

MECHANISM OF ACTION OF TETANUS TOXIN

ANNUAL REPORT

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) We examined an in vivo system to explore whether protein kinase C (PKC) activity is diminished when animals become intoxicated with tetanus toxin (TT). Homogenates of brain and spinal cord from mice manifesting generalized tetanus after receiving a 50x minimal lethal dose of TT were assayed for PKC activity. PKC activity (p-mole per mg protein per min) was similar in cytosol and membrane fractions of brain from control (C) and TT-intoxicated mice. Cytosolic PKC activity in the spinal cord was depressed in TT-intoxicated mice, 87.3±25, compared to control, 182.6±47, p<0.02, n=7. Membrane associated PKC activity in the spinal cord was similar in C- and TT-intoxicated mice. Cytosolic PKC activity was similar in C- and TT treated macrophages as well as in C- and TT-intoxicated mice from both the brain and the spinal cord. These data could be linked to our previous finding of TT-induced inhibition of lysosomal secretion in human macrophages. However, in subsequent experiments, we were unable to demonstrate any direct interaction of TT with purified PKC. Because of our recent substantial progress in purification and characterization of the human neutrophil			
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lysosomal  $\text{Ca}^{++}$  pump, we turned our investigation towards the observation that TT directly stimulates this pump. Tetanus toxin significantly increased ATP-dependent  $\text{Ca}^{++}$  uptake by human neutrophil lysosomes in a saturable and dose-dependent manner. An increase of 59% was observed at a tetanus toxin concentration as low as 1 ug/ml. An increase of 425% corresponded to a tetanus toxin concentration of 20 ug/ml, above which no further increase could be achieved. Heat-inactivated tetanus toxin or bovine serum albumin did not enhance neutrophil lysosome  $\text{Ca}^{++}$  uptake. A monoclonal antibody specific for the light chain of the toxin molecule completely inhibited the observed effect. In contrast, monoclonal antibodies for the C fragment and for the heavy chain of the B fragment did not alter the ability of tetanus toxin to augment  $\text{Ca}^{++}$  uptake. Increased ATP-dependent  $\text{Ca}^{++}$  influx was responsible for the stimulated  $\text{Ca}^{++}$  uptake;  $\text{Ca}^{++}$  efflux was unaffected. The stimulation of  $\text{Ca}^{++}$  uptake by lysosomes was irreversible only if ATP was present during the incubation of lysosomes with tetanus toxin, indicating that the toxin possibly acts as a kinase capable of either directly phosphorylating the neutrophil lysosomal  $\text{Ca}^{++}$  ATPase or indirectly activating it by phosphorylation of a regulatory intermediate.

## TABLE OF CONTENTS

Text, Annual Report . . . . .	1 - 4
Figure 1, Brain protein kinase C activity from control (C) and TT-intoxicated (TT) mice . . . . .	5
Figure 2, Spinal cord protein kinase C activity from control (C) and TT-intoxicated (TT) mice . . . . .	6
Figure 3, Dose-response curve of the effect of TT on ATP-dependent $^{45}\text{Ca}$ uptake of human PMN lysosomes . . . . .	7
Figure 4, Effect of TT, B-TT, BSA, and B-BSA (each at a protein concentration of 1 ug/ml) on ATP-dependent $\text{Ca}$ uptake of human PMN lysosomes. . . . .	8
Figure 5, Structure of the molecule of tetanus toxin and the binding domains of the monoclonal antibodies used in the presented experiments. . . . .	9
Figure 6, Effects of three neutralizing monoclonal Ab on ATP-dependent calcium uptake of lysosomes . . . . .	10
Figure 7, Effect of TT (1 ug/ml) on calcium efflux from lysosomes. . . . .	11
Figure 8, Irreversibility of the effect of TT (1 ug/ml) on ATP-dependent $^{45}\text{Ca}$ uptake of human PMN lysosomes . . . . .	12
Figure 9, TT has an irreversible effect on calcium uptake in the presence of ATP . . . . .	13
Figure 10, Inhibitory effect of TFP on ATP-dependent calcium uptake of control (C) and TT-treated (TT) lysosomes . . . . .	14
Figure 11, Effect of TFP (100 $\mu\text{M}$ ) on the irreversible action of TT (1 ug/ml) on the calcium uptake by lysosomes . . . . .	15
Reference . . . . .	16
Distribution List . . . . .	17

Since in the macrophage model we found that TT treatment resulted in a dose-dependent decrease of protein kinase C activity without measurable effect on protein kinase A activity (see 1986 annual report), we next examined the effect of TT on protein kinase activity in neural tissue obtained from mice exhibiting generalized tetanus.

TT at  $10^{-6}$  mg induced generalized tetanus with progressive rigidity, pilo- and tail erection, depressed respiration and death at less than 16 hours. These findings occurred at progressively longer times with lower TT doses. Animals given  $10^{-7}$  and  $10^{-8}$  mg of TT demonstrated generalized tetany and death at 40 and 67 hours respectively, while animals given lower doses of TT or media survived. The calculated MLD was  $10^{-8}$  or 0.6 ng per kg. Based on these findings, a 50x minimal lethal dose ( $5 \times 10^{-7}$  mg) was used for subsequent experiments (10,11).

Figure 1 illustrates the PKC activity measured from mouse brain. Cytosol PKC activity from the brain was similar for control and mice intoxicated with 50x MLD of TT holotoxin. Membrane associated (particulate) PKC activity from the brain was also not significantly different between control and animals given TT 50x MLD (n=7). In contrast, compared with control mice, cytosolic PKC activity was significantly diminished in the spinal cord (Figure 2). PKC activity from the spinal cord particulate fraction was similar in control and TT-intoxicated mice, suggesting that redistribution of PKC from the cytosol to membrane associated fraction was not the explanation for the diminished cytosolic PKC activity. To further eliminate this possibility, the percent of total PKC activity localized in the particulate fraction was calculated and the values were not significantly different between control and TT-intoxicated mice.

To examine whether the diminution of PKC activity in TT-intoxicated mice was specific, we assayed for cyclic-AMP-dependent protein kinase (PKA) in the same cytosolic fractions. PKA activity was similar in control and TT-intoxicated animals. However, in the same cytosolic fraction from spinal cords of TT-intoxicated mice, PKC activity was diminished (10,11).

