Another biological effect of tosylphenylalanylchloromethane (TPCK): it prevents p47^{phox} phosphorylation and translocation upon neutrophil stimulation

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TPCK (tosylphenylalanylchloromethane), first discovered as a serine protease inhibitor, has been described to affect in diverse systems a number of physiological events probably unrelated to its antiprotease effect, such as proliferation, apoptosis and tumour formation. In the present study, we focus on its inhibition of the neutrophil respiratory burst, an important element of non-specific immunological defence. The superoxide anion-producing enzyme, NADPH oxidase, is quiescent in resting cells. Upon cell stimulation, the redox component, membrane-bound flavocytochrome *b*₅₅₈, is activated when the cytosolic factors (p47^{phox}, p67^{phox} and p40^{phox}, as well as the small GTPase Rac) associate with it after translocating to the membrane. This requires the phosphorylation of several p47^{phox} serine residues. The signal transduction events leading to enzyme activation are not completely understood. In the past, the use of diverse protease inhibitors suggested that proteases were involved in NADPH oxidase activation. We suggested previously that TPCK could prevent

enzyme activation by the phorbol ester PMA, not due to inhibition of a protease, but possibly to inhibition of the cytosolic factor translocation [Chollet-Przednowed and Lederer (1993) Eur. J. Biochem. **218**, 83–93]. In the present work, we show that TPCK, when added to cells before PMA, prevents p47^{phox} phosphorylation and hence its translocation; moreover, when PMA-stimulated cells are incubated with TPCK, p47^{phox} is dephosphorylated and dissociates from the membrane. These results are in line with previous suggestions that the respiratory burst is the result of a series of continuous phosphorylation and dephosphorylation events. They suggest that TPCK leads indirectly to activation of a phosphatase or inactivation of a kinase, and provide the first clue towards understanding the steps leading to its inhibition of NADPH oxidase activation.

Key words: NADPH oxidase, polymorphonuclear leucocyte, p47^{phox}, respiratory burst, tosylphenylalanylchloromethane (TPCK).

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils or PMNs) constitute the first line of immunological defense against micro-organisms. Invaders are killed in phagolysosomes through the combined action of granule proteases and ROS (reactive oxygen species) [1–3]. The latter are produced by NADPH oxidase which reduces oxygen to superoxide anion, O₂^{•−}, the first chemical species from which ROS are produced. In resting cells, the molecular components of the dormant enzyme are distributed between membrane and cytosol. The redox component is membrane-bound flavocytochrome *b*₅₅₈, a heterodimer associating gp91^{phox}, a heavily glycosylated flavohaemoprotein, and p22^{phox}. In the cytosol reside the soluble factors p67^{phox}, p47^{phox} and p40^{phox} in a complex, as well as p21rac, a small GTPase. Upon cell stimulation by physiological or non-physiological stimuli, the cytosolic factors translocate to the membrane, where they form an active complex with flavocytochrome *b*₅₅₈ [1–3]. The production of superoxide anion by PMNs or respiratory burst is an abrupt production of short duration. It has previously been discovered that many non-phagocytic cells can produce superoxide anion and ROS at a much lower and more sustained level, due to flavocytochrome *b*₅₅₈ homologues [4]; in these cells, ROS play important roles in signalling. In contrast, neutrophil-derived extracellular ROS contribute to pathological conditions in inflamed tissues.

The signalling events leading to NADPH oxidase activation have been intensively investigated over the years; nevertheless, much remains to be discovered. It seems clear that signals at various membrane receptors lead to the activation of several protein kinases, in particular PKC (protein kinase C), which phosphorylate several serine residues on the p47^{phox} C-terminus. This induces a conformational change which discloses a binding site on p47^{phox} for the flavocytochrome. p47^{phox} then acts as an adapter, facilitating the functional interaction between p67^{phox} and the redox component [1–3]. The exact role of p40^{phox} and p21rac in oxidase activation is still under debate, as well as the potential role of kinases other than PKC [5,6]. Second messengers, such as phosphatidic acid and arachidonate, appear to be active players under certain conditions, which implicates enzymes such as phospholipases A₂ and D [7,8]. The role of phospholipase C is better understood, since it is activated by the βγ subunits of trimeric G-proteins, as a result of the activation of relevant receptors; phospholipase C produces diacylglycerol and inositol triphosphate, which both contribute to PKC activation. Finally, the events leading to oxidase deactivation have been less well studied. It has been suggested in particular that the burst phenomenon may result from a continuous phosphorylation–dephosphorylation cycle [9–11].

Progress in the understanding of the system was accomplished, in particular, due to the cell-free activation system. It allows

Abbreviations used: BCIP/NBT, 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium; fMLP, N-formyl-methionyl-leucyl-phenylalanine; GTP[S], guanosine 5'-[γ-thio]triphosphate; HBSS, Hanks balanced salt solution; NF-κB, nuclear factor κB; PKC, protein kinase C; PMN, polymorphonuclear leucocyte; ROS, reactive oxygen species; SOD, superoxide dismutase; TBS, Tris-buffered saline; TPCK, tosylphenylalanylchloromethane; TLCK, tosyl-lysylchloromethane.

