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Effect of the Synthetic Inhibitor Tosylamino-Phenylethyl-Chloromethylketone on Chemotactic Peptide Receptor Activation and Superoxide Production in Human Neutrophils

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ABSTRACT. It was previously shown that inhibitors such as tosylamido-phenylethyl-chloromethylketone (TPCK) inhibit superoxide production by human neutrophils. These studies suggested that a chymotrypsin-like protease inhibited by TPCK was involved in the activation of the neutrophils oxidative system. In this study, we attempted to define the step in cellular activation and/or cell function inhibited by TPCK. TPCK 10^{-5} M did not inhibit the following early events thought to be involved in the activation of oxidase. 1) f-met-leu-phe-induced activation of phospholipase C assessed by the production of inositol-tris-phosphate (IP3), 2) f-met-leu-phe-induced membrane potential changes, 3) f-met-leu-phe-induced increase in free cytosolic calcium, and 4) phorbol-myristate acetate-induced protein phosphorylation in ^{32}P labeled neutrophils. We also showed that TPCK 10^{-5} M inhibited bactericidal activity of neutrophils on *Staphylococcus aureus*, whereas it did not inhibit the ingestion rate of endotoxin-coated Oil red O particles. We conclude that 1) TPCK at the concentration of 10^{-5} M inhibits superoxide production but not ingestion of Oil red O particles and 2) TPCK inhibits superoxide production at a step distal from calcium mobilization and protein phosphorylation. Radiolabeled TPCK may therefore be a useful tool to study, whether a protease is involved in the activation of the oxidative system distal to second messenger generation. (*Pediatr Res* 20: 848–852, 1986)

Abbreviations

DMSO, dimethylsulfoxide
Di-O-C3(5), 3-3′-dipentyl-oxycarbo cyanine iodide
FMLP, f-met-leu-phe
IP3, inositoltrisphosphate
ORO particles, endotoxin coated Oil red O-particles
PMA, phorbol-myristate acetate
TPCK, tosylamino-phenylethyl-chloromethyl-ketone

One of the most important mechanisms by which neutrophil granulocytes mediate bacterial killing is the generation of oxidants such as O^-_2, OH^-, and H_2O_2 (1). The precise nature of the enzyme system producing these molecules remains unknown, as well as the mechanism of its activation. Results from studies with inhibitors and substrates for chymotrypsin-like proteases have suggested that protease(s) may be involved in the activation and activity of the oxidative system (2, 3). In one of these studies, the kinetics of inhibition of superoxide production of neutrophils by TPCK have been well defined (3). The conclusion of this study was that TPCK inhibits superoxide production by inhibiting a chymotrypsin-like protease at the neutrophil surface, which may play a role in the activation of the oxidative system, but the precise step at which TPCK inhibits superoxide production was not defined. Inhibition of superoxide production by TPCK could occur at several steps of cell activation and/or cell function. In the process of neutrophil activation, stimulation of a chemotactic receptor on the cell surface leads to activation of phospholipase C (4) and subsequently breakdown of membrane phosphoinositides. Thereby, two second messengers are generated: IP3 (5), which mobilizes free cytosolic calcium (4), and diacylglycerol, which is thought to activate protein kinase C (6). Once activated, protein kinase C phosphorylates neutrophil proteins (10, 11), and both, the rise in free cytosolic calcium and activation of protein kinase C are required to initiate cell function (5, 7, 8). Another event occurring early during cell activation is the depolarization of the neutrophil membrane (9).

In this study, we decided to define conditions, in which the potential unspecific effects of TPCK (4) were minimal and subsequently, to determine the level at which TPCK inhibits receptor mediated superoxide production.

MATERIALS AND METHODS

Sources or reagents. TPCK, zymosan, Cytochrome C, FMLP, PMA, ORO, and phenylmethylsulfonylfluoride were from Sigma Co, St. Louis, MO. LPS P *Escherichia coli* 026 B6 was from Difco Laboratories, Detroit, MI. Pertussis toxin was from List Biological Laboratories Inc. Cambell, CA. Myo-(2-^{3}H)inositol and quin2 acetoxymethylester (quin2/AM) was supplied by Amersham, England. Dextran T 500 and Ficoll-Hypaque was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. DI-O-C^3(5) was from Molecular Probes Inc., Junction City, OR. 4-Methylumbelliferyl substrates were from Koch Laboratories, Haverhill, England. RPMI medium 164D was from Amimed AG, Basel, Switzerland. All other reagents used were of analytical grade.