Protein kinase C has distinct regulatory and catalytic domains. Phorbol esters bind specifically to the PKC regulatory domain resulting in increased enzymatic activity. We used  $^3\text{H}$ -PdBu to determine whether the differences in PKC activity were due to a decrease of total PKC. In four separate experiments, cytosolic PKC assayed by bound  $^3\text{H}$ -PdBu was similar in brain and spinal cords of control and TT-intoxicated mice. These data suggested that the diminished PKC activity in the spinal cord was not due to a diminution of the phorbol binding domain of PKC. The data were presented at the 1987 National Meeting of the AAP/ASCI/AFCR (10) and are all published in The Journal of Infectious Diseases (11).

While these data provided a possible causal link between the ability of TT to inhibit secretion (1) and to decrease PKC activity, there were several alternative explanations for these observations. Perhaps of greatest concern was fulfillment of the generally held requirement for TT to behave in an enzymatic fashion since relatively few molecules are necessary to intoxicate a cell. We therefore sought evidence that TT directly interacted with PKC (but not PKA) in a series of experiments using crude preparations containing either MO cytosolic or membrane associated PKC activity. Despite successful

demonstration of specific  $^3\text{H}$ -PCBu binding to the membrane fraction and the presence, in low concentration, of PKC activity in concentrated cytosolic fractions, we were unable to inhibit PKC activity with TT (holotoxin). Because these negative results could have been because of a requirement for "intracellular processing" of the toxin molecule, toxin fragments resulting from limited papain digestion were also tried, but also failed to inhibit PKC activity. We next examined whether an inhibitor might be present which interfered with the TT-PKC interaction. Purified PKC was the generous gift of Dr. Kuo at the CDC. However, even with the purified enzyme, in the presence of phospholipid and  $\text{Ca}^{++}$ , TT did not inhibit PKC activity. Because we had recently made substantial progress in purifying and characterizing the calcium uptake pump which we described in human neutrophil lysosomes, we turned our attention towards further investigation of the observation that TT directly stimulated this pump (8,9,13).

Despite our finding that TT inhibited secretion in MO, we were strongly urged to focus on the interaction of TT with a relatively clean system such as isolated (neutrophil) lysosomes instead of an ill defined system of broken cell fragments, mitochondria, and other cellular detritus such as the theoretical microsomal pellet of macrophages. Furthermore, as part of our ongoing studies under a NIH grant (AI16732), we have been actively studying this lysosomal ATP-dependent  $\text{Ca}^{++}$  uptake pump, have made substantial progress towards purifying this enzyme, and we are investigating the role of lysosomes in calcium storage and homeostasis (9,12,13). While TT did not inhibit secretion by means of altered cytosolic calcium homeostasis, at low concentrations of ionomycin, there was a decrease in the rise in cytosolic  $\text{Ca}^{++}$  (2). For all of these reasons our efforts have been directed at closely examining the interaction of TT with the lysosome  $\text{Ca}^{++}$  ATPase.

In a series of experiments, whose methods are detailed in the appendix manuscript, we found that incubation of human neutrophil lysosomes at  $37^\circ\text{C}$  for 10 min with pure TT increased their ATP-dependent  $\text{Ca}^{++}$  uptake in a dose-dependent manner (Figure 3). At a TT concentration of 1  $\mu\text{g}/\text{ml}$ ,  $^{45}\text{Ca}$  uptake was  $59.40 \pm 6.73\%$  ( $n=15$ ) greater than the uptake by lysosomes incubated with buffer alone. At a TT concentration of 20  $\mu\text{g}/\text{ml}$ , the uptake increased by  $425.30 \pm 38.22\%$  ( $n=4$ ). Higher concentrations of TT did not further increase  $\text{Ca}^{++}$  uptake. The background uptake of  $^{45}\text{Ca}$  in the absence of ATP was unaffected by TT.

We next examined whether the augmentation of  $\text{Ca}^{++}$  uptake could be produced by heat-inactivated tetanus toxin. As shown in Figure 4, boiled TT (B-TT) at 1  $\mu\text{g}/\text{ml}$  decreased  $^{45}\text{Ca}$  uptake by  $0.46 \pm 7.06\%$  ( $n=9$ ). The difference between B-TT and TT at 1  $\mu\text{g}/\text{ml}$  ( $65.15 \pm 9.94\%$ ,  $n=9$ ) was statistically significant ( $P<0.001$ ). In the same set of experiments, we examined whether the effect of TT could be produced by another protein, i.e. bovine serum albumin (BSA). As illustrated in Figure 3, BSA (1  $\mu\text{g}/\text{ml}$ ) decreased  $^{45}\text{Ca}$  uptake by  $12.26 \pm 13.79\%$  ( $n=3$ ). Boiled BSA (B-BSA) produced essentially the same effect as BSA, i.e. a decrease by  $9.71 \pm 22.61\%$  ( $n=3$ ). The differences between the effect of TT and those of BSA and B-BSA were statistically significant ( $P<0.005$  and  $P<0.02$ , respectively).

The next set of experiments was designed to study whether monoclonal antibodies provided by Dr. William Habis (Chief, Laboratory of Bacterial Toxins, FDA) would block the observed effect of TT. The binding domains of the

monoclonal Ab used in our experiments are indicated in Figure 5. As shown in Figure 6, TT at 1 ug/ml elicited an increase in  $^{45}\text{Ca}$  uptake by  $60.63 \pm 7.11\%$  ( $n=14$ ), whereas in the presence of Ab 21.83.4 (specific for the light chain) a decrease of  $6.97 \pm 5.79\%$  ( $n=10$ ) was observed. The difference between these two conditions was found to be statistically significant ( $P<0.001$ ). Two other monoclonal antibodies, one (18.1.7) specific for C fragment and one (21.76.10) specific for the heavy chain of B fragment, did not change the effect of TT. When the lysosomes were incubated with each of the antibodies alone, no effect was observed. In addition, when lysosomes were incubated with TT in the presence of BSA instead of an Ab, the effect of TT was unchanged (Figure 6).

Termination of the action of ATP on  $\text{Ca}^{++}$  uptake by the addition of a dextrose/hexokinase mixture allowed us to demonstrate that the effect of TT was due to increased  $\text{Ca}^{++}$  uptake and not to depressed  $\text{Ca}^{++}$  efflux. As illustrated in Figure 7, lysosomes treated with TT (1 ug/ml) showed no significant difference in  $^{45}\text{Ca}$  efflux from control lysosomes both at 30 sec ( $36.01 \pm 5.77\%$ ,  $n=3$ , and  $32.31 \pm 10.01\%$ ,  $n=3$ , respectively) and at 60 sec ( $46.35 \pm 7.85\%$ ,  $n=3$ , and  $36.93 \pm 11.47\%$ ,  $n=3$ , respectively).

