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A DUAL EFFECT OF L-1-TOSYLAMIDE-2-PHENYLETHYL CHLOROMETHYL KETONE ON THE RESPIRATORY METABOLISM OF GUINEA PIG PHAGOCYTES

EFFET DOUBLE DE LA L-1-TOSYLAMIDE-2-PHÉNYLÉTHYL CHLORORMÉTHYL CÉTONE SUR LE MÉTABOLISME RESPIRATOIRE PHAGOCYTAIRE DU COBAYE

P. Dri *, G. Zabucchi *, P. Bellavite **

ABSTRACT : The protease inhibitor, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), stimulated the O_2^- production, H_2O_2 generation, oxygen consumption and the hexose monophosphate shunt of guinea pig peritoneal polymorphs. Maximum stimulation was obtained at 100μM concentration of the compound. None of these effects was seen in human blood polymorphs even at concentrations higher than those effective on guinea pig polymorphs. TPCK also stimulated the oxidative metabolism of guinea pig blood polymorphs and guinea pig resident peritoneal macrophages. The TPCK induced metabolic burst was rapid in onset and was short lived. At concentrations which did not stimulate the oxidative metabolism of guinea pig polymorphs, TPCK inhibited the O_2^- production induced in these cells by treatment with phorbol myristate acetate (PMA). Inhibition of the respiratory burst induced by PMA was also obtained with other protease inhibitors. These substances, however, had no direct stimulatory effect on guinea pig peritoneal leucocyte metabolism. The inhibitory effect of TPCK on PMA-induced stimulation had a brief lag time and increased with time for up to 10 min at least. It is concluded that TPCK exerts two effects on the metabolism of guinea pig phagocytes, which are probably mediated by different mechanisms. The inhibitory effect on PMA stimulated respiratory burst seems to be mediated by the antiprotease activity of TPCK since it is also obtained with other protease inhibitors. The mechanism of the stimulatory effect is still unknown.

Guinea pig phagocytes ; L-1-tosylamide-2-phenylethyl chloromethyl ketone ; oxidative metabolism ; protease inhibitors.

It has ben suggested that proteolytic enzymes play a role in a number of phagocyte functions including chemotaxis [9, 26], exocytosis [3] and phagocytosis [18, 19]. Recently it has been shown that serine protease inhibitors inhibit superoxide anion (O_2^-) production by human neutrophils and monocytes sti-

* Istituto di Patologia Generale, Università di Trieste, via A. Fleming, 22, 34127 Trieste, Italy.

** Istituto di Patologia Generale, Università di Padova, Sede di Verona, Strada Le Grazie, 37100 Verona, Italy.

mulated by various soluble agents [10, 13-15, 24]. Among the compounds tested, the most effective, on a molar basis, was L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK). These results have been taken as an evidence for a requirement of an intact protease function for the activation of the burst of oxygen metabolism following stimulation of phagocytes.

In this paper, we report a comparative study of the effects of TPCK on the oxidative metabolism of guinea pig phagocytes and human polymorphonuclear leucocytes. We show that the effects of TPCK on phagocytes are polymorphic and that the type of response of phagocytes to this compound depends on the concentration of TPCK and on the type of phagocyte used.

METHODS

Materials

Cytochrome c (type VI), horseradish peroxidase (type VI), superoxide dismutase (type I), phorbol 12-myristate 13-acetate (PMA), L-1-tosylamide-2-phenylethyl chloromethyl ketone, soybean trypsin inhibitor (type I-S), phenylmethylsulphonyl fluoride (PMSF), p-tosyl-L-arginine methyl ester HCl (TAME), N-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose-1-^{14}C and glucose-6-^{14}C were purchased from the Radiochemical Center, Amersham, England. Other reagents were of reagent grade. PMA, TPCK, BTEE and PMSF were dissolved in dimethyl sulfoxide.

Preparation of the cells

Human blood polymorphonuclear leucocytes were isolated from heparinized venous blood by dextran sedimentation followed by centrifugation of the resulting supernatant on Ficoll-Paque (Pharmacia Fine Chemicals AB, Sweden) [5]. Guinea pig blood neutrophils were isolated, in the same manner, from blood obtained by cardiac puncture. Guinea pig peritoneal neutrophils were obtained from peritoneal inflammatory exudates, induced by casein, as described [21]. Guinea pig peritoneal resident macrophages were obtained by peritoneal lavages with sterile 0.9 % NaCl. After a brief hypotonic treatment to eliminate contaminating erythrocytes, all cell preparations were suspended in Krebs-Ringer phosphate buffer pH 7.4 containing 0.5mM $CaCl_2$ and 5mM glucose (KRP).