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NADPH oxidase activation with the use of a mixture of cytosolic and membrane fractions from resting PMNs, together with NADPH, GTP[S] (guanosine S'-[γ -thio]triphosphate) and an amphiphile, such as SDS or arachidonate, as the activator. This system led to the identification of the molecular components mentioned above and to the elucidation of many of their molecular interactions [2,3]. It is now possible to reconstitute the cell-free activity with recombinant cytosolic proteins and purified flavo-cytochrome. It is, however, generally accepted that the system does not reproduce all facets of activation *in vivo*; for example, p47^{phox} can be omitted from the reconstitution system, but its absence *in vivo* causes chronic granulomatous disease, a severe and sometimes fatal immunological deficiency arising from the inability of PMNs to mount a respiratory burst [2,3].

Among pharmacological agents, a variety of protease inhibitors has been used over the past 25 years for probing the system at the cellular level. A number of them, in particular chloromethane derivatives such as TPCK (tosylphenylalanylchloromethane) and TLCK (tosyl-lysylchloromethane), were shown to inhibit superoxide production elicited with various cell stimuli [12–20]. It was found that TPCK was generally more potent than TLCK and that addition of the inhibitors to the cells before a variety of physiological or non-physiological stimuli prevented superoxide production; in a few cases, it was also shown that inhibitor addition after activation stopped the production [12,14]. Nevertheless, no correlation was ever established with a specific protease, and, moreover, doubts were expressed as to the interpretation of the results. In particular, it was reported that the reagents decreased the intracellular GSH content [18]. It was also remarked that, since di-isopropyl fluorophosphate, a serine protease inhibitor with broad specificity, does not inhibit the burst elicited by immune complexes, it was unlikely that the inhibition due to TPCK and TLCK could be exerted via inhibition of a protease [16].

This laboratory studied the effect of TPCK and TLCK on the burst elicited by PMA in human PMN [21–23]. Kinetic studies indicated, as found by previous authors, that TPCK was effective at lower concentrations than TLCK, and both reagents could prevent superoxide generation when added before PMA, or suppress the burst when added after the stimulus. The concentration-dependence curves showed a saturation effect, suggesting a specific target. As the incubations were carried out in the presence of PMSF and leupeptin, which by themselves had no effect on superoxide production, it was concluded that the target could not be a protease. TPCK and TLCK can inhibit PKC *in vitro* [21,24], but the phosphatidylserine- and calcium-dependent PKC activity of TPCK-treated cells was normal, and translocated normally to the membrane upon PMA stimulation. Furthermore, enzymes of the hexose monophosphate shunt were not inhibited either and, contrary to a previous report [18], the cell glutathione content was unaltered [21]. Further studies restricted to TPCK indicated that burst inhibition was due to an effect on the activation and not on the activity of NADPH oxidase. Indeed, in the cell-free system, fractions from TPCK-treated cells yielded the same activity as control ones [22,23]. It was concluded that the reagent was interfering with some signalling step which is not operative in the cell-free system.

Ambruso et al. [25] found that the cytosol from PMA-activated neutrophils shows decreased activating capacity in the cell-free activation system, compared with cytosol from control cells. This was ascribed to the cytosolic factors partial translocation, which decreases their concentration in the cytosol. In our previous study [23], we showed that the cytosol from TPCK-treated cells displayed the same activating capacity as that from control cells; but, when neutrophils had been treated with PMA after TPCK, or with TPCK after PMA, their cytosols had an activating

capacity higher than that of PMA-treated cells. This suggested that TPCK was preventing the PMA-induced translocation of cytosolic factors to the membrane, and was able to dissociate the factors from the membrane after they had formed the active NADPH oxidase complex. In the present work, we verified the suggestion by testing the effect of TPCK before and after PMA on superoxide production in parallel with p47^{phox} translocation. We shed some light on the signalling steps affected by TPCK, by showing that the reagent, when added before PMA, inhibits both phosphorylation and translocation of p47^{phox}; when added after cell stimulation, it induces dephosphorylation of p47^{phox} and its loss from the membrane.