One of the consistent in vivo features of intoxication with TT is its long duration of action. In vitro, the effect of TT on neural tissue preparations is so longlasting that new neurite outgrowth is required before neurotransmitter release can again occur. It was therefore important to determine the reversibility of the interaction of TT with the lysosomal pump. In initial experiments, lysosomes were incubated with TT, washed and then incubated with  $^{45}\text{Ca}$  and ATP. As illustrated in Figure 8, lysosomes incubated in the absence of TT, washed, and then incubated in the presence of TT (column A) showed an increase in  $^{45}\text{Ca}$  uptake by  $90.25 \pm 12.85\%$  ( $n=11$ ). Similarly, lysosomes incubated in the presence of TT, washed, and then incubated again in the presence of TT (column B) showed an increase in  $^{45}\text{Ca}$  uptake by  $95.54 \pm 31.34\%$  ( $n=5$ ). In contrast, lysosomes incubated in the presence of TT, washed, and then incubated in the absence of TT (column C) did not show any difference in  $^{45}\text{Ca}$  uptake from control lysosomes ( $-0.50 \pm 9.87\%$ ,  $n=11$ ). Washing lysosomes which had been preincubated with TT completely reversed the effect of the toxin on  $\text{Ca}^{++}$  uptake. Because our current understanding of the lysosomal  $\text{Ca}^{++}$  ATPase involves an integral regulatory protein which is activated by phosphorylation, we next examined whether lysosomes preincubated with TT plus a phosphate donor, like ATP, would show the effect of TT even after washing. Consistent with our hypothesis, the TT effect was completely irreversible when ATP was included in the preincubation. As shown in Figure 8, lysosomes incubated in the presence of TT and ATP (1 mM), washed, and then incubated in the absence of TT (column D) showed an increase in  $^{45}\text{Ca}$  uptake by  $225.38 \pm 60.31\%$  ( $n=9$ ). In fact, the effect of TT on  $\text{Ca}^{++}$  uptake was accentuated by preincubation with ATP because ATP alone had a stimulatory effect. We believe that this is related to an endogenous protein kinase in the lysosomal membrane which is capable of phosphorylating the regulatory substrate similar to the protein kinase activity previously described in SR membranes (3-6). That the effect of TT could be made irreversible by including ATP in the preincubation media strongly suggests that TT mediates phosphorylation of a regulatory subunit of the  $\text{Ca}^{++}$  ATPase. The methods used for all of the above experiments are described in detail in the appendix manuscript.

Our hypothesis was strengthened by experiments using TT preincubated with lysosomes in the presence of an ATP analogue, in which the phosphates are not

hydrolyzable and therefore unavailable for substrate phosphorylation (7). As shown in Figure 9, preincubation of lysosomes with TT and ATP markedly increased  $\text{Ca}^{++}$  uptake, whereas TT plus the nonhydrolyzable analogue AMP-PNP produced a modest decrease in uptake. AMP-PNP alone had the same effect as TT+AMP-PNP and was different from preincubation of lysosomes with ATP alone. This latter result also strengthens our concept of the intrinsic lysosomal membrane kinase which can regulate the activity of the lysosomal  $\text{Ca}^{++}$  uptake pump.

Our ongoing study, "Purification and Characterization of the Neutrophil Lysosomal  $\text{Ca}^{++}$  ATPase", revealed that calmodulin copurifies with the lysosomal  $\text{Ca}^{++}$  ATPase. Attempts to dissociate calmodulin from the enzyme destroys virtually all of its activity which is not restorable by exogenous calmodulin (13). This is reminiscent of the sarcoplasmic reticulum  $\text{Ca}^{++}$  ATPase (3,5). We therefore asked whether the calcium binding protein inhibitor trifluoroperazine (TFP) could block the activation of the  $\text{Ca}^{++}$  ATPase by TT. As shown in Figure 10, TFP at concentrations (50 and 100  $\mu\text{M}$ ) which inhibit calmodulin dependent reactions (including kinase activity), completely blocked the calcium uptake and therefore the TT effect. However, preincubation of lysosomes with TT, ATP, and TFP, followed by washing, did not alter the irreversible effect induced by preincubation of the lysosomes with TT and ATP (Figure 11). The above findings suggest that  $\text{Ca}^{++}$  uptake is a calmodulin-dependent process but that the effects of TT (with ATP) proceed by a calcium binding protein independent reaction which activates the pump. More precisely, at this point we can say that the irreversible modification of the calcium pump induced by TT plus ATP is TFP insensitive and we will further explore the role of calcium binding proteins and cations in future experiments.

We believe it is important to confront and comment directly on the relationship between the effects of TT on the lysosomal calcium ATPase and the action of TT in the central nervous system. We in no way mean to imply that identifying how TT modifies the  $\text{Ca}^{++}$  ATPase can be immediately extended to a causal relationship with inhibited secretion from neurons. We do, however, know of no other intracellular biochemical effect which has been defined for TT and believe that this system can provide clues pointing to the mechanism of tetanus toxin action. While the intracellular substrates may differ between cell types, the mechanisms by which these substrates are modified by TT should be shared. Indeed, there is substantial evidence, which we reviewed in the previous application, that the overall cascade of events leading to secretion is similar among neurons, phagocytes, and chromaffin cells (all of which can be intracellularly intoxicated by TT), as well as in secretory cells which have yet to be tested for their susceptibility to tetanus intoxication.



