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CALMODULIN INHIBITORS BLOCK NEUTROPHIL DEGRANULATION AT A STEP DISTAL FROM THE MOBILIZATION OF CALCIUM

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SUMMARY: Two calmodulin inhibitors, trifluoroperazine and N-(6-aminohexyl-5-chloro-1-naphtalene sulfonamide, were found to be potent inhibitors of the chemotactic factor plus cytochalasin B induced lysosomal enzyme release from rabbit peritoneal neutrophils. On the other hand, these inhibitors did not affect the chemotactic factor induced intracellular calcium redistribution and membrane permeability increase. These results strongly suggest that calmodulin is involved in the expression of the various neutrophil functions. In addition, these compounds provide a unique experimental tool for the dissection of the excitation-secretion coupling sequence in neutrophils and other secretory cells.

INTRODUCTION

Although calcium is the generally accepted second messenger in polymorphonuclear leukocytes (1,2), very little is known about the ways it is able to initiate chemotaxis, secretion, aggregation and the other functions these cells are capable of.

By analogy with current evidence from a variety of cell types, it appears probable that some, if not all, of the biochemical events that are initiated upon the binding of chemotactic factors to their receptors are mediated or amplified by calmodulin (3-5).

The experiments whose results are reported here were designed to examine such a possibility. The approach used was to take advantage of calmodulin - specific inhibitors such as trifluoroperazine (TFP) (6,7) and N-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide (W7) (8-10) and to examine their effects on lysosomal enzyme release from neutrophils. In addition, the effects of these

Abbreviations: Formyl-Methionyl-Leucyl-Phenylalanine = f-Met-Leu-Phe Trifluoroperazine = TFP; N-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide = W7

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inhibitors on the chemotactic factor stimulated ^{45}Ca fluxes have been investigated in an attempt to locate the step(s) at which these compounds may be acting.

MATERIALS AND METHODS

Chemotactic factor and cytochalasin B induced lysosomal enzyme release from rabbit peritoneal neutrophils was performed as previously described (11). Only the results obtained with lysozyme will be reported. β Glucuronidase was measured in a few instances and its release paralleled that of lysozyme. In all the experiments to be reported and unless specified otherwise, the release of lactate dehydrogenase, a marker of cell death, was less than 5% of the total cell content.

Calcium fluxes were measured using the rapid sampling silicone oil method previously described in detail (2,12).

F-Met-Leu-Phe was generously provided by Dr. R. J. Freer, Department of Pharmacology, Medical College of Virginia, Richmond, VA., W7 was kindly provided by Dr. H. Hidaka, Department of Pharmacology, School of Medicine, Mie University, Ebashi, Tsu 519, Japan and TFP was a gift from Dr. Harry Greene, Smith Kline and French Labs., Philadelphia, PA. Cytochalasin B was obtained from Aldrich Chemical Co., Milwaukee, WI, and ^{45}Ca, as $CaCl_2$ in water, from New England Nuclear, Boston, MA. All other reagents were analytical grade.

RESULTS AND DISCUSSION

We have tested for the possible involvement of calmodulin in neutrophil functions by examining the effects of two calmodulin inhibitors on lysosomal enzyme release, a secretory event, from these cells.

The effect of 10 μM TFP on the f-Met-Leu-Phe plus cytochalasin B induced degranulation are illustrated in Figure 1. In this and similar experiments, the thermally equilibrated cells were pretreated for 1 minute with TFP before being transferred to tubes containing f-Met-Leu-Phe and cytochalasin B. As evident in Figure 1, TFP is a powerful inhibitor of lysosomal enzyme release caused in this manner. In addition, as evidenced in Figure 1, TFP reduces the degranulation that is observed in the presence of cytochalasin B alone. About 15-20% of rabbit neutrophil cell suspensions thus "spontaneously" release lysosomal enzymes ("spontaneous" because it is observed in the absence of chemotactic factor). This result indicates that the cytochalasin B sensitive step(s) may be related to one or more of the calmodulin dependent enzymatic activities.

