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ENHANCEMENT OF CHEMILUMINESCENCE OF THE NEUTROPHILS BY LOW CONCENTRATIONS OF TRIFLUOPERAZINE

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Summary: One to 10 μM trifluoperazine was found to potentiate luminol-dependent chemiluminescence of neutrophils induced by n-formyl-methionyl-leucyl-phenyalanine. It did not potentiate chemiluminescence induced by A23187 or by phobor myristate acetate. Low concentrations of another calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, an intracellular Ca^{++} antagonist 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate, and a local anesthetic dibucaine, were found to possess similar activity. It is suggested that trifluoperazine potentiates chemiluminescence by acting on certain cellular processes that follow after stimulation by n-formyl-methionyl-leucyl-phenylalanine, but not by A23187 or by phorbor myristate acetate, and that this effect may be calmodulin-independent.

Introduction: TFP is known to be a potent calmodulin antagonist, and there have been a number of reports on the inhibitory effects of this tranquilizing agent on calmodulin-dependent processes of a variety of cells (1). In addition to calmodulin antagonism, it has long been established that TFP possesses phospholipid-interacting properties and exhibit a number of effects independent of calmodulin antagonism (2). As for neutrophils, the inhibitory effects of TFP on functions such as chemotaxis, degranulation, and superoxide production have been reported (3,4), and these inhibitory effects have been attributed mostly to the drug's calmodulin antagonism.

It has been established that the levels of CL of neutrophils represent the amount of oxygen radicals produced by them. Van Dyke et al. developed luminol-dependent CL as a simple and sensitive method for evaluating the activity of neutrophils (5), and it is now widely used for clinical as well as fundamental

Abbreviations: TFP, trifluoperazine; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate; fMLP, n-formyl-methionyl-leucyl-phenylalanine; PMA, phorbor myristate acetate; CL, chemiluminescence;W-7, N-(6-aminohexyl)5-chloro-1-naphthalenesulfonamide

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research. With the use of luminol-dependent CL of neutrophils, we found that low concentrations of TFP significantly potentiated fMLP-induced CL production by neutrophils, instead of inhibiting it. Further study was undertaken with other types of stimulators and with other Ca^{++} modulating agents, such as TMB-8, dibucaine, and W-7, in order to characterize the nature of this interesting phenomenon.

Materials and methods. 1: Preparation of the neutrophil suspension. For the measurement of CL, human neutrophil-rich suspensions were prepared as previously described (6), and the cell count was adjusted to $1x10^6$ cells/ml in the HEPES-buffered salt solution (pH 7.4) containing (in mM) NaCl, 148; KCl, 5; $MgSO_4$, 1.2; glucose, 10; HEPES, 10.
2: Measurement of luminol-dependent CL. Half a ml of $1x10^{-4}$ M luminol solution in the HEPES-based buffer was added to 0.5 ml of the neutrophil-rich suspension in a polyethylene vial, and incubated at 37°C for five minutes. Ten µl of various concentrations of Ca^{++} modulating drugs were then added and incubated for another five minutes. The reaction was initiated with the addition of 1 µl of various concentrations of fMLP, A23187, or PMA, and the measurement was started immediately afterward. CL was measured by a Monolight 401 photometer (Analytical Luminescence Laboratory Inc., Ca., USA), equipped with a temperature control device. The levels of CL were expressed as voltage, and the averaged CL data for the first 10 minutes after the initiation of the reaction were presented as CL data (V/min).
3: Production of CL in a cell-free system. Half a ml of $1x10^{-4}$ M luminol solution in the HEPES-buffered salt solution, 0.4 ml of the HEPES buffer, 0.1 unit of xanthine oxidase, and 1 µl of 10^{-4} M fMLP in a polyethylene vial were incubated at 37°C for five minutes. Ten µl of various concentrations of TFP, TMB-8, or dibucaine were then added, and incubated for another five minutes. The reaction was initiated with the addition of 100 µl of 1 mM hypoxanthine to the vial and the measurement was started immediately afterward by a Monolight 401 photometer.

Results: TFP, at concentrations of more than 15 µM, inhibited CL induced by 10^{-7} M fMLP dose-dependently. Paradoxically, TFP at concentrations of 1-10 µM enhanced fMLP-induced CL production by neutrophils. Five µM of TFP showed the highest level of CL enhancement. The magnitude of enhancement was variable, and occasionally this phenomenon was not demonstrated. The mean value (n=30) of CL enhancement by 5 µM TFP was 60% (Fig.1).

On the other hand, TFP failed to increase the levels of CL induced by other soluble stimuli such as A23187 or PMA (Fig.2). CL enhancement by 5 µM TFP occurred at virtually all concentrations of fMLP, but this phenomenon was not demonstrated at any concentration of A23187 or PMA (Fig.3).