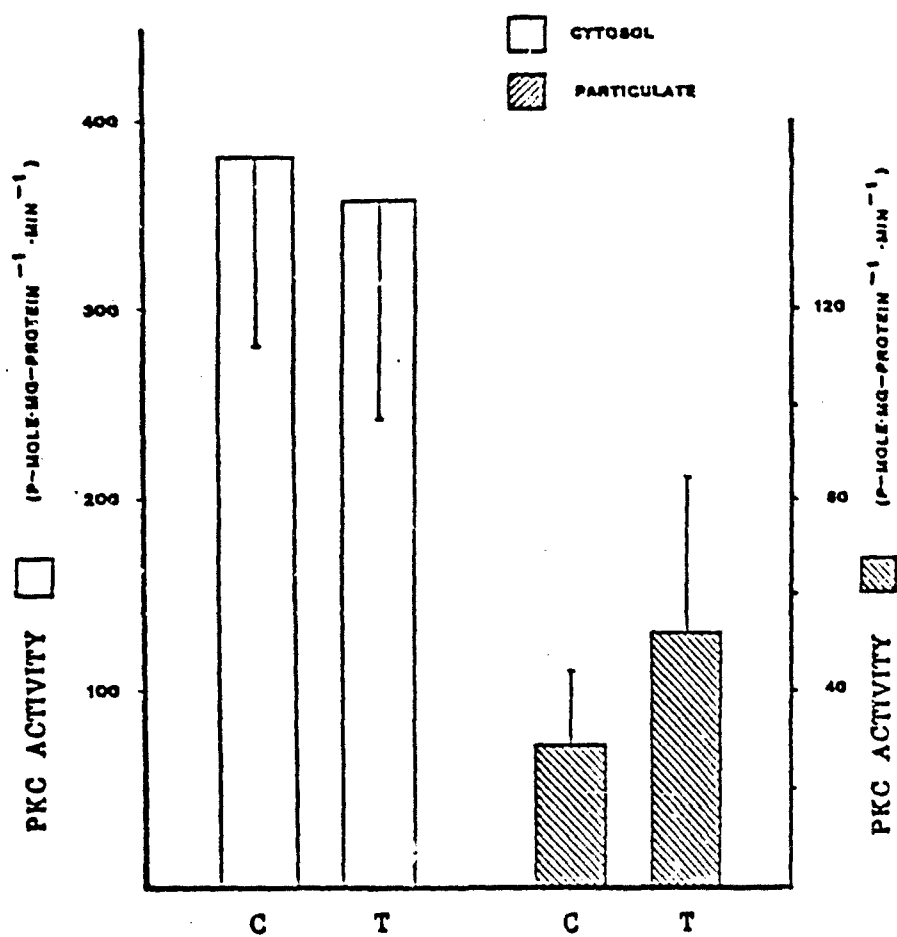


Figure 1. Brain protein kinase C activity from control (C) and TT-intoxicated (T) mice. Results represent the mean  $\pm$  SEM of 7 separate experiments run in duplicate.

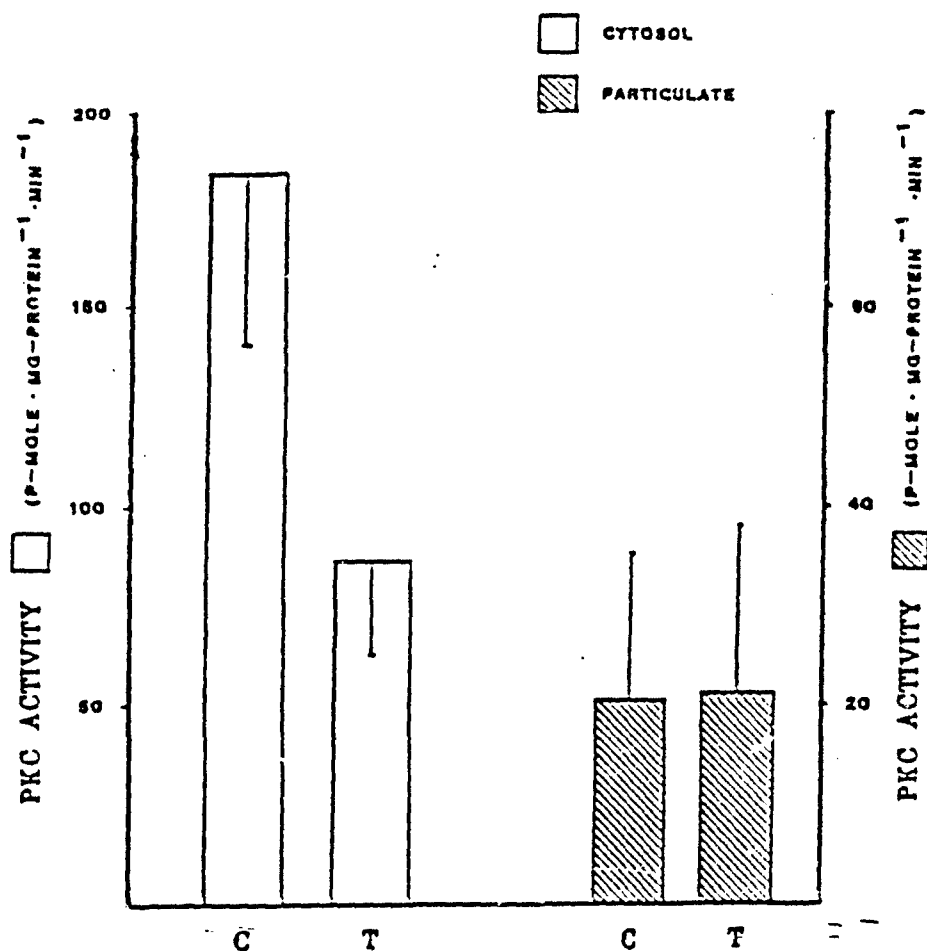


Figure 2. Spinal cord protein kinase C activity from control (C) and TT-intoxicated (TT) mice. Results represent the mean  $\pm$  SEM of 7 separate experiments run in duplicate.