EXPERIMENTAL

Isolation of human neutrophils and preparation of subcellular fractions

Heparinized peripheral blood was obtained from healthy adult volunteers who gave their informed consent. Neutrophils were isolated by the standard technique of dextran sedimentation, Ficoll-Hypaque density centrifugation and hypotonic lysis of residual erythrocytes [26]. Cells were resuspended in HBSS (Hanks balanced salt solution) at 10^8 cells/ml, except when stated otherwise. After the various treatments described below, they were resuspended in the same volume of homogenization buffer (0.34 M sucrose, 7 mM MgSO₄, 1 mM PMSF and 50 μ M leupeptin in 20 mM K⁺/Na⁺ phosphate buffer, pH 7.4), except for metabolic labelling (see below), and sonicated on ice with a Branson sonifier 250 (6 $\times$ 10 s pulses at 30 % efficiency, power 3). The sonicated material was then centrifuged at 4 °C for 10 min at 10 000 g. The supernatant (homogenate) was usually stored at –70 °C until use. Cytosol and membrane fractions were obtained by centrifuging the homogenate at 175 000 g (4 °C) for 90 min in a Beckman TL-100 ultracentrifuge. The pellet (membrane fraction) was resuspended in homogenization buffer by a gentle sonication. When the membrane fraction was used for immunoblots, it was first washed by resuspending in the same buffer supplemented with 200 mM NaCl. Protein concentrations were determined according to the method of Bradford [27].

Superoxide anion production by cells

Neutrophils (10^6 cells/ml) were equilibrated at 37 °C in 500 μ l of HBSS supplemented with 200 μ M horse heart cytochrome *c* (Sigma). SOD (superoxide dismutase) was added to the reference cuvette (20 μ g/ml). Activation was started by adding to both cuvettes 5 μ l of a PMA solution in DMSO (final concentration 1.6 μ M). Cytochrome *c* reduction was monitored at 550 nm with a Uvikon 930 spectrophotometer ($\epsilon_{\text{reduction}} - \epsilon_{\text{oxidation}} = 21 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Activities (nmol/min per 10^6 cells) were determined as the tangent to the maximum slope.

Cell-free assays

Cytosol (40 or 20 μ g of protein) and membrane fractions (20 or 10 μ g of protein respectively), prepared as described above, were activated at 25 °C in a total volume of 110 μ l of 20 mM K⁺/Na⁺ phosphate buffer, pH 7.4, in the presence of 10 μ M GTP[S] and 7 mM MgSO₄. Activation was carried out by addition of different volumes of 2.2 mM arachidonate in 25 % ethanol, according to [28], so as to determine the activity at the maximum of the bell-shaped curve (usually between 80 and 120 μ M arachidonate). After a 4-min incubation, 50 μ l of the mixture were transferred to each of the assay cuvettes equilibrated at 25 °C containing 200 μ l of 20 mM K⁺/Na⁺ phosphate buffer, 7 mM MgSO₄, 100 μ M ferri-cytochrome *c* and 200 μ M NADPH at pH 7.4; the reference

Table 1 Effect of time and PMA concentration on neutrophil activation

Neutrophils (10^8 cells/ml) were incubated in HBSS at 37 °C in the presence of PMA. At various times, the cells were diluted with 5 buffer vols, centrifuged immediately at room temperature at 300 *g*, then washed twice. Their activity was then determined in assay buffer, in the absence of PMA. The data are from a single experiment.

PMA (μ M)	Activation (min) . . .	Superoxide production (nmol/min per 10^6 cells)					
		0	1	2	3	4	5
1.64		0.1	0.4	0.5	0.3	0.25	0.2
3.25		0.2	—	1.4	1.8	1.7	—
15.1		0.1	2.1	3.4	4.5	2.5	0.7

cuvette also contained 20 μ g/ml SOD. Activities were calculated as described for whole cells. The activities of membrane fractions (particulate NADPH oxidase) were determined in the same way, after incubation of 50 μ l of membrane proteins for 4 min at 37 °C in 110 μ l of 20 mM K^+/Na^+ phosphate buffer, pH 7.4, in the presence of 7 mM $MgSO_4$.

Cell treatments

For activity measurements and translocation studies, cells were suspended in HBSS at 10^8 cells/ml. Based on our initial kinetic studies [21], we established satisfactory conditions for cell treatment. Cells were incubated with several PMA concentrations for various periods of time, then washed, and their superoxide production was measured in the absence of activator (Table 1). Cells were more active at higher than at lower PMA concentrations, in agreement with previous reports concerning the dependence on cell concentration of dose–response relationships for a number of effectors, including PMA [14,29–31]. We chose an incubation time of 3 min in the presence of 15.1 μ M PMA at 37 °C and 1 h at room temperature (20 °C) with 0.5 mM TPCK dissolved in 75 % aqueous acetonitrile (the 3 % final acetonitrile concentration was shown previously not to affect cell viability [21,23]). The reactions were stopped by diluting the mixtures 5-fold in HBSS and centrifugation for 10 min at 300 *g*; after two washes with HBSS, one half of each suspension was treated with the second reagent, PMA for the TPCK-treated cells or TPCK for the PMA-activated cells, under conditions identical to those of the first treatment. Assays for superoxide production were carried out immediately after the cells had been washed; the samples not undergoing treatment with the second reagent were left standing at room temperature with gentle shaking occasionally. The cells from all samples were then sonicated as described above and the homogenates, after centrifugation at 10000 *g*, were stored at –70 °C until use.