Preparation of neutrophils. Neutrophils were prepared from fresh citrate blood from healthy adult volunteers. They were purified by dextran sedimentation followed by centrifugation through a Ficoll layer as described previuosly (10) and contami-

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nating erythrocytes were removed by hypotonic lysis. The neutrophils were then suspended in a medium called calcium medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1,1 mM $CaCl_2$, 100 μM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5,5 mM glucose, and 20 mM Hepes, pH 7.4. The so-called calcium-free medium is identical to this medium, without $CaCl_2$ and with addition of 1 mM EGTA.

Superoxide production was measured continuously in a double beam spectrophotometer, thermostated at 37° C as previously described (11). Data are presented as nmol O^{-2} produced per 10^6 neutrophils per min. The stimuli used to assess superoxide production by neutrophils were FMLP, PMA, and opsonized zymosan, which was prepared as previously described (12).

Phagocytic bactericidal assay. This assay was performed as previously described (13). Briefly, the killing of *Staphylococcus aureus* strain Wood 46 by isolated neutrophils in a medium containing 10% pooled human serum was assessed after 60 min. Surviving intra- and extracellular bacteria were quantitatively determined after osmotic lysis of neutrophils with distilled water, serial dilutions of the samples, and plating on Muller Hinton agar to determine colony forming units. A control containing heat inactivated human serum was always run simultaneously. The effect of TPCK on the growth *in vitro* of *S. aureus* Wood 46 was also assessed simultaneously.

Phagocytic assay. The phagocytic assay was performed as previously described (14).

Degranulation. Neutrophils, 1.25×10^6, were suspended in 50 μl calcium medium containing 2.5 μg cytochalasin B and warmed at 37° C for 5 min. FMLP was added as a stimulus and the incubation continued for 5 min. The reaction was stopped by rapid cooling on ice and centrifugation at $8000 \times g$/min. Vitamin B_{12} binding protein (as a marker of specific granules) and β-glucuronidase (as a marker of azurophile granules) were assayed in the supernatant. β-Glucuronidase was measured fluorimetrically (15) and vitamin B_{12}-binding protein with a slight modification of the method of Kane (15). The release of granules is expressed as percent release in the supernatant/total enzyme content of neutrophils lysed with triton.

Depolarization. Changes in neutrophil membrane potential were measured in a fluorimetric assay using the membrane potential-sensitive cyanine dye Di-O-C_3 as described by Seligman *et al.* (9). A Perkin-Elmer LS-3 spectrofluorimeter was used. Excitation and emission wavelength were 460 and 520 nm, respectively. Di-O-C_5 (3) was added at a final concentration of 10^{-7} M into a cuvette containing calcium medium warmed continuously to 37° C and 5×10^5 neutrophils were added. The cell suspension was magnetically stirred. Depolarization is expressed as change in fluorescence.

Measurement of inositolphosphates. For the determination of the stimulus-induced changes in IP3 and its breakdown products (IP2 and IP1) cells were incubated in RPMI medium containing myo-(2-^{3}H inositol) (1 μCi/ml) and 3% heat inactivated serum for 18 h as described by Di Virgilio *et al.* (5) in order to label phosphoinositides and inositolphosphates. The labeled cells (12×10^6 cells for each condition) were washed twice at 37° C in RPMI medium without inositol. They were then resuspended in calcium medium and warmed to 37°C for 5 min and incubated with or without TPCK for 1 min followed by addition of FMLP for 20 s. Incubations were terminated by addition of 10% (v/v) trichloracetic acid and the samples were kept on ice and centrifuged thereafter. The supernatants were washed three times with a 5-fold excess of diethylether. The washed extract was adjusted to pH 7.5 with Tris 0.2 M and inositolphosphates separated by stepwise elution from Dowex (formate) columns as described (16). The radioactivity in the fractions was determined by liquid scintillation counting with 67% (v/v) aquasol.