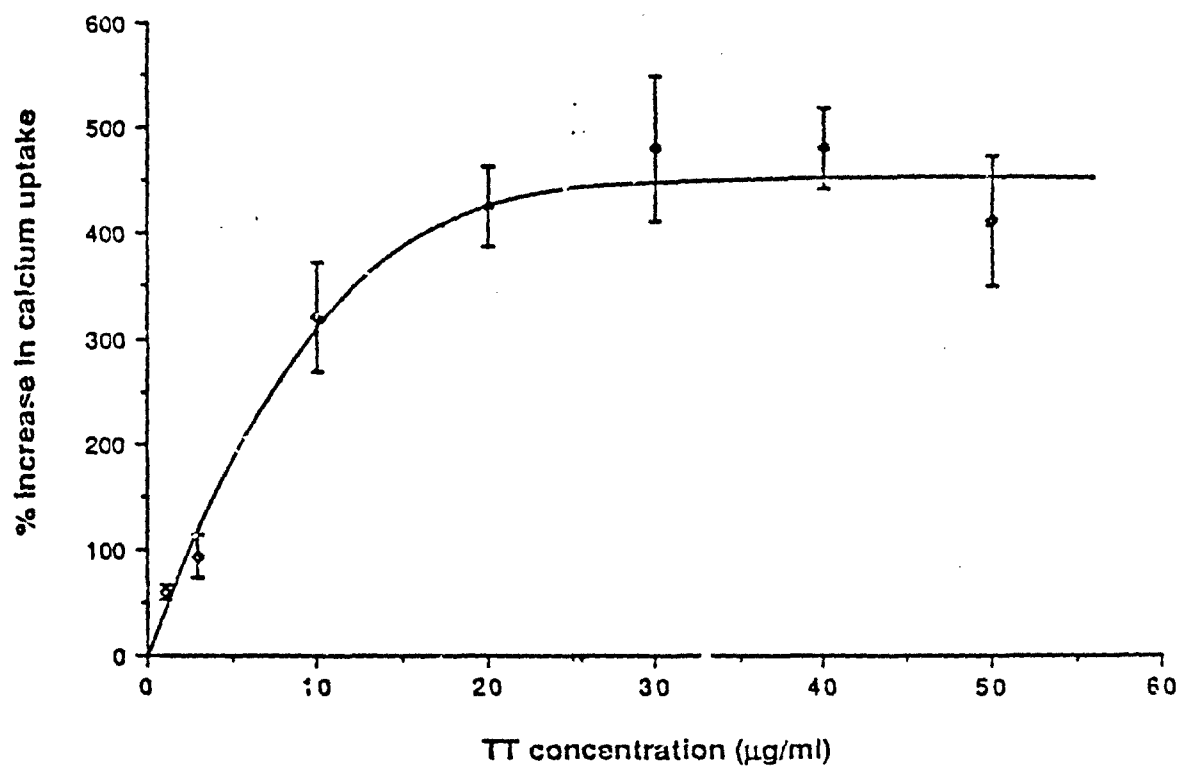


Figure 3. Dose-response curve of the effect of TT on ATP-dependent <sup>45</sup>Ca uptake of human FMN lysosomes. Results represent the mean  $\pm$  SEM of 3-15 experiments run in duplicate.

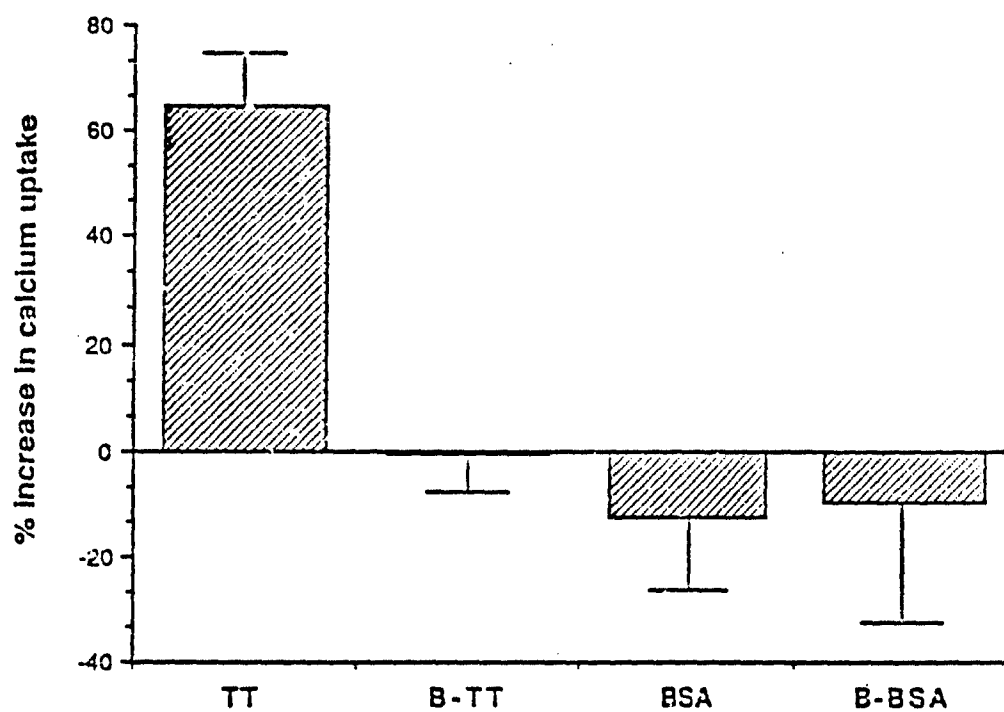


Figure 4. Effect of TT, B-TT, BSA, and B-BSA (each at a protein concentration of 1 ug/ml) on ATP-dependent  $^{45}\text{Ca}$  uptake of human PMN lysosomes. Results represent the mean  $\pm$  SEM of 3-9 experiments run in duplicate.

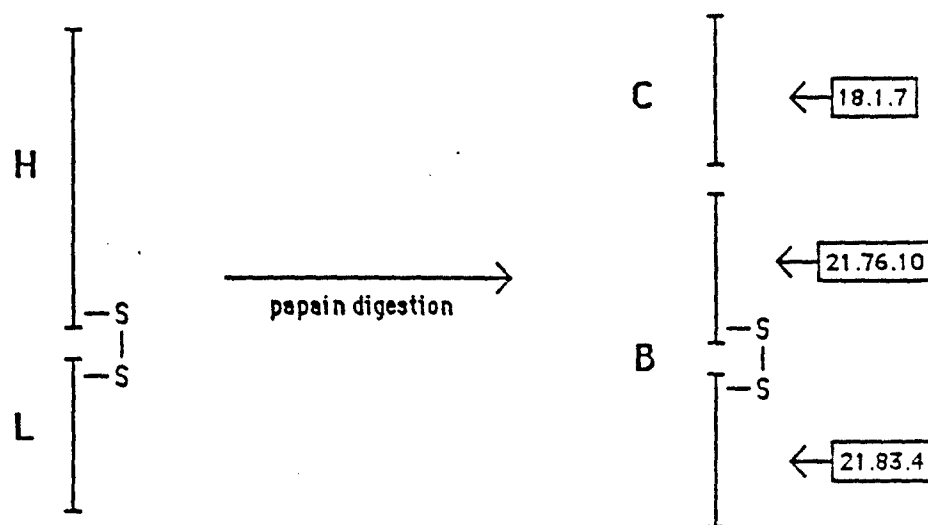


Figure 5. Structure of the molecule of tetanus toxin and the binding domains of the monoclonal antibodies used in the presented experiments.