Electrophoresis and blotting

Samples (4 μ g of cytosolic proteins and 8 μ g of membrane proteins per lane) were submitted to SDS/PAGE in 12 % polyacrylamide gels and electrotransferred on to nitrocellulose or Immobilon membranes at 170 mA for 90 min with a Trans Blot SD semi-dry transfer apparatus from Bio-Rad. The protein load and transfer duration were established by trial and error until transfer from the gel appeared complete in the relevant molecular-mass area, and no protein was transferred to a second membrane. After blocking with 3 % non-fat milk in TBS containing 0.1 % Tween and three washes with the buffer, the membranes were incubated for 1 h with rabbit anti-p47^{phox}. The secondary antibody (1-h incubation) was mouse anti-rabbit IgG coupled to either alkaline phosphatase or horseradish peroxidase (Sigma), for detection with either BCIP/NBT (5-bromo-4-chloroindol-3-yl phosphate/Nitro

Blue Tetrazolium) or the Pierce SuperSignal West Pico ECL[®] reagent respectively; the blots were then scanned and the bands were quantified. The anti-p47^{phox} antibodies had been raised against recombinant p47^{phox} prepared from GST (glutathione S-transferase)–p47^{phox}. The immunoglobulin fraction was obtained after precipitation of the antiserum at 50 % ammonium sulphate saturation and dialysis against PBS.

Immunoprecipitation

Approx. 60 μ l of packed Protein A–agarose beads (Sigma) were equilibrated in TBS and incubated in 300 μ l of TBS for 2 h at 4 °C, with 250 μ g of whole immunoglobulins, or 30 μ g of anti-p47^{phox} immunoglobulins purified on Protein A–Sephrose. After washing with the same buffer supplemented with 0.1 % Triton X-100, the beads were incubated overnight with the cell homogenates (0.5 to 1.5 mg of protein) at 4 °C. After washing, the proteins were eluted with 80 μ l of electrophoresis sample buffer. The samples were then submitted to SDS/PAGE (12 %, gel) electrotransferred and analysed as described above.

Phosphorylation experiments

After erythrocyte lysis, the neutrophils were suspended and washed in a phosphate free-buffer (10 mM Hepes, 5.4 mM KCl, 137 mM NaCl, 0.8 mM $MgCl_2$, 5.5 mM glucose, 0.05 % BSA); they were then incubated for 1 h at 30 °C at a concentration of 10^8 cells/ml with 0.5 to 1 mCi/ml carrier-free [³²P]P_i (NEN or ICN). After washing, they were resuspended in the same phosphate-free buffer, and incubated with TPCK and PMA as described above. Control experiments indicated that the buffer change relative to previous conditions did not alter the cell responses. For sonication, they were resuspended in 10 mM Hepes, 5.4 mM KCl, 137 mM NaCl, 0.8 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 8 % sucrose, 200 μ M leupeptin, 1 mM PMSF, 25 mM NaF and 5 mM sodium vanadate. The homogenate, after the 10-min centrifugation at 10000 *g*, was submitted to immunoprecipitation with anti-p47^{phox} antibodies and Western blotting as described above. The membrane was subjected to autoradiography on a Kodak BioMax MR film and the protein was immunodetected with anti-p47^{phox} antibodies. For some experiments, the cells were treated with 2.7 mM di-isopropyl fluorophosphate for 15 min at 4 °C before labelling, sonicated after the incubations in a buffer containing 1 % Triton X-100 and directly submitted to ultracentrifugation (100000 *g*). The results were similar under the two sets of conditions.

RESULTS

Effect of cell treatment with TPCK on PMA-elicited superoxide production

Cells were treated with either PMA or TPCK alone; they were also submitted to the second reagent after the first one, as described in the Experimental section. The effect of the incubations on whole-cell superoxide production is shown in Table 2. Cells pre-treated with TPCK only produced a little superoxide when challenged with PMA in the assay cuvette. Cells activated with PMA then washed and assayed in the absence of activator were not as active as cells activated in the assay cuvette (Table 2, line 3), but this is to be expected, since in the latter case, the full time-course is monitored so that the maximal activity can be determined. Finally, after treatment with the two reagents in either order, neutrophils hardly produced any superoxide. Thus TPCK prevents activation by PMA and, interestingly, inactivates preactivated neutrophils (Table 2, bottom line). The same phenomena had been observed

Table 2 TPCK effect on the PMN respiratory burst

Control and TPCK-treated cells were activated with PMA in the assay cuvette as described in the Experimental section. The cells that had been incubated with PMA, with or without additional TPCK treatment (last 3 rows), were assayed in the absence of PMA in the spectrophotometer cuvette. Control cells did not undergo any treatment. PMA-only and TPCK-only treated cells were tested for superoxide production immediately after the washing procedure, and were left standing at room temperature with gentle occasional shaking until the end of the second incubation for the other samples. They were all sonicated together. Other details for the various incubations are described in the Experimental section. The numbers in parentheses are the number of independent cell preparations tested.