Metabolic assays with intact cells

Superoxide anion production was assayed by measuring spectrophotometrically the superoxide dismutase inhibitable reduction of ferricytochrome c [2]. Both the reference and the sample cuvette contained 150μM ferricytochrome c and 0.5-1.5 $\times$ 10^6 cells. The reference cuvette contained, in addition, 30 μg of superoxide dismutase. The final volume was 1 ml and the temperature was 37 °C. The basic reaction medium was KRP. The reduction of cytochrome c was followed at 550 nm with a Perkin Elmer 576 spectrophotometer.

Oxygen consumption was measured polarographically with a Clark-type oxygen electrode as previously described [23].

Hydrogen peroxide production was measured fluorimetrically by following the conversion of the non fluorescent compound homovanillic acid to the highly fluorescent 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′diacetic acid, as previously described [22].

The rate of oxidation of glucose-1-^{14}C and glucose-6-^{14}C was measured as previously described [8] with the following modifications : the cell suspensions (10^7 leucocytes in 1 ml KRP containing 0.5mM cold glucose) were preincubated for 10 min with 1 μCi of glucose-1-^{14}C or with 2.5 μCi of glucose-6-^{14}C at 37 °C and the reaction was then started by the addition of 100μM TPCK through the rubber cap that sealed the Erlenmeyer flasks.

In each study, control samples contained the solvent used to solubilize the inhibitor.

Cell activation and preparation of cell-free particles

Cell suspensions (10^7/ml) were prewarmed at 37 °C and then incubated with 100μM TPCK for 90 s under continuous stirring. Control cells were incubated under identical conditions in the presence of dimethylsulfoxide. After dilution with ice cold KRP, the cells suspensions were centrifuged at 250 *g* for 7 min. The packed cells were suspended in ice cold 0.34M sucrose buffered at pH 7.0 with Na-bicarbonate and were then homogenized. The homogenization procedure and the preparation of the cell-free particles have been described elsewhere [21].

Determination of the NADPH oxidase activity of cell-free particles

The O_2^- formation by the cell-free particles in the presence of NADPH was measured. The method is based on the spectrophotometric determination of the superoxide mediated cytochrome c reduction as described by BABIOR *et al.* [1]. The assays were carried out in a double beam Perkin Elmer 576 spectrophotometer at 37 °C. Both the reference and the sample cuvette contained 65mM Na-K phosphate buffer, pH 7.0, 170mM sucrose, 150μM cytochrome c, 2mM NaN_3 and 0.15mM NADPH, in a final volume of 1 ml. The reference cuvette contained in addition 30 μg of superoxide dismutase. The reaction was started by adding the particles to both cuvettes, and the absorbance change at 550 nm was followed.

RESULTS

Figure 1 shows the stimulation of O_2^- generation observed when guinea pig peritoneal neutrophils are exposed to optimally effective concentrations of TPCK and PMA. The burst of O_2^- generation induced by TPCK is rapid and terminates within 2-4 min, whereas that induced by PMA is longer lasting. After termination of the short burst induced by TPCK, the cells could be stimulated again by addition of PMA, indicating that the TPCK-induced burst was due neither to a toxic effect of the compound nor to a reduced availability of oxygen or cytochrome c. The increased generation of O_2^- was done dependent as shown in figure 2.

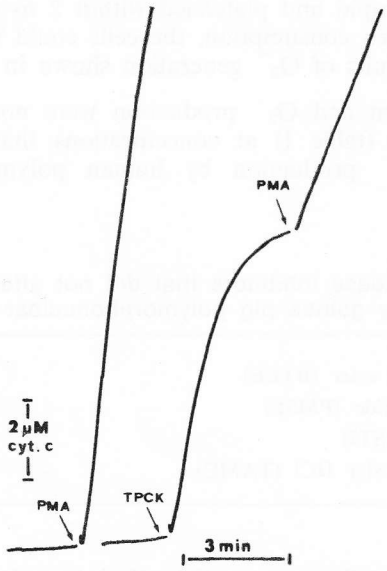

Fig. 1. — O_2^--dependent cytochrome c reduction by guinea pig peritoneal neutrophils, stimulated by TPCK or PMA. In each assay 1.5×10^6 PMN/ml were used. PMA (0.1 μg/ml) or TPCK (100μM) were added at the arrows.