Measurement of cytosolic-free calcium. Free cytosolic calcium was determined in neutrophils loaded with the fluorescent dye quin2 as previously described (6).

Phosphorylation of proteins. Phosphorylation of neutrophil phosphoproteins was determined by incubating neutrophils with ^{32}P in a phosphate-free medium as previously described (7, 8). In brief, 2.5×10^7 neutrophils suspended in 4 ml of phosphate-free calcium medium were incubated with 1 mCi of ^{32}P for 2 h at 37° C. The cells were then washed three times in phosphate-free medium and adjusted to a concentration of 2.5×10^6 cells/ml. Aliquots of 1 ml of cell suspension were warmed at 37° C for 5 min and the following conditions tested: stimulation of labeled cells with PMA 3×10^{-8} M for 5 min; preincubation of cells with TPCK 10^{-5} M for 4 min; and incubation with PMA for 5 min. Controls included incubation of cells with TPCK alone and with DMSO alone. The reaction was stopped by cooling on ice and simultaneous addition of trichloracetic acid 36% (v/v) 0.5 ml. After centrifugation, the supernatants were discarded and the pellets suspended in SDS-sample buffer 25 μl (containing 1% 2-mercaptoethanol, 2% SDS, 80 mM Tris-HCl pH 6.8, 10% glycerol, and 0.05% bromophenolblue). The samples were boiled for 3 min and run on discontinuous slab gels with 5% acrylamide (w/v) in the running gel. Monitoring of molecular weights was performed with standard proteins of known molecular weights. Radiolabeled proteins were revealed by autoradiography with a Kodak X-omat S film with exposure at −70° C for 4 days.

Statistical evaluation. The results are mean ± SD and for comparisons, the Mann-Whitney test was used. The results represent the mean values of three experiments in triplicates, except for the phagocytic test for which samples were tested in duplicates.

RESULTS

Effect of TPCK on superoxide production. We first determined the lowest concentration of TPCK leading to inhibition of superoxide production in neutrophils stimulated with a particulate stimulus, opsonized Zymosan particles (12), and with FMLP 10^{-7} M. In the absence of TPCK, neutrophils stimulated with opsonized Zymosan particles produced 5.37 ± 0.53 nmol O^-_2/min/10^6 neutrophils. When preincubated with TPCK 10^{-5} M for 1 min, superoxide production was inhibited by 99%. When the concentration of TPCK was lowered to 10^{-6} M, superoxide production was 3.42 ± 0.53 nmol/min/10^6 neutrophils. Increasing the time of preincubation of cells with TPCK 10^{-6} M resulted in a linear decrease of superoxide production, reaching an inhibition of superoxide production of 86% after 30 min preincubation. TPCK 10^{-5} M, when added to stimulated neutrophils at the moment of maximum superoxide production, led to cessation of superoxide production within 15 s. These results indicate that TPCK inhibits superoxide production in a dose-dependent and time-dependent manner. Similar results were found, when FMLP was used as a stimulus. The superoxide production of neutrophils preincubated with TPCK 10^{-5} M for 1 min was inhibited by 97%.

It had been suggested, that the effect of TPCK on superoxide production could be due to unspecific inhibition of nonprotein sulfhydryl groups (17). This effect was obtained with much higher TPCK concentrations and longer preincubation periods and was shown to be reversible by incubation of neutrophils with glutathion (17). We thus determined the effect of reduced glutathion on the inhibition of superoxide production achieved with TPCK 10^{-5} M. When 10^6 neutrophils preincubated with TPCK 10^{-5} M for 5 min (which abolished superoxide production) were incubated with glutathion 10^{-3} M for further 10 minutes, the superoxide production was 0.79 ± 0.01 nmol O^-_2/min, which corresponded to 16% of the superoxide production of untreated cells. When TPCK-treated cells were washed three times before incubation with glutathion under similar conditions, the results were identical to those in unwashed cells. All further experiments were therefore performed in cells preincubated with TPCK 10^{-5} M for 1 min, *i.e.* under conditions in which reduced glutathion did not release the almost total inhibition of superoxide production by TPCK.