Cell treatment	PMA	Superoxide production (nmol/min per 10 ⁶ cells)	Activity (%)	
			Relative to control	Relative to PMA
Control	+	9.9 ± 2.0 (10)	100	—
TPCK	+	1.3 ± 0.7 (10)	13 ± 7	—
PMA	—	4.7 ± 1.4 (10)	47 ± 14	100
TPCK then PMA	—	0.5 ± 0.6 (5)	5 ± 6	11 ± 13
PMA then TPCK	—	0.3 ± 0.1 (5)	3 ± 1	6 ± 2

Table 3 NADPH oxidase activity in the membrane fractions

Membrane proteins (50 µl) were incubated in 110 µl of 20 mM phosphate buffer, 7 mM MgSO₄, pH 7.4, for 4 min at 25 °C. Then 50 µl of the incubation mixture were added to each of the spectrophotometer cells containing 200 µl of 100 µM cytochrome *c* and 200 µM NADPH. The reference cuvette in addition contained 20 µg/ml SOD. The numbers in parentheses are the number of independent cell preparations tested.

Cell treatment	Superoxide production (nmol/min per mg)	Activity (%)
Control	1.1 ± 0.6 (11)	7 ± 0.7
TPCK	1.4 ± 0.8 (11)	9 ± 5
PMA	16 ± 9 (11)	100
TPCK then PMA	4.5 ± 1.9 (7)	28 ± 12
PMA then TPCK	4.2 ± 2.4 (5)	26 ± 15

Table 4 Activating capacity of cytosol from treated cells

Cytosolic proteins (20 µg) and control membrane proteins (10 µg) were incubated for 4 min at 25 °C, together with 10 µM GTP[S] and various concentrations of arachidonate in a total volume of 110 µl made up with 20 mM phosphate buffer, 7 mM MgSO₄, pH 7.4. Then 50 µl was added to each of the spectrophotometer cells containing 200 µl of 100 µM cytochrome *c* and 200 µM NADPH in the same buffer. The reference cuvette in addition contained 20 µg/ml SOD. The rates are those determined at the arachidonate concentration that gave the highest activity. The numbers in parentheses are the number of independent cell preparations tested.

Cell treatment	Cell-free activity (nmol/min per mg of membrane proteins)	Activity (%)
Control	35 ± 9 (7)	100
TPCK	32 ± 6 (5)	91 ± 17
PMA	15 ± 3 (7)	43 ± 7
TPCK then PMA	24 ± 2 (3)	69 ± 7
PMA then TPCK	21 ± 3 (4)	60 ± 9

when PMA or TPCK were added directly in the presence of the other reagent [21].

The superoxide production by membrane fractions from treated neutrophils followed the same trend (Table 3). The membranes from control and TPCK-treated cells were not very active. Those from double-treated cells were much less active than those from PMA-treated cells and a little more active than control and TPCK-treated cells. Finally, the activating capacity of the cytosols from the treated cells with respect to control membranes was analysed, using arachidonate as activator (Table 4). The cytosols from TPCK-treated cells behaved as the control cells. Those from PMA-activated cells, as expected, had partially lost their

Figure 1 Effect of TPCK on p47^{phox} translocation

Cytosolic proteins (4 µg) and membrane proteins (8 µg) were submitted to SDS/PAGE. Electrotransfer to a nitrocellulose membrane was carried out as described in the experimental section, as was immunodetection with anti-p47^{phox} rabbit antibodies and BCIP/NBT.

Table 5 Influence of TPCK on p47^{phox} translocation to the membrane

Western blots, such as those in Figure 1, were scanned for quantification by densitometry. Details of the various treatments and of the electrophoretic procedures are described in the Experimental section. The numbers in parentheses are the number of independent cell preparations tested.

Cell treatment	Membrane fraction (% translocation)	Cytosolic fraction (% remaining)
Control	9 ± 5 (11)	100 (8)
TPCK	5 ± 2 (11)	109 ± 17 (7)
PMA	100 (11)	66 ± 7 (7)
TPCK then PMA	20 ± 8 (7)	84 ± 1 (3)
PMA then TPCK	3 ± 2 (5)	112 ± 25 (4)

activating capacity. The cytosols from double-treated neutrophils displayed an efficiency intermediate between that of the control and of the PMA-activated samples; they had lost some activating capacity, or regained some. Together with the data from Table 3, these results suggest that, with the incubation duration chosen by us, the reaction between the cells and TPCK does not reach complete inhibition.

All these results are in agreement with initial studies from this laboratory [23]. They suggest that TPCK prevents translocation of cytosolic factors to the membrane upon neutrophil stimulation with PMA, and that TPCK treatment of pre-activated cells may lead to dissociation of the factors from the membrane. This hypothesis has now been tested by analysing p47^{phox} translocation to the membrane on a number of the cell preparations described in Tables 2–4.