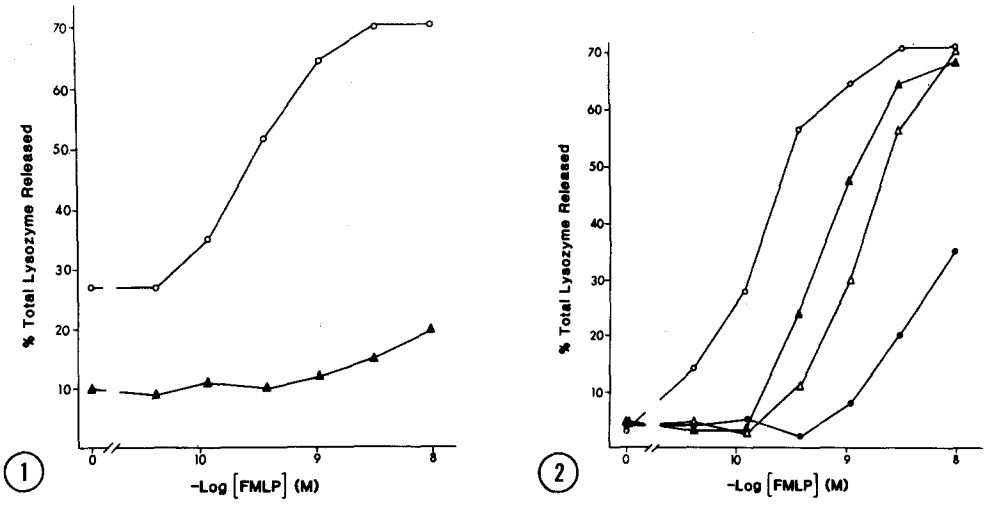


Figure 1: Effect of TFP on the f-Met-Leu-Phe plus cytochalasin B induced lysosomal enzyme release from rabbit peritoneal neutrophils. Open circles: control cells; closed triangles: cells pretreated for 1 minute with 10^{-5}M TFP.

Figure 2: Effect of W7 on the f-Met-Leu-Phe plus cytochalasin B induced lysosomal enzyme release from rabbit peritoneal neutrophils. Open circles: control cells; closed triangles: cells pretreated for 1 minutes with 1.25 x 10^{-5}M W7; open triangles: cells pretreated for 1 minute with 2.5 x 10^{-5}M W7; closed circles: cells pretreated for 1 minute with 5.0 x 10^{-5}M W7.

No significant amounts of lysozyme were found to be released over the whole concentration range of f-Met-Leu-Phe tested indicating a shift in the dose-response curve of at least two orders of magnitude.

However, possibly because it is so powerful an inhibitor, the experimental usefulness of TFP is seriously limited. Concentrations equal to or larger that 20 μM or incubation times longer than 2 minutes at 10 μM were found to cause large leakage (up to 70%) of the cells' content of lactate dehydrogenase into the extracellular medium.

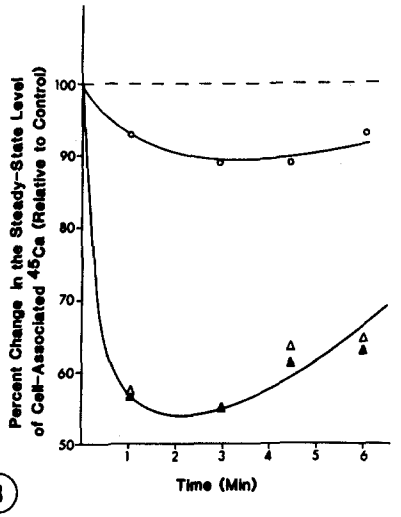
Seeking to confirm and extend the results described above, we have examined the effects of another calmodulin inhibitor, W7, which has been characterized in detail by Hidaka et al. (8-10). As shown in Figure 2, W7 inhibits in a dose-dependent manner the f-Met-Leu-Phe plus cytochalasin B induced enzyme release. In this experiment involving a 1 minute preincubation with W7 no leakage of lactate dehydrogenase was detected. The release of β-glucuronidase was found

to be inhibited in a manner analogous to that shown for lysozyme. Longer preincubation times (2 - 5') with 50 μM W7 were found to cause large leakage of lactate dehydrogenase. The lytic effects of W7 can however be easily avoided in rabbit neutrophils by using lower concentrations of W7 (10-20 μM) and short incubation times (1 - 2'). The effect of 10 μM W7 is rapid with very little additional inhibition with preincubation times longer than 1 - 2 minutes (results not shown).

The inhibitory effects of W7 are not limited to f-Met-Leu-Phe. In experiments not shown here W7 (10^{-5}M) was found to inhibit lysosomal enzyme release induced by arachidonic acid and cytochalasin B and by A23187 by 87 ± 5 and 28 ± 17 percent respectively as judged from the displacement of the dose-response curve (mean ± standard error of the mean of three separate experiments). In addition, both of these compounds severely decrease the aggregatory response and the oxygen consumption burst that follow the addition of f-Met-Leu-Phe to rabbit neutrophils (Alobaidi et al., manuscript in preparation).