Enhancement of CL by 5 µM TFP decreased in intensity as the incubation time with TFP before the application of stimuli was lengthened. After 60 minutes of incubation, the enhancement of CL by TFP was almost completely abolished (Fig.4).

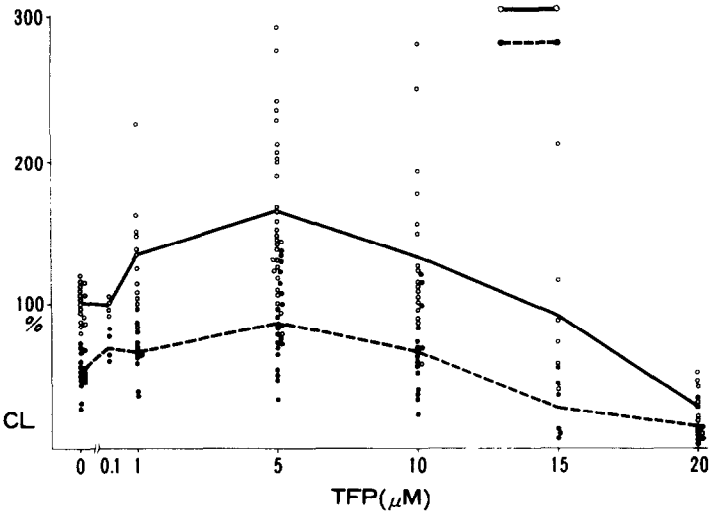

Fig.1 Effect of various concentrations of TFP on fMLP-induced CL. The levels of CL of the neutrophil suspensions with 4 mM Ca^{++} induced by 10^{-7} M fMLP were set as controls, and expressed as 100 %. Various concentrations of TFP were added 5 min before stimulation by 10^{-7} M fMLP. CL data with 4 mM Ca^{++} (o). CL data without Ca^{++} (●). (—) represents the mean of CL data with Ca^{++}. (- - -) represents the mean of CL data without Ca^{++}. (Maximum number of determinations = 30 at the concentration of 5 μM)

W-7, which is another calmodulin antagonist, TMB-8, which is an intracellular Ca^{++} blocker, and dibucaine, which is a local anesthetic, all showed biphasic effects on fMLP-induced CL, quite similar to those of TFP. At the concentrations of 10-100 μM, 10-200 μM and 10-500 μM, respectively, W-7,

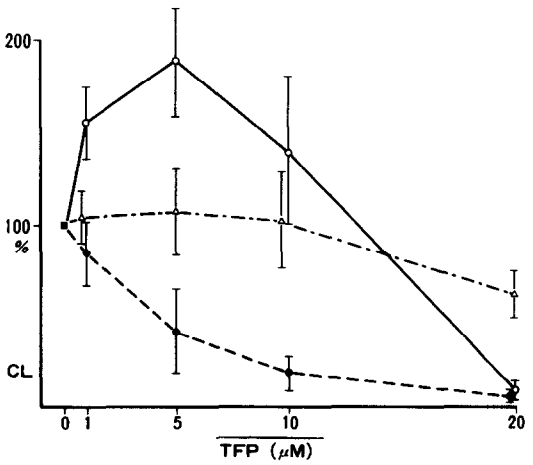

Fig.2 Effect of TFP on CL induced by 10^{-7} M fMLP, $2x10^{-7}$ M A23187, and 100 ng/ml PMA. The levels of CL induced by each stimulator in the presence of TFP were presented as percentages of the control CL level without TFP. TFP was added to the neutrophil suspension five minutes before the addition of stimulators. CL data presented are the means ±SD of four determinations.

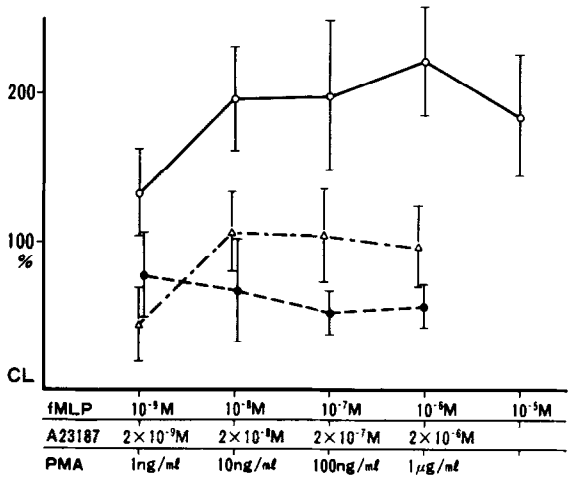

Fig.3: Effect of TFP on CL induced by various concentrations of stimulators. TFP at the concentration of 5 μM was added to the neutrophil suspension five minutes before various concentrations of fMLP, A23187 or PMA were added. The levels of CL induced by a stimulator at a certain concentration in the presence of 5 μM TFP were presented as percentages of the control CL level induced by the same stimulator at the same concentration without TFP. (——), fMLP; (- - -), A23187; (-·-·-), PMA. CL data presented were means ±SD of four determinations.