Effect of incubation with TPCK on PMA-elicited p47^{phox} translocation

The presence of p47^{phox} in both membrane and cytosol from treated cells was analysed after gel electrophoresis, electrotransfer and immunodetection, as described in the Experimental section. Figure 1 shows the Western blots for a representative experiment. The blots were scanned and spots quantified for a number of independent experiments (Table 5). It is clear that TPCK by itself did not induce p47^{phox} translocation, contrary to PMA. But PMA

Figure 2 Comparison between the effects of TPCK on superoxide production and p47^{phox} translocation

Open bars, superoxide production; closed bars, p47^{phox} content. The data used in this Figure are those from Tables 2–5. (A) Relative membrane superoxide production and p47^{phox} content. (B) Relative cytosol activating capacity and remaining p47^{phox}.

treatment after cell incubation with TPCK elicited little translocation; moreover, as expected from superoxide production tests (Table 4), most of the p47^{phox} that had translocated to the membrane during the first incubation with PMA was removed from the membrane upon subsequent cell treatment with TPCK, and returned to the cytosol, as indicated by Figure 1 and by quantification. Indeed, knowing the protein amounts of each fraction loaded on to the gels, the spot intensities were brought back to the total protein amount for the same number of cell equivalents, and the sum of cytosol and membrane p47^{phox} for each treatment compared with that for control cells. The average for three independent cell preparations was $99 \pm 11\%$ for the double treatment with TPCK first, and $107 \pm 16\%$ for the double treatment with PMA first. Thus it is not because of degradation that p47^{phox} disappears from the membrane. One can then conclude that the reagent not only prevents PMA-induced p47^{phox} translocation, but reverses its translocation after activation.

Figure 2 compares the relative p47^{phox} content and activation capacity of cytosols from treated cells, on one hand, and the relative p47^{phox} content and superoxide producing capacity of membranes, on the other hand. The correlation is generally satisfactory, although there is a tendency for the relative cytosol activating capacities to be lower than the relative p47^{phox} content. This could possibly be due to the known p67^{phox} instability [32].

Under our conditions, the p47^{phox} translocation level is about 35%, whereas previous data usually lie between 10 and 20% [33–35]. In these studies, the relative PMA to cell concentrations were between 10- and 50-fold lower than the ones used in the present work. Tsunawaki and Yoshikawa [36], on the other hand, reported that one-third of cytosolic p47^{phox} translocated to the membrane,

Figure 3 Effect of TPCK on p47^{phox} phosphorylation

After metabolic labelling, cells underwent the usual treatments, as described in the Experimental section and Table 2. Homogenates were immunoprecipitated using anti-p47^{phox} antibodies, and the results analysed by autoradiography after electrotransfer; the proteins were then immunodetected using BCIP/NBT on to a nitrocellulose membrane (A) or ECL[®] detection on an immobilon membrane (B). For further details, see the Experimental section. The blots are representative of three independent experiments.

using a relative PMA to cell concentration only 2-fold lower than ours. Thus it seems reasonable to assume that, just as the ratio of PMA to cell concentration influences the level of elicited NADPH oxidase activity, it influences the amount of translocation, a phenomenon also described by others [33,35].

Effect of TPCK on p47^{phox} phosphorylation

Since p47^{phox} phosphorylation is considered a prerequisite for translocation and oxidase activation [1–3], we tested if TPCK was affecting p47^{phox} phosphorylation. After metabolic labelling of cells with [³²P]P_i, the homogenates were treated with immobilized anti-p47^{phox} antibodies, and the immunoprecipitated proteins were analysed for radioactivity. The results are shown in Figure 3. As expected, PMA treatment induced p47^{phox} phosphorylation; control cells showed no or weak p47^{phox} labelling, and so did cells exposed to TPCK. But, clearly, when PMA came after TPCK, phosphorylation was prevented; interestingly, when TPCK came after PMA, p47^{phox} was largely dephosphorylated.

DISCUSSION

The results presented above provide the first lead towards understanding the inhibitory effect of TPCK on NADPH oxidase activation. Pre-incubating neutrophils with TPCK inhibits PMA-elicited activation by preventing p47^{phox} phosphorylation and hence p47^{phox} translocation. This phenomenon cannot be ascribed to inhibition of a conventional PKC, since this laboratory has shown previously that the phosphatidylserine- and calcium-dependent PKC activity is not affected by cell incubation with TPCK, and this activity translocates normally to the membrane upon cell stimulation with PMA after pre-incubation with the inhibitor [21]. When TPCK is added to PMA-stimulated superoxide-producing cells, either in the presence of the activator [21] or after it has been washed away ([23] and the present work), it inhibits O₂⁻ production. We can now conclude that this is due to p47^{phox} dephosphorylation leading to p47^{phox} dissociation from the membrane. These effects of TPCK on p47^{phox} should lead to the inhibition of p67^{phox} translocation to and its dissociation from the membrane, depending on the order of reagents addition, and we have preliminary evidence for this.