Effect of TPCK on the release of specific and azurophile granules. The release of specific and azurophile granules by neutrophils stimulated with FMLP was measured with and without preincubation of neutrophils with TPCK 10^{-5} for 1 min. Specific granule release, measured by released vitamin B_{12} binding protein was 19.8 ± 1.2%. Inhibition of specific granule release was 34%. The release of azurophile granules measured by the release of β-glucuronidase was 13.6 ± 1% in these experiments (cells were not preincubated with cytochalasin B before stimulation). Inhibition of azurophile granule release by TPCK was 46%.

Effect of TPCK 10^{-5} M on generation of IP3 after stimulation of cells with FMLP (Table 1). Since IP3 generated by receptor activation of phospholipase C is thought to function as a second messenger (4, 5) we also determined the generation of IP3 and its degradation products, IP2 and IP1, in neutrophils stimulated with FMLP 10^{-7} M, with and without preincubation of cells with TPCK 10^{-5} M for 1 min. The results are expressed in percent of basal values, which correspond to the radioactivity of fractions of unstimulated cells (Table 1). In control neutrophils stimulated with FMLP 10^{-7} M IP3 levels rose to 269 ± 16% of basal levels within 20 s. These results were similar to those obtained with neutrophils preincubated with TPCK (244 ± 11%). Since recently, pertussis toxin was shown to abolish the FMLP-induced generation of IP3 in neutrophils (4), we used pertussis toxin as a control. Preincubation of cell with 250 ng/m of pertussis toxin before stimulation of neutrophils with FMLP abolished the FMLP-induced rise in IP3.

Effect of TPCK 10^{-5} M on FMLP stimulated membrane depolarization, rise of free cytosolic calcium, and release of intracellular calcium stores (Fig. 1). The membrane depolarization which follows stimulation of neutrophils with FMLP 10^{-7} M is shown in Figure 1*A* in neutrophils preincubated with TPCK 10^{-5} M as well as control neutrophils. Both curves are identical. The increase in free cytosolic calcium in calcium medium (Fig. 1*B*) and the release of intracellular calcium stores in the absence of extracellular calcium (Fig. 1*C*) on stimulation of cells with FMLP 10^{-7} M were also identical when cells preincubated with TPCK 10^{-5} M were compared to control cells.

When neutrophils were preincubated with higher concentrations of TPCK (10^{-4} M), the membrane depolarization as well as the rise in free cytosolic calcium were inhibited significantly. As a control, the superoxide production of quin2 loaded cells (control or preincubated with TPCK 10^{-5} M) was also determinated in parallel with the same batch of cells. In these cells, superoxide production was totally abolished by preincubation with TPCK 10^{-5} M.

Effect of TPCK 10^{-5} M on PMA-stimulated phosphorylation of neutrophil proteins (Fig. 2). We tested the effect of preincubation of neutrophils with TPCK 10^{-5} on phosphorylation in neutrophils labeled with ^{32}P and stimulated with PMA 10^{-7} M. As shown in Figure 2, six new protein bands appeared in neutrophils stimulated with PMA and one band disappeared after stimulation in comparison to unstimulated cells. After preincubation with TPCK 10^{-5} M prior to stimulation, the same pattern of newly phosphorylated bands as in stimulated cells was observed and the same band disappeared. Thus, TPCK at the used concentration did not interfere with PMA-induced protein phosphorylation.

Effect of TPCK on phagocytosis and bactericidal activity of neutrophils (Fig. 3). In order to exclude an unspecific effect of TPCK 10^{-5} M on cell function, we performed a phagocytic assay (12) in the presence and absence of TPCK 10^{-5} M. Whereas the ingestion of endotoxin coated ORO particles was 88 ± 6% of that of control cells (p = NS), the killing of *S. aureus* strain Wood 46 was only 11 ± 2% of that achieved in control cells ($p < 0.01$). These results show that preincubation of neutrophils with TPCK 10^{-5} M significantly inhibits bactericidal activity on *S aureus*, whereas the complement mediated phagocytosis was not affected.

