedebergs Arch. Pharmacol. 316,244-51.
8. Wendon, L.M.B., and Gill, D.M. (1982) Brain Res. 238,292-7.
9. Bizzini, B. (1977) In: Cuatrecasas P., ed. The specificity and action of animal, bacterial and plant toxins. Receptors and recognition. Series B. Vol. 1. pp. 177-218, Chapman and Hall, London.
10. Habermann, E., (1978) In: Vinken, P.J., Bruyn, G.W., eds. Part 1. Handbook of clinical neurology. Vol. 33. pp. 491-547, Amsterdam, North Holland.
11. Ho J.L., and Klempner, M.S. (1985) J. Infect. Dis. 152,922-29.
12. Kanamimoff, R., Erulkar, S.D., Lev-Tor, A. and Meir, H. (1978) Ann. N.Y. Acad. Sci. 307,583-98.
13. Neering, I.R., and McBurney, R.N. (1984) Nature 309,158-160.
14. Berridge, M.J. (1982) In: Cheung, W.Y. ed. Calcium and cell function. Vol. 3. pp. 1-26, Academic Press, New York.
15. Nishizuka, Y. (1984) Science 225,1365-9.
16. Wright, D. (1982) In: Gallin J.I., Fauci, A.S., eds. Phagocytic cells. Advances in host defence mechanisms. Vol. 1. pp. 75-110, Raven Press, New York.
17. Tsien, R.Y., Possan, T., and Rink, T.L. (1982) J. Cell. Biol. 94,325-34.
18. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21 (suppl), 77-89.
19. Klempner, M.S. (1985) J. Clin. Invest. 76,303-10.
20. Edelson, P.J. (1980) In: Weissman, G., ed. Handbook of inflammation. Vol. 2., pp. 469-95, Elsevier North Holland Biomedical Press, Amsterdam.
21. Litwick, G. (1955) Proc. Soc. Exp. Biol. Med. 89,401-5.
22. Bergmeyer, H.U., Bernt, E., and Hess B. (1963) In: Bergmeyer, H.U., ed. Methods of enzyme analysis. pp. 737-9, Academic Press, New York.
23. Takai, Y., Kikkawa, U., Kaibuchi, K, and Nishizuka Y. (1984) In: Greengard, P., and Robison, G.A. Adv. Nucleotide Res. pp.119-58, Raven Press, New York. Rasmussen, H., Kojima, I., Kojima, K., Zawalich, W., and Apfeldorf, W. pp. 159-195, Ibid.
24. Scully, S.P., Segel, G.B., and Lichtman, M.A. (1984) J. Clin. Invest. 74,589-99.
25. Sugimoto, N., Higashida, H., Ozutsumi, K., Miki, N., and Matsuda, M. (1983) Biochem. Biophys. Res. Comm. 115,788-93.
26. Korchak, H.M., Vienne, K., Rutherford, L.E., Wilkenfeld, Finkelstein, M.C., and Weissmann, G. (1984) J. Biol. Chem. 259:4076-82.
27. DiVirgilio, F., Lew, D.P., and Pozzan, T. (1984) Nature 310,691-93.
28. McNeil, P., McKenna, M.P., and Taylor, D.L. (1985) J. Cell Biol. 101,372-79.