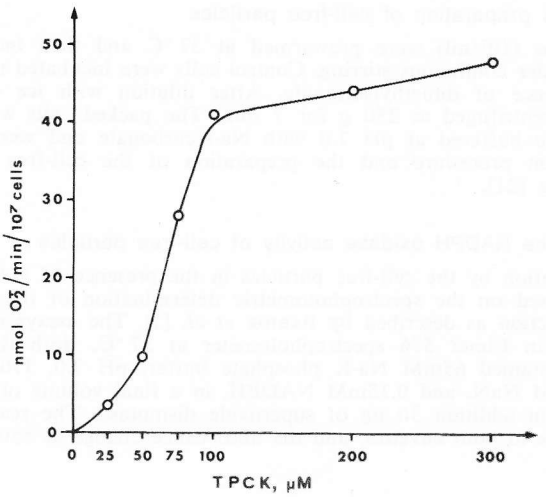

Fig. 2. — O_2^--dependent cytochrome c reduction by guinea-pig peritoneal neutrophils as a function of TPCK concentration. In each assay 1.5×10^6 PMN/ml were used.

Figure 3 shows that TPCK also stimulated three other classical parameters of the respiratory burst of granulocytes, that is, oxygen consumption, hydrogen peroxide production and $^{14}CO_2$ production from glucose labelled on carbon one. $^{14}CO_2$ production from glucose labelled on carbon six was not appreciably affected by TPCK. The kinetics of the stimulation of oxygen consumption, of H_2O_2 and $^{14}CO_2$ production was similar to that described for O_2^- generation, that is, the burst was rapid and plateaued within 2 to 4 min. After termination of TPCK-induced oxygen consumption, the cells could be restimulated by PMA, in keeping with the results of O_2^- generation shown in figure 1.

Oxygen consumption and O_2^- production were not affected by four other inhibitors of proteases (table I) at concentrations that were found to almost completely inhibit O_2^- production by human polymorphonuclear leucocytes [13, 15].

Table I. — Protease inhibitors that did not affect the generation of O_2^- by guinea pig polymorphonuclear leucocytes

N-benzoyl-L-tyrosine ethyl ester (BTEE)	0.2-0.4mM
Phenylmethylsulfonyl fluoride (PMSF)	0.5-1mM
Soybean trypsin inhibitor (STI)	0.1-0.25mM
p-tosyl-L-arginine methyl ester HCl (TAME)	5-10mM

As a further step in the investigation of the mechanism of the stimulatory effect of TPCK, we measured the NADPH oxidizing activity by cell free particles, obtained from resting and TPCK-treated cells, since this enzymatic activity is