TMB-8 and dibucaine enhanced fMLP-induced CL, whereas they all inhibited CL production at higher concentrations (Fig.5). Enhancement of CL induced by A23187 or PMA was not demonstrated by any of these agents (Fig.6).

The effects of TFP, TMB-8 and dibucain on luminol-dependent CL in a cell-free system were evaluated using hypoxanthine and xanthine oxidase. TFP

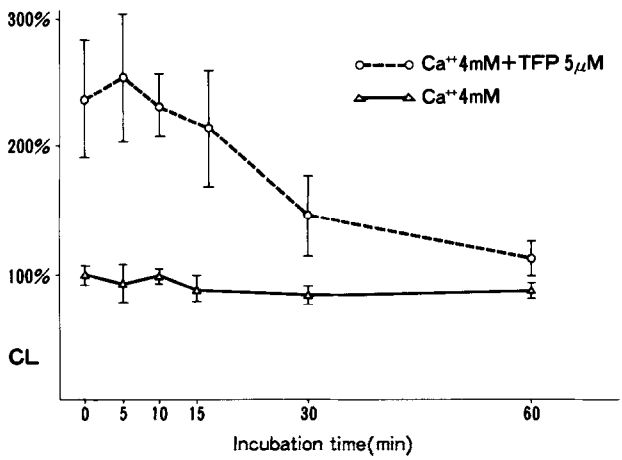

Fig.4: Effect of incubation on enhancement of CL by TFP. 5 μM TFP was added to the neutrophil suspension, and incubated at room temperature for various periods of time before fMLP at the concentration of 10^{-7} M was added. (——) represents CL data without TFP, and (- - -) represents CL data with 5 μM TFP. CL data with TFP and those without TFP were presented as percentages of the control CL produced by the neutrophil suspension freshly prepared without TFP. (mean ±SD of four determinations)

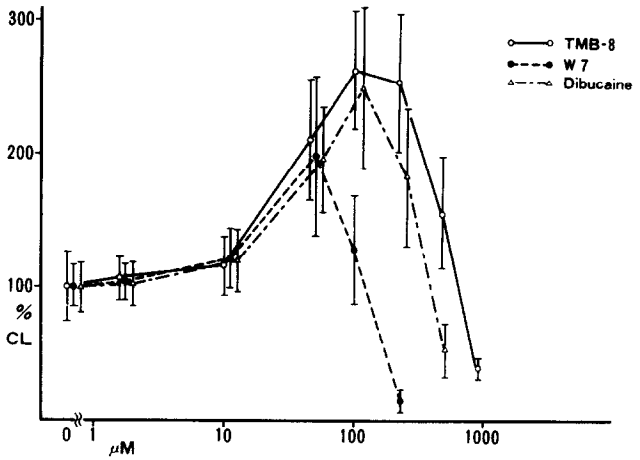

Fig.5: Effect of W-7, TMB-8, and dibucaine on fMLP-induced CL. Various concentrations of W-7, TMB-8, and dibucaine were added to the neutrophil suspension before stimulation by fMLP. CL data were presented as percentages of the control CL induced by fMLP in the absence of any of these agents. (-----), TMB-8; (- - -), W-7; (-·-·-), dibucaine. (n=4. mean ±SD)

partially inhibited CL at concentrations of 20 μM. Dibucaine and TMB-8, at any concentrations tested, had no effect on CL produced in a cell-free system. In no case, was enhancement of CL by these agents demonstrated (Fig.7).

Discussion: We report in this paper that TFP at low concentrations paradoxically enhances fMLP-induced CL production by neutrophils. Most reports dealing with the effects of TFP on the neutrophil functions confirmed the inhibitory nature of TFP, as could be deduced by its calmodulin antagonism. We could find only one report that referred to a paradoxical effect of TFP on the functions of the

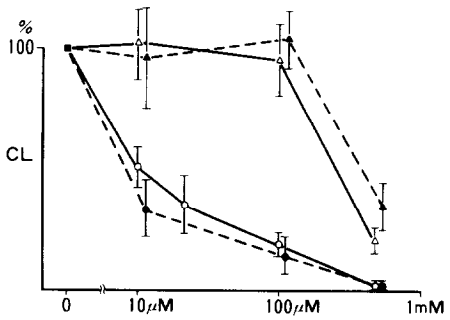

Fig. 6: Effect of TMB-8 and dibucaine on CL induced by A23187 or PMA. Various concentrations of TMB-8 or dibucaine were added to the neutrophil suspension five minutes before 2×10^{-7} M A23187 or 100 ng/ml PMA was added for stimulation. CL data were presented as percentages of the control CL induced by each stimulator in the absence of TMB-8 and dibucaine. Ca^{++} modulating agents and stimulators: (Δ—Δ), dibucaine and PMA; (▲- -▲), TMB-8 and PMA; (o—o), dibucaine and A23187; (●- -●), TMB-8 and A23187. Each point and bar represents the mean ±SD of four determinations.