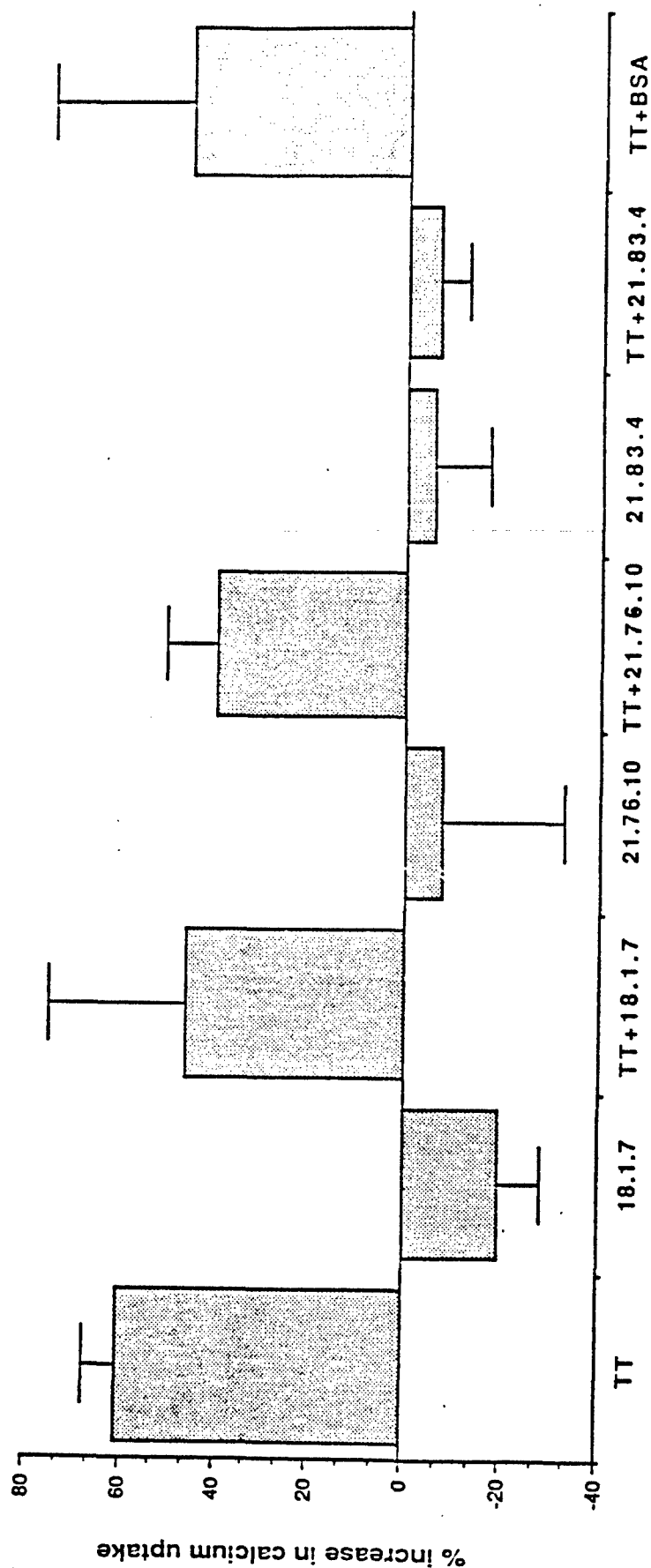


Figure 6. Effects of three neutralizing monoclonal Ab on ATP-dependent calcium uptake of lysosomes. Ab 18.1.7 is directed against the C fragment, Ab 21.76.10 against the heavy chain of the B fragment, and Ab 21.83.4 against the light chain of the B fragment. Last column shows the effect of TT with BSA instead of an Ab. Results represent the mean ± SEM of 3-14 experiments run in duplicate.

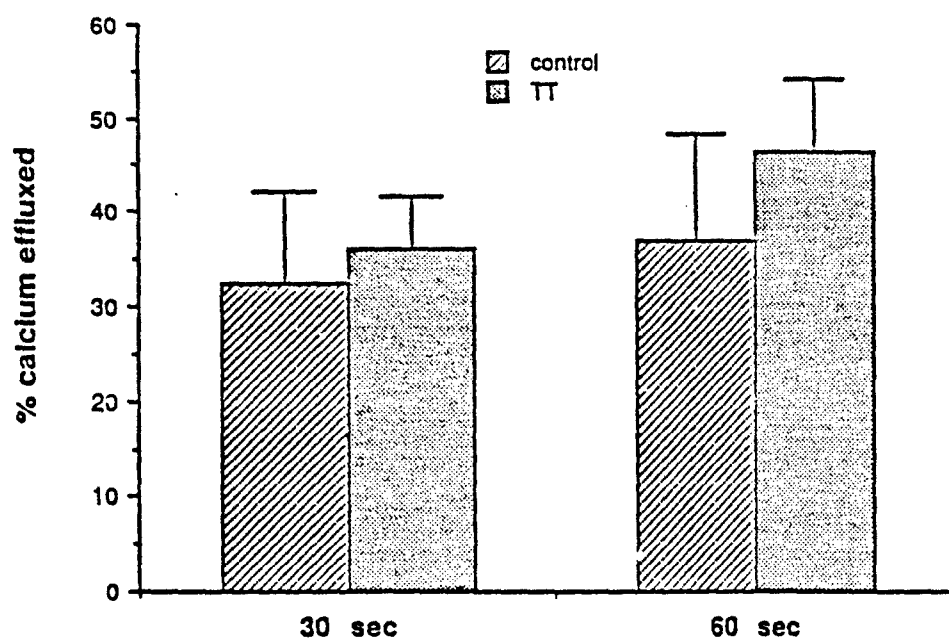


Figure 7. Effect of TT (1 ug/ml) on calcium efflux from lysosomes. Calcium uptake was stopped after 5 min by depletion of ATP from the reaction mixture (addition of glucose/hexokinase) and lysosome-associated  $^{45}\text{Ca}$  was measured. Results represent the mean  $\pm$  SEM of three separate experiments run in duplicate.