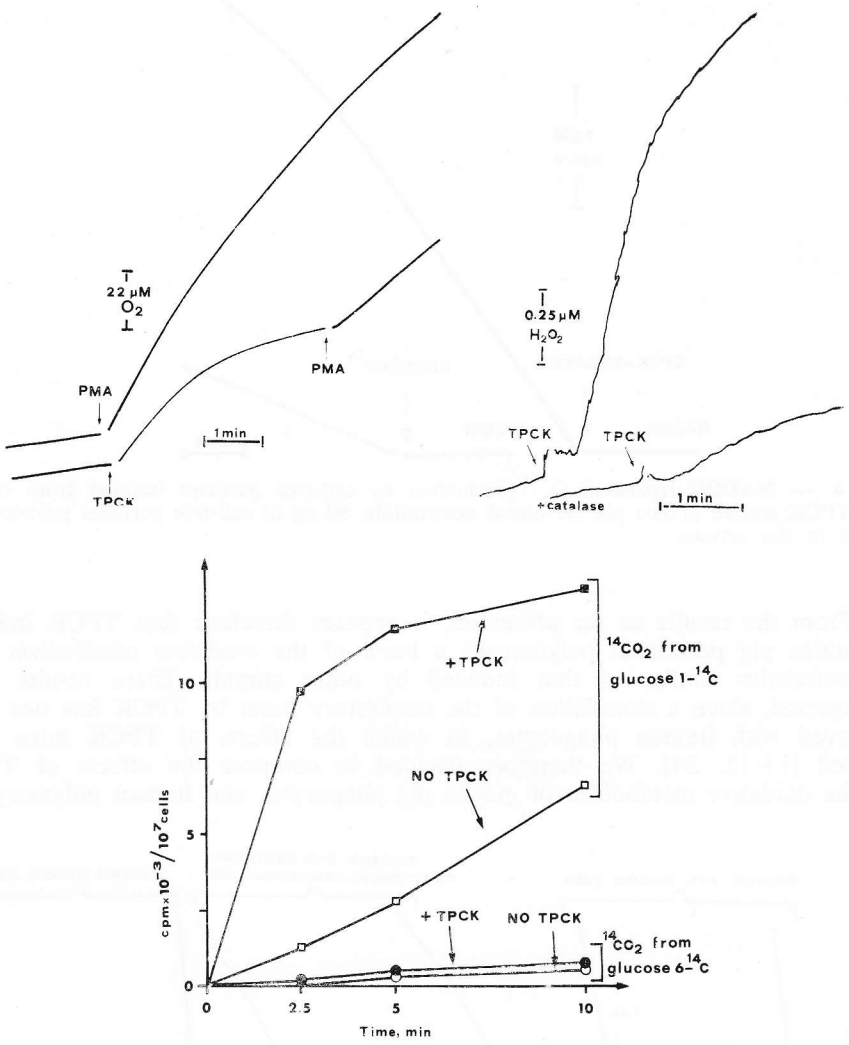

Fig. 3. — Oxygen consumption (top, left), hydrogen peroxide production (top, right) and $^{14}CO_2$ production from ^{14}C-glucose (bottom) by guinea pig peritoneal neutrophils stimulated by TPCK. The oxygen consumption induced by PMA is also shown. 5×10^6 PMN/ml were used for the assay of O_2 consumption ; 0.33×10^6 PMN/ml were used for the assay of H_2O_2 production ; a control with exogenously added catalase (2,600 U/ml) is also included to demonstrate that the increase in fluorescence is specifically due to H_2O_2. 10×10^6 PMN/ml were used for the measurement of $^{14}CO_2$ production from ^{14}C-glucose. For further details, see Methods.

considered by most authors as the basis of the increased respiratory burst of phagocytes [1, 6, 11, 20]. Figure 4 shows that the rate of oxidation of NADPH by cell-free particles isolated from TPCK treated cells is considerably higher than that of particles isolated from untreated cells.

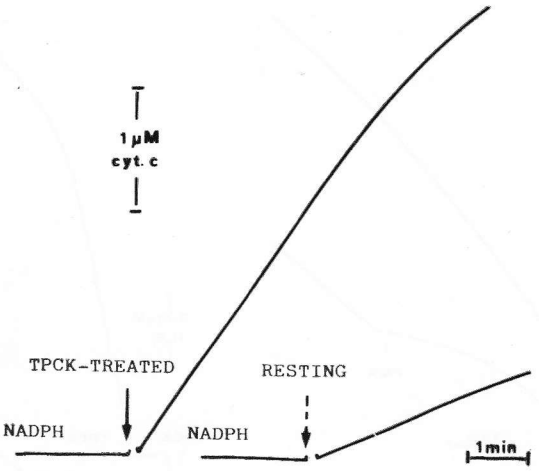

Fig. 4. — NADPH-dependent O_2^- production by cell-free particles isolated from resting and TPCK treated guinea pig peritoneal neutrophils. 80 µg of cell-free particles protein were added at the arrows.

From the results so far presented, it appears therefore that TPCK induces in guinea pig peritoneal polymorphs a burst of the oxidative metabolism with characteristics similar to that induced by other stimuli. These results were unexpected, since a stimulation of the respiratory burst by TPCK has not been observed with human phagocytes, in which the effects of TPCK have been studied [13-15, 24]. We therefore decided to compare the effects of TPCK on the oxidative metabolism of guinea pig phagocytes and human polymorphs.