Table 1. *Effect of TPCK 10^{-5} M on generation of IP3, IP2, and IP1 in neutrophils stimulated with FMLP 10^{-6} M**

	IP3	IP2	IP1
Unstimulated neutrophils	100 ± 2%	100 ± 3%	100 ± 2%
Stimulated neutrophils	269 ± 16%	183 ± 10%	215 ± 15%
Stimulated neutrophils preincubated with TPCK 10^{-5} M	244 ± 11%	186 ± 11%	205 ± 9%
Stimulated neutrophils preincubated with Pertussis toxin 250 ng/ml	110 ± 3%	99 ± 5%	103 ± 3%

* Preincubation of cells with inhibitors was 1 min for TPCK and 2 h for Pertussis toxin (250 ng/ml) followed by FMLP for 20 s. Results are percent dpm of basal values in the corresponding fraction. Results are mean ± SD.

DISCUSSION

In this study we showed that when neutrophils were preincubated with TPCK 10^{-5} M and stimulated with FMLP (or opsonized zymosan as a particulate stimulus), the superoxide production was completely inhibited. We then performed various ex-

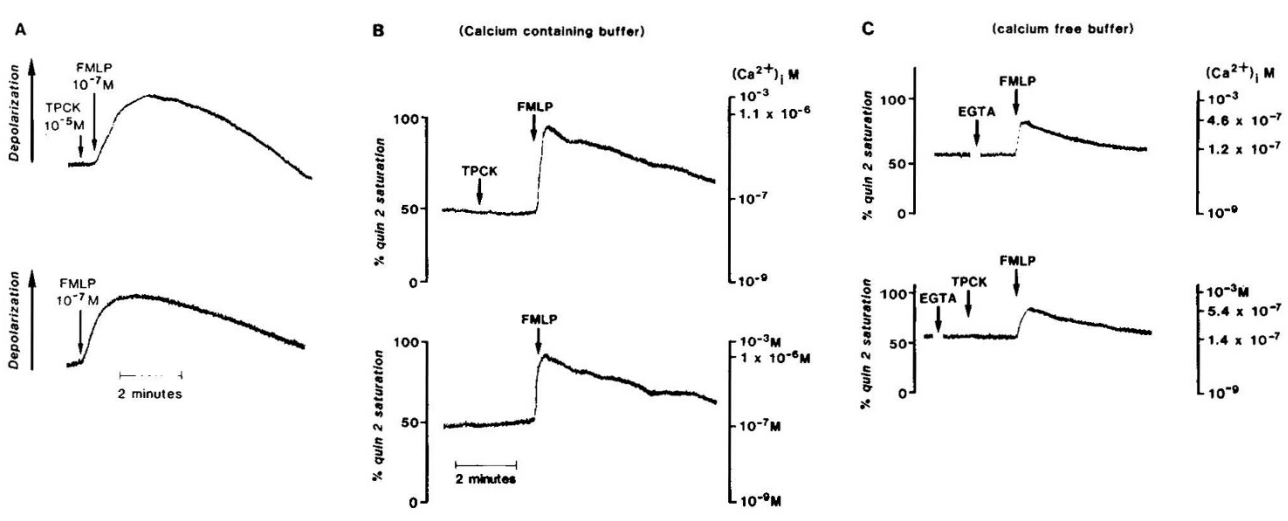

Fig. 1. Effect of preincubation of neutrophils with TPCK 10^{-5} M on the FMLP-induced membrane depolarization assessed with the fluorescent dye di-0-C5 (3) according to Seligman *et al.* (9) (*A*), the FMLP-induced calcium transient in a calcium containing buffer (*B*), and the FMLP-induced release of intracellular calcium stores (*C*) assessed in neutrophils loaded with quin2 as previously described (6, 11). Figure 1 shows a representative curve of three experiments performed in triplicates.