That W7 and TFP act in this system by interacting with calmodulin is suggested by the findings that these two compounds are active at concentrations similar to their respective binding constants to purified calmodulin (7,10). In addition, earlier reports have shown that chloropromazine, a weak calmodulin inhibitor (6) and local anesthetics, a class of compounds with recently described anti-calmodulin activity (13) inhibit lysosomal enzyme release from human and rabbit neutrophils (14,15). The additional specificity of the two compounds tested here strengthens the hypothesis that these various agents act the same way, i.e., through their effect on calmodulin, the presence of which in neutrophils has recently been reported (16).

We also examined the effects of W7 and TFP on the f-Met-Leu-Phe induced changes in calcium homeostasis. As previously described in detail (17), chemotactic factors are thought to transiently increase the steady-state level of exchangeable, and by extrapolation, free calcium. This is brought about both by an intracellular release of previously unexchangeable calcium



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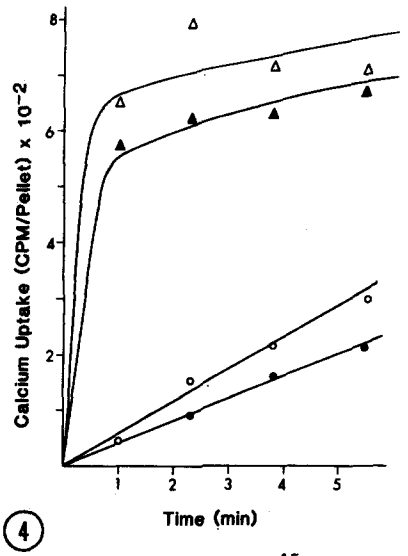

(4)

Figure 3: Effect of W7 on the steady-state level of cell-associated ^{45}Ca in the absence of added extracellular calcium. The cells were pre-incubated for 50 minutes prior to the various additions. W7 ($10^{-5}M$) was added 1 minute before f-Met-Leu-Phe ($10^{-9}M$). The results were normalized to the control cells at each time point. Open circles: W7 alone; filled triangles: f-Met-Leu-Phe alone; open triangles: f-Met-Leu-Phe and W7.

Figure 4: Effect of W7 on the rate of uptake of ^{45}Ca by rabbit peritoneal neutrophils. W7 ($10^{-5}M$) was added to thermally equilibrated cells suspended in the presence of 0.5 mM $CaCl_2$ 1 minute before ^{45}Ca or f-Met-Leu-Phe. Filled circles: control cells; open circles: W7 alone, filled triangles: f-Met-Leu-Phe ($10^{-9}M$) alone; open triangles: f-Met-Leu-Phe and W7.

and by an increase in the inward permeability of the plasma membrane to calcium. The effects of W7 on the experimental parameters reflecting these two events were therefore studied.

F-Met-Leu-Phe has previously been shown to transiently decrease the steady-state level of cell-associated ^{45}Ca in the absence of added extracellular calcium. On the basis of this and other experiments, this decrease in the steady-state level of cell-associated ^{45}Ca has been thought to be a reflection of a stimulus-induced intracellular release of previously unexchangeable calcium (2,17). As shown in Figure 3, the f-Met-Leu-Phe induced intracellular calcium redistribution is unaffected by the presence of W7.

Figure 4 similarly shows that W7 does not affect the chemotactic factor induced change in membrane permeability to Ca^{2+}, as evidenced by the stimulated

rate of ^{45}Ca uptake. TFP (10^{-5}M) was also found, in preliminary experiments, not to inhibit the f-Met-Leu-Phe induced ^{45}Ca uptake.

The results presented above clearly show that calmodulin inactivators inhibit neutrophil functions by acting, principally, at a step subsequent to the ones leading to the increase in the cytoplasmic level of free calcium. The calcium and calmodulin sensitive enzymes of the neutrophils have not as yet been described and thus any speculation about the exact step or steps which might be inhibited by W7 and TFP must remain conjectural at present. Nevertheless, the clear cut differentiation between stimulated ^{45}Ca fluxes and subsequent functional responses that these inhibitors permit provides a unique experimental tool for the dissection of the sequence of events constituting the excitation-response coupling in the neutrophils and possibly in other secretory cells. In particular, it allows one to examine the effects of secretory stimuli on various biochemical events in the absence of possible degranulation artifacts; for example, the effects of cytochalasin B on cation fluxes can now be studied independently of the usually attendant secretion.

ACKNOWLEDGMENTS

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