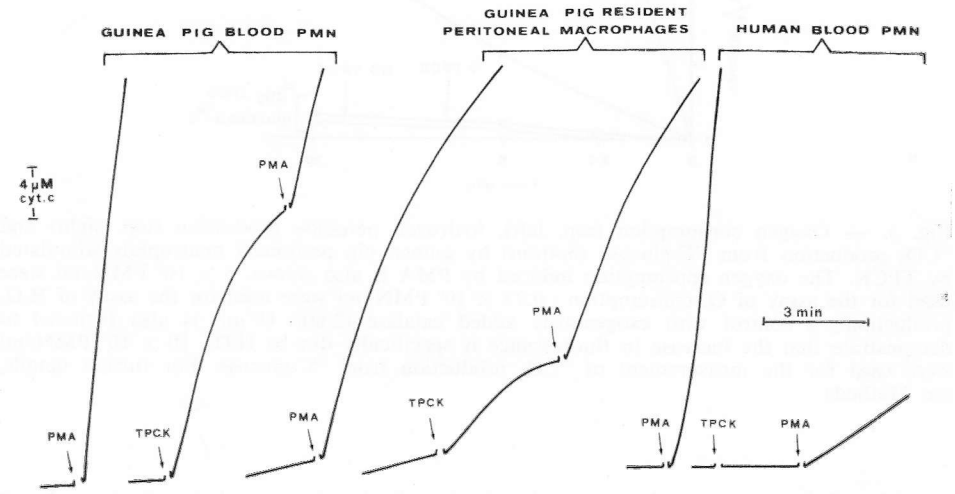

Fig. 5. — O_2^--dependent cytochrome c reduction by guinea pig blood polymorphs, guinea pig resident peritoneal macrophages and human blood polymorphs stimulated by TPCK or PMA. In each assay 10^6 cells/ml were used. TPCK (100 µM) or PMA (0.1 µg/ml) were added at the arrows.

The results are shown in figure 5 and demonstrate that TPCK did not stimulate the basal rate of O_2^- generation by human blood polymorphs at a concentration which gave maximal stimulation in guinea pig peritoneal polymorphs. However, TPCK inhibited the stimulation of O_2^- generation induced by PMA in human blood polymorphs, as reported by other authors [14]. In guinea pig blood polymorphs and in resident guinea pig peritoneal macrophages, TPCK induced a short burst of O_2^- production similar to that previously described for guinea pig peritoneal polymorphs.

It appears therefore that the stimulatory effect of TPCK does not depend on the type or the source of phagocytes, but, rather, on the animal species, although more extensive comparative studies are required in support of this suggestion.

We have already pointed out that guinea pig polymorphonuclear leucocytes could be restimulated by PMA after the termination of the TPCK induced respiratory burst. We observed, however, that the rate of PMA stimulation was always lower than that obtained with cells which had not been prestimulated by TPCK. This raised the possibility that TPCK could have an inhibitory effect on the burst induced by PMA, as it has been already described for human neutrophils [14]. It was found that O_2^- generation by peritoneal and blood guinea pig polymorphonuclear leucocytes stimulated by PMA was inhibited by TPCK at concentrations showed to be slightly or no stimulatory of the oxidative metabolism. Figure 6 compares the kinetics of the stimulatory effect of TPCK which has ben described previously, with the inhibition of the O_2^- generation by guinea pig peritoneal polymorphs treated with PMA. The stimulatory effect reached its maximum after about 1 min and then rapidly decreased, whereas the inhibitory effect started later, then progressively increased and tended to plateau after about 10 min.