Our results are in line with previous suggestions that the oxidative burst could be the result of a continuous cycle of phosphorylation and dephosphorylation events, the latter taking precedence

as time goes on and ensuring burst termination [9,11,37]. It is tempting to conclude from our findings that TPCK treatment results in the activation of a protein phosphatase, rather than in the inhibition of a protein kinase. Indeed, the use of inhibitors, such as calyculin and okadaic acid, also indicated the importance of serine/threonine protein phosphatases in burst regulation [3,11,38–40]. But it was also reported that addition of protein kinase inhibitors, such as H-7 or staurosporine, to pre-activated cells stopped superoxide production and led to p47^{phox} dissociation from the membrane, as well as to loss of ³²P from the factor [9,11,37]. Therefore, at this point, we cannot exclude that TPCK treatment leads to the inactivation of a protein kinase, which would not be a conventional PKC. Kinases other than PKC, such as ERK (extracellular-signal-regulated kinase), p38 MAPK (mitogen-activated protein kinase) and Akt, have been proposed to play a role in NADPH oxidase activation [3,5,6]. But the TPCK-mediated Akt inactivation [41] described in other cells (see below) should not come into play in the present case, since an Akt inhibitory peptide had no effect on PMA-elicited superoxide production [6].

So far, we have only used PMA to stimulate neutrophils. Nevertheless, previous data suggest that the burst elicited with more physiological activators is also inhibited by pre-incubation with TPCK; this is the case for activation with fMLP (*N*-formyl-methionyl-leucylphenylalanine) [15,17], with an immune complex [13], with opsonized zymosan [17] and with concanavalin A plus cytochalasin E [12,17]. In a few cases, TPCK was added after stimulation, with the same inhibitory effect as found in the present study after PMA [12,14]. Therefore, it would appear that the TPCK inhibitory effect is not restricted to stimulation with PMA.

TPCK is a reagent with interesting chemical reactivity. It was first elaborated as an affinity label for chymotrypsin [42]. It was later elegantly demonstrated that it actually behaves as a mechanism-based inactivator in a two-step reaction [43]. The inactive enzyme presents a reversible covalent bond to Ser¹⁹⁵ and an irreversible one to His⁵⁷, as do other serine proteases with chymotryptic-like activity. TPCK is also now known to affinity label cysteine proteases with the same specificity, this time by directly forming a bond with the active site cysteine residue. In addition, the chloromethane function is endowed with an intrinsic susceptibility to second-order attack by nucleophilic groups (in particular sulphhydryl groups), with rates depending on conditions. It can, for example, react more rapidly with protein side chains that, for structural reasons, would have acquired special nucleophilicity due to a *pK_a* change. The chloromethane function can also be attacked more rapidly by a nucleophile which would be located by chance close to an affinity site for the hydrophobic phenylalanyl moiety. Thus a number of proteins were reported to be labelled or affinity labelled by TPCK *in vitro*, such as protein kinases {PKC [21,24], PKA (on a thiol group) [44]}, aldehyde dehydrogenase (on a glutamate) [45], fibroblast interferon (possibly on a histidine) [46], transcription factor TFIIIC [47], EFTu [48], luciferase [49] and a viral oncoprotein [50] (the latter four proteins on thiol groups). These findings indicate clearly that TPCK does not act only as a protease inhibitor.

In vivo, the TPCK reactivity may be altered relative to when it is freely accessible in solution, because of compartmentalization, the conformational state of proteins, their inclusion in complexes, etc. Conseiller and Lederer [21], for example, showed that the conventional PKC activity in PMN cytosol can be inhibited by TPCK and TLCK *in vitro*, but not in whole PMNs. TPCK was shown to have anti-proliferative, pro-apoptotic and anti-tumorigenic effects, as well as anti-apoptotic properties (for references, see [41]). A number of these effects may be linked to the inhi-

bition of NF- κ B (nuclear factor κ B) activation, which is due to inhibition of I κ B (inhibitor κ B) phosphorylation [51], a prerequisite for its degradation. Consequently, the reagent has been widely used as an inhibitor of NF- κ B activation (for example, see [41]). TPCK was also reported to inhibit taxol-induced c-Raf-1 and Bcl-2 phosphorylation in human breast carcinoma cells [52], to block the activation of several PDK-1 (phosphoinositide-dependent kinase 1)-dependent kinases, such as Akt, S6K1 and RSK, but not PKA, in a number of cell lines [41] and to dephosphorylate p53 during apoptosis in human colorectal carcinoma cells [53]. These results also suggest an effect of TPCK on phosphorylation/dephosphorylation events in other cell types. Whether the reagent target is the same for all phenomena studied, or whether there might be different targets depending on the cell type is a question that remains to be solved. Whether the reagent could inhibit activation of NADPH oxidase homologues in other cell types would also be worth examining. In any case, it is very difficult to draw conclusions from the doses used in the various systems, since, as discussed above, the effect of a pharmacological agent depends on cell concentration. We have ourselves shown with neutrophils that the TPCK IC₅₀ for burst inhibition after a 5-min incubation is 90 μ M at 10⁸ cells/ml and 2 μ M at 10⁶ cells/ml [21]; the effect may also depend on the cell culture media, since they may contain compounds that could, more or less rapidly, unspecifically react with the chloromethane function and thus decrease the reagent actual concentration. So far, to our knowledge, no intracellular TPCK target has been identified that would be responsible for any of the observed effects, but the papillomavirus E7 oncoprotein was labelled both *in vitro* and *in vivo* with *in vitro* loss of its affinity for the retinoblastoma susceptibility protein [50].