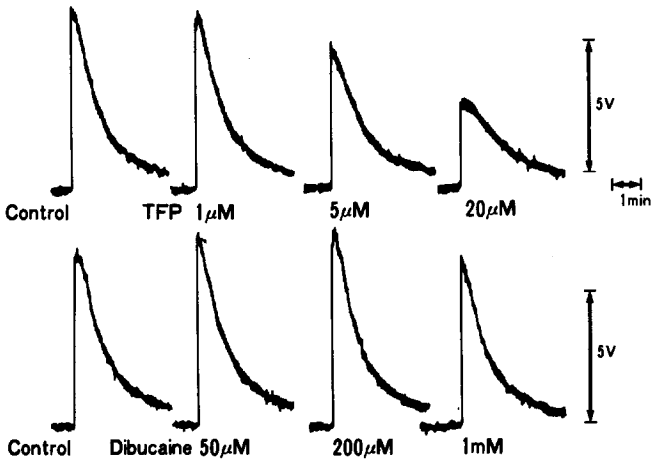

Fig. 7: Effect of TFP and dibucaine on CL produced in a cell-free system. To a vial containing $5x10^{-5}$ M luminol, 0.1 unit of xanthine oxidase and 10^{-7} M fMLP, each at the final concentration, in the HEPES-buffered salt solution, various concentrations of TFP or dibucaine were added and incubated for five minutes. The final concentrations of luminol and fMLP were equal to those in the neutrophil suspension. The reaction was initiated with the addition of 100 μM hypoxanthine and CL levels expressed as voltage (V) were continuously recorded.

neutrophils. Alobaidi et al. noted that TFP at concentrations of 5-10 μM increased the rate of fMLP-induced oxygen consumption by 30% and enhanced fMLP-induced aggregation of the rabbit neutrophils, though they did not seem to consider this finding important (7). Their findings are fundamentally consistent with ours, since the levels of CL are closely related to oxygen radical production and hence oxygen consumption. Our finding of TFP enhancement of CL cannot be discarded as non-specific for two reasons. Firstly, this phenomenon was observed only in the presence of neutrophils. In a cell-free oxygen radical generating system, TFP did not enhance CL at concentrations of 1-10 μM. Secondly, this effect was stimulator-specific, since enhancement was induced only when fMLP was used as a stimulator, but not when A23187 or PMA was used. The fact that TFP enhanced CL induced by various concentrations of fMLP argues against the hypothesis that TFP created the optimal stimulator-receptor ratio for CL production by acting as a blocker for the binding of fMLP. From the observations obtained in this study, it is assumed that TFP enhances fMLP-induced CL production by neutrophils by acting on a certain cellular process that follows after stimulation of neutrophils by fMLP, but not by A23187 or PMA. fMLP

activates neutrophils by binding to receptors on the neutropil membrane, and Ca^{++} transport ensues, whereas A23187 activates neutrophils by directly introducing Ca^{++} into the cells (8). The mechanism of activation of neutrophils by PMA is still largely unknown. Whether the process on which TFP acts is located distal to the Ca^{++} transport or not awaits further evaluation.

We have shown in this study that not only W-7, another calmodulin antagonist, but TMB-8 and dibucaine, which lack calmodulin-antagonistic activity, had potentiating effects on fMLP-induced CL at relatively low concentrations, just as TFP did. Interestingly, Singh et al. reported quite similar findings, though the cell employed in their study was different from ours. They observed respiratory stimulation and motility activation of bovine sperms treated with TFP, TMB-8, and local anesthetics (9). Recently, the surface-acting natures of several agents of different catergories, one of which is TFP, have drawn the attention of workers in Ca^{++} research (10). These agents include Ca^{++} blockers, local anesthetics, β-blockers and calmodulin antagonists. Several intriguing effects of TFP and other phenothiazine derivatives recently reported, which are hard to explain by calmodulin antagonism, may be derived from the phospholipid-interacting property. From these observations, it is feasible that TFP potentiates fMLP-induced CL independently of its calmodulin antagonistic activity, though a confirmative study with purified calmodulin may be needed to draw a conclusion.

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