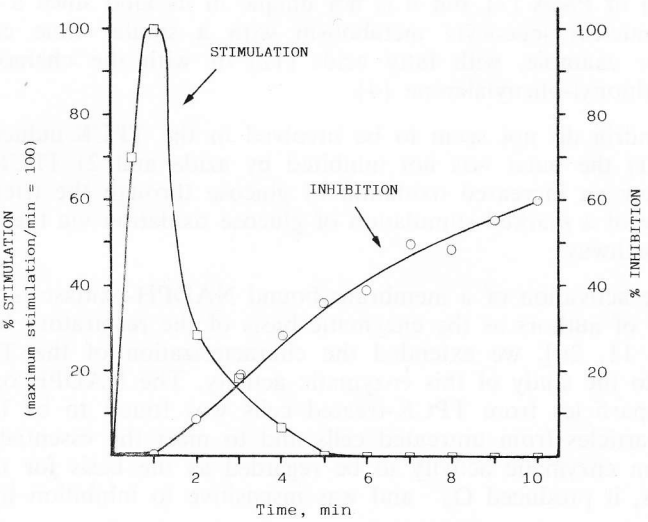

Fig. 6. — Comparison of the kinetics of the stimulation by TPCK of the O_2^- production by guinea pig peritoneal PMN and of the inhibition by TPCK of the PMA-stimulated O_2^- production by guinea pig peritoneal polymorphonuclear leucocytes.

Table II shows that other protease inhibitors, in addition to TPCK, inhibited the generation of O_2^- by guinea pig peritoneal polymorphs stimulated by PMA.

Table II. — Effect of protease inhibitors on the O_2^- production by guinea pig peritoneal PMN stimulated by PMA

		% inhibition of O_2^- production *
BTEE	0.3mM	20-30
PMSF	1.0mM	40-50
TAME	10mM	15-25
TPCK	0.02mM	25-40

* The cells (10^6/ml) were preincubated with the inhibitor for 5 min before PMA (0.1 μg/ml) was added.

DISCUSSION

The results reported in this paper demonstrate that TPCK has a two fold effect on the oxidative metabolism of guinea pig polymorphonuclear leucocytes: 1) stimulation of the basal respiratory metabolism, and 2) inhibition of the respiratory metabolism stimulated by PMA.

The stimulation of the respiratory metabolism by TPCK included an increased oxygen uptake, an increased production of O_2^- and H_2O_2 and an increased oxidation of glucose through the hexose monophosphate pathway. This stimulation is short lived as compared with that induced by other agents such as bacteria [28] or PMA [7], but it is not unique in its kind since a stimulation of polymorphonuclear leucocyte metabolism with a similar time course can be obtained, for example, with fatty acids [12] or with the chemotactic peptide N-formylmethionyl-phenylalanine [4].

Mitochondria do not seem to be involved in the TPCK-induced respiratory burst since 1) the burst was not inhibited by azide and 2) TPCK-treated cells failed to show an increased oxidation of glucose through the tricarboxylic acid cycle in spite of a marked stimulation of glucose oxidation via the hexose monophosphate pathway.

Since the activation of a membrane-bound NADPH-oxidase is considered by the majority of authors as the enzymatic basis of the respiratory burst of leucocytes [1, 6, 11, 20], we extended the characterization of the TPCK induced stimulation to the study of this enzymatic activity. The NADPH-oxidase activity of cell-free particles from TPCK-treated cells was found to be increased with respect to particles from untreated cells and to meet the essential requirements needed for an enzymatic activity to be regarded as the basis for the respiratory burst, that is, it produced O_2^- and was insensitive to inhibition by azide.

It can be, therefore, concluded that the metabolic stimulation of guinea pig polymorphonuclear leucocytes by TPCK and the mechanism of stimulation are basically similar to those induced by other stimuli.

Since a stimulatory effect of TPCK has not ben observed by other authors in human blood polymorphs and monocytes [13-15, 24], we have repeated the experiments with human blood polymorphs and have fully confirmed the results reported in the literature : 1) TPCK had no stimulatory effect on the oxidative metabolism of these cells, and 2) TPCK strongly inhibited the PMA-stimulated metabolism of human blood polymorphs at the concentrations which stimulated guinea pig polymorphs. The inhibition was still evident, although less marked, at concentrations which had no stimulatory effect on guinea pig polymorphs. Whether or not TPCK also inhibited the PMA-induced burst of guinea pig phagocytes could not be decided in our experiments where high concentrations of the compound were used, since such an effect would be masked by the stimulation induced by TPCK at these concentrations. However, it was possible to dissociate the two effects using low concentrations of the compound which did not induce an appreciable stimulation of the cells. At these concentrations, TPCK had an evident inhibitory effect on the PMA-induced burst in guinea pig polymorphs.