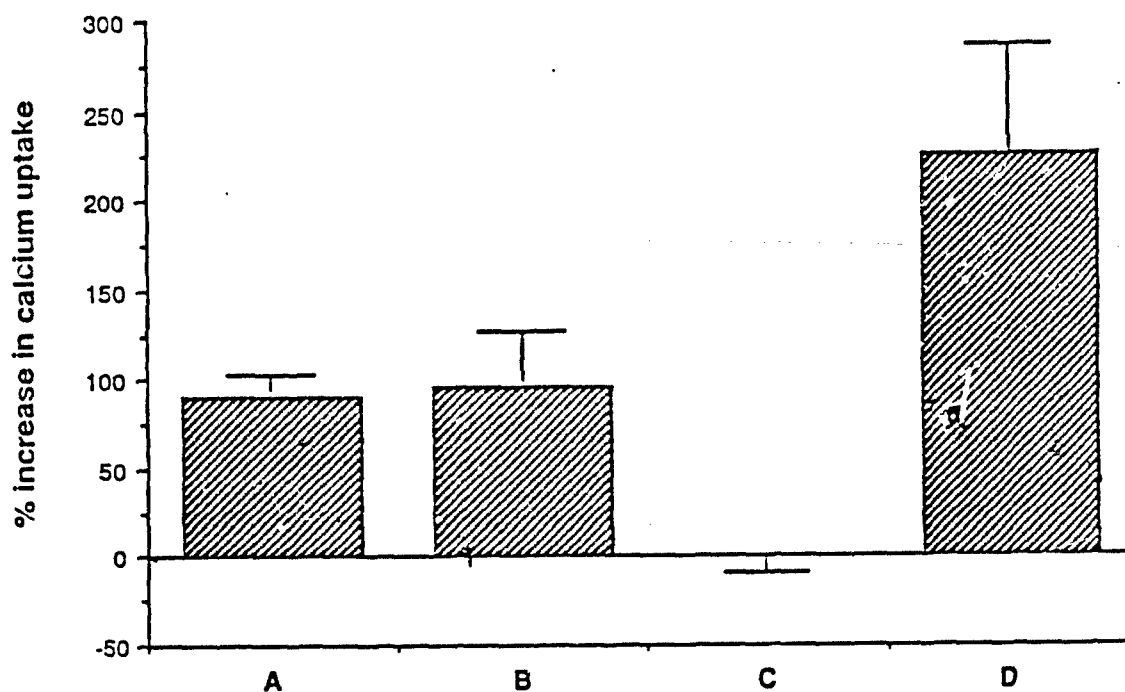


Figure 8. Irreversibility of the effect of TT (1  $\mu$ g/ml) on ATP-dependent  $^{45}\text{Ca}$  uptake of human PMN lysosomes. (A) Lysosomes incubated without TT, washed, and then incubated with TT; (B) Lysosomes incubated with TT, washed, and then incubated again with TT; (C) Lysosomes incubated with TT, washed, and then incubated in buffer alone; (D) Lysosomes incubated with TT and ATP, washed, and then incubated in buffer alone. Results represent the mean  $\pm$  SEM of 5-11 experiments run in duplicate.



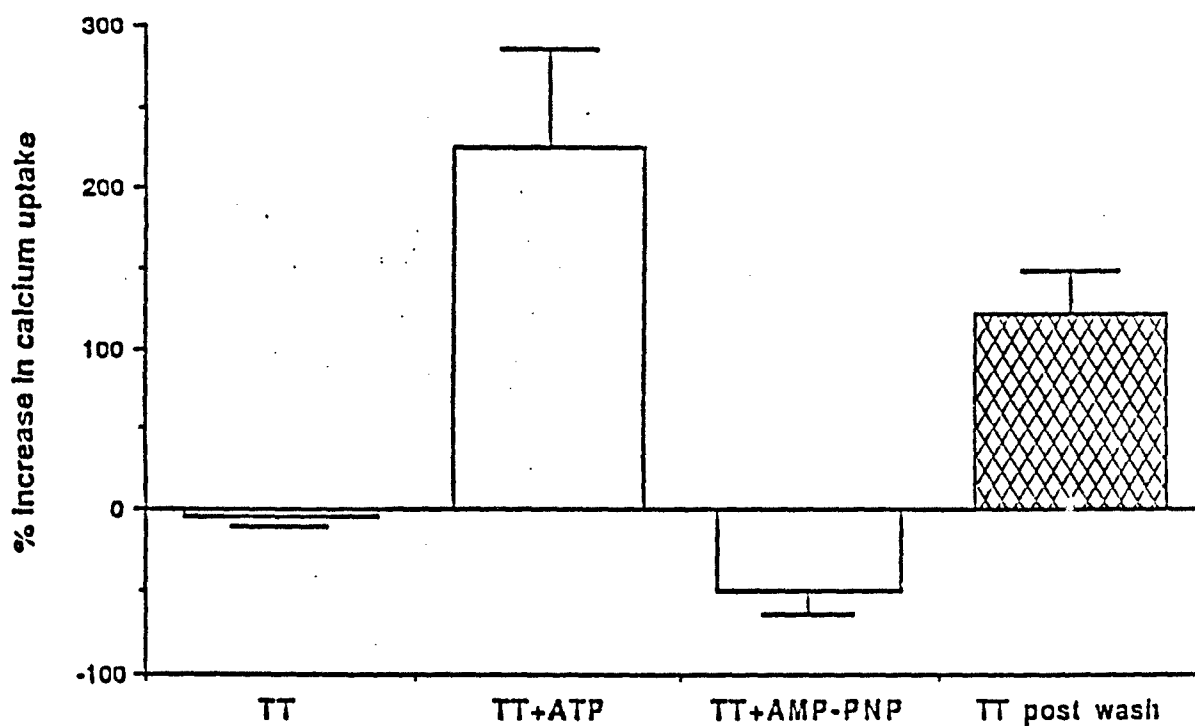


Figure 9. TT has an irreversible effect on calcium uptake in the presence of ATP. Lysosomes were preincubated with or without TT (1  $\mu$ g/ml), TT + ATP, and TT + AMP - PNP, washed, and resuspended in toxin-free buffer. For comparison (▨), lysosomes were preincubated in buffer, washed, and resuspended in buffer with TT. To all conditions,  $^{45}\text{Ca}$  + ATP was then added and ATP-dependent calcium uptake was measured.