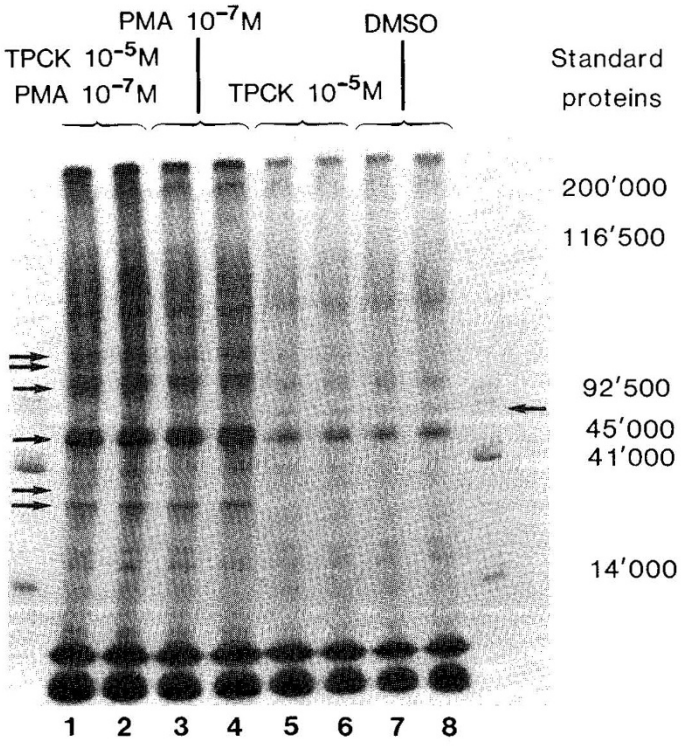

Fig. 2. Autoradiography of ^{32}P-labeled neutrophil proteins separated by molecular weights with polyacrylamide-gel-electrophoresis. The molecular weights of standards run simultaneously are indicated on the *right* of Figure 2. *Lanes 1–4* phosphorylated proteins of neutrophils stimulated with PMA; *lanes 1 and 2*, cells preincubated with TPCK 10^{-5} M for 1 min before stimulation with PMA. *Lanes 5–8*, phosphorylated proteins of unstimulated control cells. *Lanes 5 and 6*, cells preincubated with TPCK. *Arrows* on the *left* indicate newly phosphorylated proteins in stimulated cells, and the *arrow* at the *right* indicates one protein band that disappears in stimulated cells.

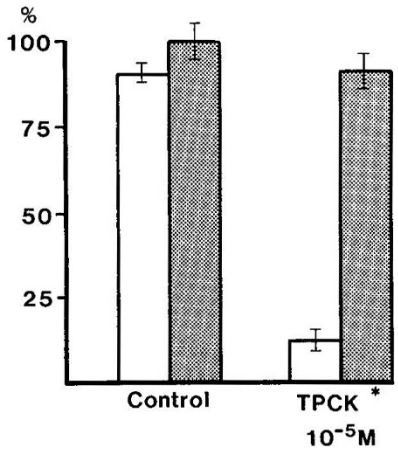

Fig. 3. Effect of preincubation of neutrophils with TPCK 10^{-5} M for 5 min on the ingestion rate of endotoxin-coated ORO particles (*shaded bars*) (14) and the phagocytic-bactericidal activity (12) on *S. aureus* Wood 46. The results correspond to mean ± SD of three separate experiments (in duplicates for the ingestion rate ORO particles and in triplicates for the bactericidal activity or *S. aureus*). The ingestion rate of control cells was considered as 100% and the bactericidal activity is expressed in percent killing of *S. aureus* Wood 46 by neutrophils.

periments to detect and quantify the levels of second messengers in neutrophils stimulated with the chemotactic peptide FMLP in the presence or absence of TPCK. These experiments showed that the enzyme(s) inhibited by TPCK were not involved in the following. 1) FMLP-induced activation of phospholipase C, which we assessed by measuring IP3 production (Table 1). 2) FMLP-induced membrane depolarization (Fig. 1*A*), an early event in cell activation which follows receptor stimulation (9). 3) FMLP-induced Ca^{++} influx from the extracellular medium (Fig. 1*B*) and release of calcium from intracellular calcium stores (Fig. 1*C*). 4) PMA-stimulated protein phosphorylation (Fig. 2) which is thought to be mediated by the activation of protein kinase C (8, 9, 20).