The characteristics of the two effects of TPCK on the oxidative metabolism of phagocytes are shown in table III. The inhibitory effect of TPCK on the respiratory burst of human polymorphs and monocytes has been taken as an evidence of the involvement of proteases in this leucocyte function. By following the same line of reasoning, we should be forced to conclude that the inhibition by TPCK of the PMA-induced respiratory burst of guinea pig and human phagocytes is mediated through the antiprotease activity of the compound, since this effect is also obtained with other protease inhibitors. On the contrary, the stimulation of the respiratory burst of guinea pig phagocytes by TPCK would not seem to recognize such a mechanism since it cannot be obtained with other protease inhibitors.

Table III. — Characteristics of the two effects of TPCK on the respiratory metabolism of phagocytes

	Stimulation of the respiratory burst	Inhibition of the respiratory burst induced by PMA
Human blood PMN	no	yes
Guinea pig blood PMN	yes	yes
Guinea pig peritoneal PMN	yes	yes
Guinea pig peritoneal resident macrophages	yes	yes
Low concentrations	no	yes
High concentrations	yes	yes
Onset	rapid	delayed
Duration	short	long
Mimicked by other protease inhibitors	no	yes

We believe, however, that more direct evidence is needed to conclude that proteases are involved in the respiratory burst of phagocytes. In fact TPCK may affect cell functions through mechanisms different from its antiprotease activity. For example, chloromethyl ketones are strong alkilating agents [27] and are capable of inhibiting transport across the plasma membrane [17, 25] and enzymes functionally unrelated to proteases [16]. It cannot be ruled out, therefore, that TPCK may affect the respiratory metabolism of phagocytes through one of these mechanisms.

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RÉSUMÉ : L'inhibiteur de protéases L-1-tosylamide-2-phényléthyl chlorométhyl cétone (TPCK) stimule la production d'O_2^- et de H_2O_2, la consommation d'oxygène et le shunt de l'hexose monophosphate chez les polynucléaires péritonéaux de cobaye. La stimulation maximum est obtenue pour une concentration de 100µM de ce composé. Aucun de ces effets n'est observé pour les polynucléaires sanguins humains, même à des concentrations supérieures à celles qui sont efficaces sur les polynucléaires de cobaye. TPCK stimule également le métabolisme oxydatif des polynucléaires sanguins de cobaye et les macrophages péritonéaux fixés de cobaye. L'explosion métabolique induite par TPCK est d'emblée rapide et de courte durée. A des concentrations qui ne stimulent pas le métabolisme oxydatif des polynucléaires de cobaye, TPCK inhibe la production d'O_2^- induite dans ces cellules par traitement au phorbol myristate acétate (PMA). L'inhibition de l'explosion respiratoire induite par PMA peut être aussi obtenue avec d'autres inhibiteurs de protéases. Cependant ces substances n'ont pas d'effet stimulant direct sur le métabolisme des leucocytes péritonéaux de cobaye. L'effet inhibiteur de TPCK sur la stimulation induite par PMA a un bref temps de latence, puis s'accroît au moins pendant 10 min. En conclusion, TPCK exerce sur le métabolisme des phagocytes de cobaye deux effets aux mécanismes probablement différents. L'effet inhibiteur de l'explosion respiratoire stimulée par PMA semble dépendre de l'activité antiprotéasique de TPCK, car il est également obtenu par d'autres inhibiteurs de protéases. Le mécanisme de l'effet stimulant reste encore inconnu.

Discussion

AUTOR. — Is it possible to overcome the inhibitory effect of TPCK on the PMA stimulation of oxygen consumption by the addition of more PMA to the treated neutrophils ?

PATRIARCA. — If saturating doses of PMA have been used to induce the burst, then the inhibition by TPCK cannot be overcome by addition of PMA.

SINGH. — Do you have any information on the toxicity to animal systems of the protease inhibitors that you have used ?

PATRIARCA. — In polymorphonuclear leukocytes and mononuclear leukocytes, TPCK and other protease inhibitors we have tested are not toxic. The cells are viable after exposure to concentrations of TPCK up to 200 μM as indicated by the fact that they exclude trypan blue and can respond with a second burst of oxygen consumption after another stimulation.

VAN BERKEL. — You showed the effect of protease inhibitors upon the respiratory burst. Did you try compounds which change the pH of lysosomes — like ammonia ?

PATRIARCA. — No, we have not tested the effect of ammonia.