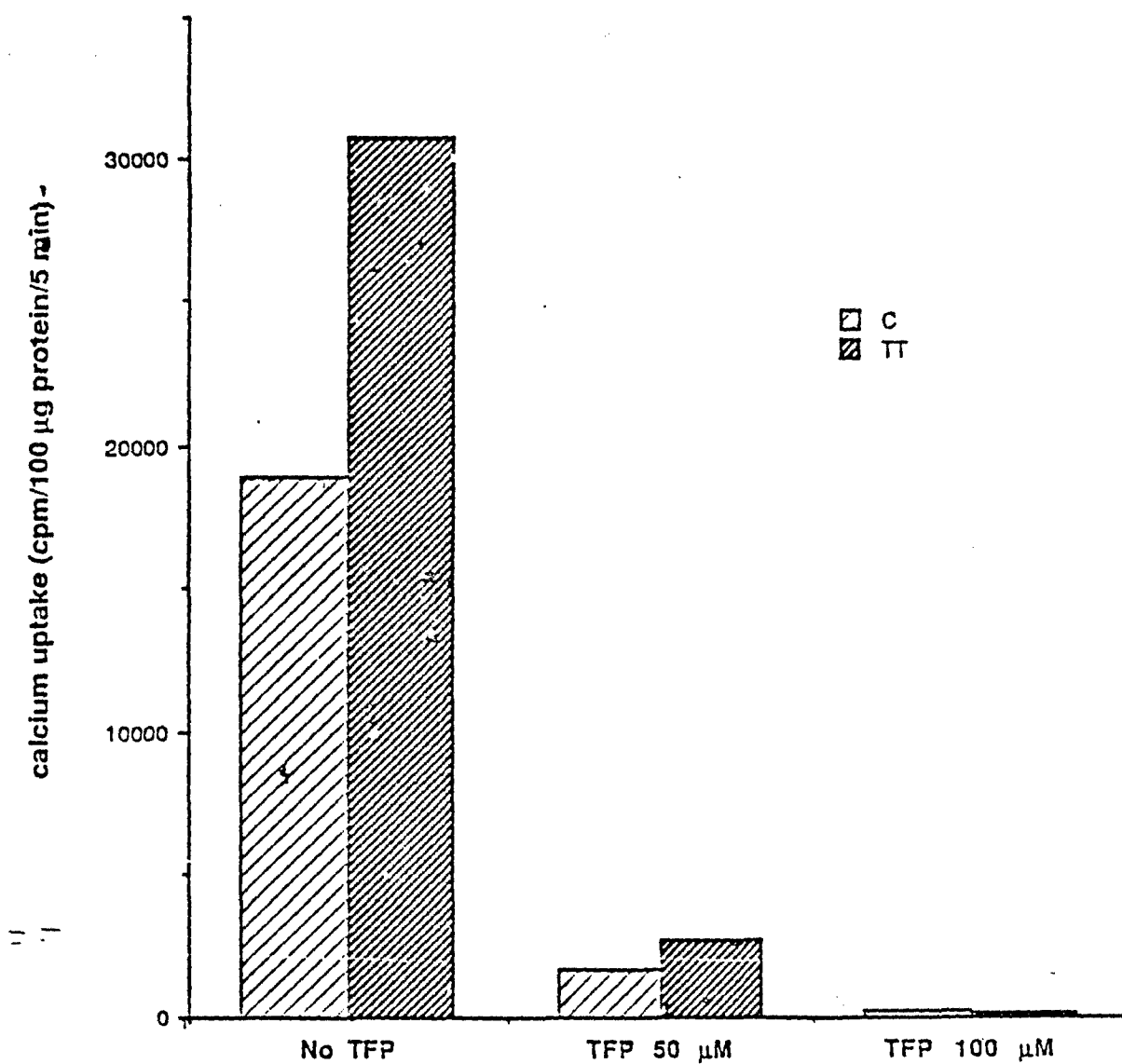


Figure 10. Inhibitory effect of TFP on ATP-dependent calcium uptake of control (C) and TT-treated (TT) lysosomes.

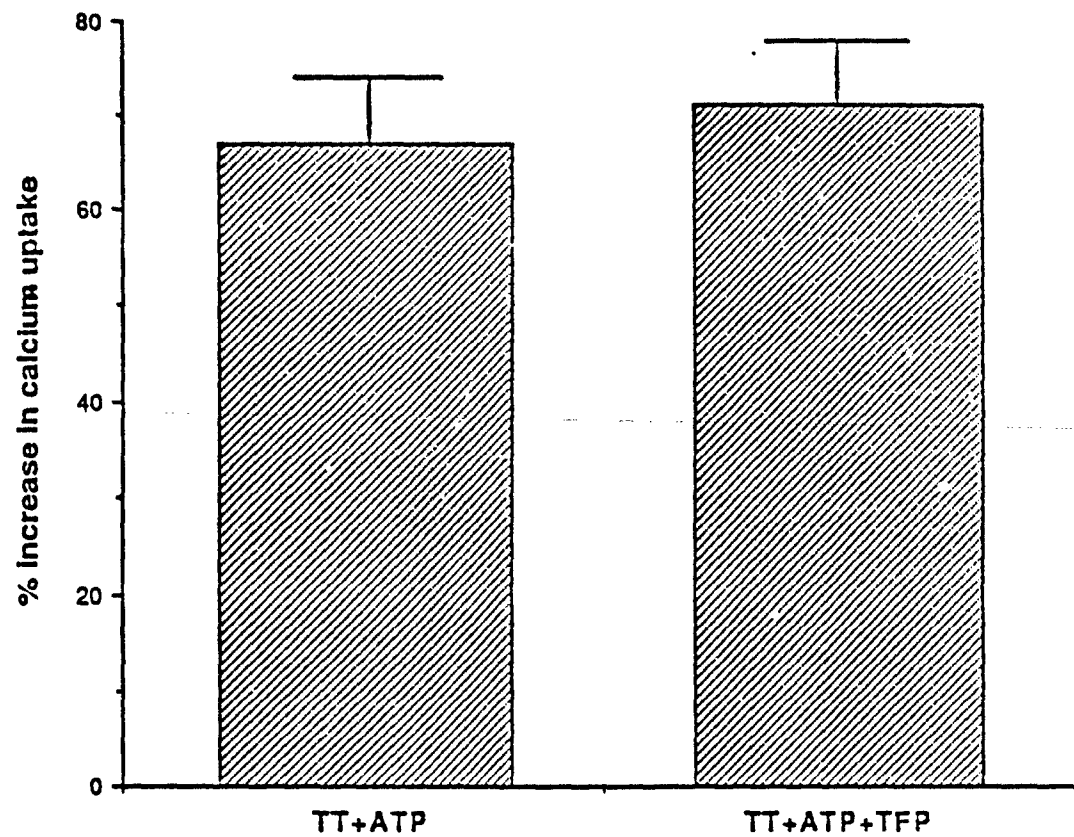


Figure 11. Effect of TFP (100  $\mu$ M) on the irreversible action of TT (1  $\mu$ g/ml) on the calcium uptake by lysosomes. PMN lysosomes were preincubated with or without TT + ATP, and with or without TFP, washed, and resuspended in toxin-free buffer.  $^{45}\text{Ca}$  + ATP was then added for ATP-dependent calcium uptake measurements.

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