Thus, the step at which TPCK inhibits superoxide production must be distal to the rise in intracellular calcium and protein phosphorylation, two second messengers required to initiate cell function (6, 7, 8, 11).

Results from a previous study on inhibition by TPCK of membrane potential changes and degranulation in rat neutrophils had suggested that the TPCK-inhibited step was located in the early events following cell stimulation. In that study by Duque *et al.* (18) the membrane potential changes assessed by a slightly different fluorescent probe were inhibited by TPCK 10^{-5} M. The authors also found that increasing the extracellular calcium concentration could overcome the inhibition of membrane potential changes by TPCK 10^{-5} M and they concluded that the TPCK-inhibited step preceded a calcium-dependent step. Since in the studies by Duque *et al.* (18) and Mackin and Becker (19) the inhibition of membrane potential changes by TPCK was dose dependent, it is conceivable that interspecies differences between rat and human neutrophils as well as the use of a slightly different fluorescent probe may explain the observed difference. In human neutrophils, we found that at a 10-fold higher concentration, TPCK also significantly inhibited phagocytosis, membrane potential changes, and the FMLP-induced rise in free cytosolic calcium. According to Tsan (17), the unspecific inhibition of human neutrophil sulfhydryl groups becomes significant at concentrations above 10^{-5} M, but it may well be that in rat neutrophils the concentration at which TPCK has unspecific effects is lower.

Another study on rabbit neutrophils showed that after preincubation of rabbit neutrophils with TPCK 2.5 ± 10^{-5} M the number of FMLP receptors decreased and simultaneously the affinity of the receptors for FMLP increased (19). If in our experiments with human neutrophils TPCK 10^{-5} M had an effect on FMLP receptors, this effect did not interfere with second messenger generation.

As mentioned above, TPCK at high concentration has unspecific effects on neutrophil sulfhydryl groups (17). To determine whether nonspecific toxic effects of TPCK were responsible for inhibition of superoxide production, we tested the effect of TPCK 10^{-5} M on complement-mediated phagocytosis (14) and complement-mediated killing of *S. aureus* (13) by human neutrophils (Fig. 3). The phagocytosis of complement-coated ORO particles was unaffected, whereas the killing of *S. aureus*, which is known to be mediated by superoxide production (1), was significantly inhibited. These results indicate that under conditions leading to complete inhibition of superoxide production, the cells were able to phagocyte normally and thus at the concentration of 10^{-5} M TPCK was devoid of unspecific side effects. In addition, reduced glutathion, which was shown to reverse the unspecific effects of TPCK on human neutrophils (17) had no significant effect on the inhibition of superoxide production by TPCK 10^{-5} M.

We also observed a partial inhibition of granule release in neutrophils preincubation with TPCK 10^{-5} M. From our data we cannot conclude, if the mechanism, by which TPCK inhibits superoxide production and granule release is the same or if it is different.

In summary we conclude that the step at which TPCK 10^{-5} M inhibited superoxide production is located at a site distal from calcium mobilization and protein phosphorylation, both events required for the activation of the oxidative system. The events distal to calcium mobilization and the nature of the proteins phosphorylated by protein kinase C are presently poorly understood. Recently, it has been shown that botulinic toxin is capable

of inhibiting exocytosis at cytosolic calcium concentrations that trigger exocytosis (20). Thus TPCK, botulinic toxin, and hopefully other probes will be useful tools to study the effector system in cellular activation. Also, a novel serine-esterase expressed by cytotoxic T lymphocytes was recently revealed by using a radiolabeled protease inhibitor (21). Since TPCK at 10^{-5} M selectively inhibits the oxidative system, whose components are thought to be located in at least two cellulars compartments (22, 23), it is possible that this compound synthesized with radiolabeled precursors may be a useful tool to localize the chymotrypsin-like enzymatic activity that is thought to be involved in the activation of the oxidase.

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