With respect to neutrophils, the present work is the first one that gives a lead towards understanding the effect of TPCK on the respiratory burst. The reagent appears as an interesting new tool for analysing links between some of the signalling steps leading to oxidase activation and deactivation. We believe that no extrapolation can be made from our results to the origin of the effects of other protease inhibitors on neutrophils. Some protease inhibitors may indeed act as antiproteases and others not, depending on the reagent functionality, the cell stimulus and the signalling steps involved.

With tritiated TPCK, the majority of the label was found in the cytosolic fraction, associated with a 15-kDa band on SDS/PAGE [23]. This molecular mass is too small for corresponding either to a protein kinase or phosphatase, suggesting an indirect effect of the reagent if this protein is the relevant TPCK target. Its identity is under investigation in our laboratory. Whatever the case, identifying the target may open the possibility of devising more specific reagents that could be used to locally inhibit ROS production by neutrophils at sites of inflammation.

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REFERENCES

- 1 Cross, A. R. and Segal, A. W. (2004) The NADPH oxidase of professional phagocytes – prototype of the NOX electron transport chain systems. *Biochim. Biophys. Acta* **28**, 1–22
- 2 Vignais, P. V. (2002) The superoxide-generating NADPH oxidase: structural aspects and activation mechanisms. *Cell. Mol. Life Sci.* **59**, 1428–1459
- 3 Babior, B. M. (1999) NADPH oxidase: an update. *Blood* **93**, 1464–1476
- 4 Lambeth, J. D., Cheng, G., Arnold, R. S. and Edens, W. A. (2000) Novel homologs of gp91^{phox}. *Trends Biochem. Sci.* **25**, 459–461

- 5 Dewas, C., Fay, M., Gougerot Pocidalo, M. A. and El Benna, J. (2000) The mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway is involved in formyl-methionyl-leucyl-phenylalanine-induced p47^{phox} phosphorylation in human neutrophils. *J. Immunol.* **165**, 5238–5244
- 6 Chen, Q. D., Powell, D. W., Rane, M. J., Singh, S., Butt, W., Klein, J. B. and McLeish, K. R. (2003) Akt phosphorylates p47^{phox} and mediates respiratory burst activity in human neutrophils. *J. Immunol.* **170**, 5302–5308
- 7 Dana, R., Leto, T. L., Malech, H. L. and Levy, R. (1998) Essential requirement of cytosolic phospholipase A₂ for activation of the phagocyte NADPH oxidase. *J. Biol. Chem.* **273**, 441–445
- 8 Dana, R. R., Eigsti, C., Holmes, K. L. and Leto, T. L. (2000) A regulatory role for ADP-ribosylation factor 6 (ARF6) in activation of the phagocyte NADPH oxidase. *J. Biol. Chem.* **275**, 32566–32571
- 9 Heyworth, P. G. and Badwey, J. A. (1990) Continuous phosphorylation of both the 47 and the 49 kDa proteins occurs during superoxide production by neutrophils. *Biochim. Biophys. Acta* **1052**, 299–305
- 10 Yamaguchi, M., Sasaki, J. I., Kuwana, M., Sakai, M., Okamura, N. and Ishibashi, S. (1993) Cytosolic protein phosphatase may turn off activated NADPH oxidase in guinea pig neutrophils. *Arch. Biochem. Biophys.* **306**, 209–204
- 11 Ding, J. and Badwey, J. A. (1992) Effects of antagonists of protein phosphatases on superoxide release by neutrophils. *J. Biol. Chem.* **267**, 6442–6448
- 12 Kitagawa, S., Takaku, F. and Sakamoto, S. (1980) Evidence that proteases are involved in superoxide anion production by human polymorphonuclear leukocytes and monocytes. *J. Clin. Invest.* **65**, 74–81
- 13 Washida, W., Sagawa, A., Tamoto, K. and Koyama, J. (1980) Comparative studies on superoxide anion production by polymorphonuclear leukocytes stimulated with various agents. *Biochim. Biophys. Acta* **631**, 371–379
- 14 Hoffman, M. and Autor, A. P. (1982) Effect of cyclooxygenase inhibitors and protease inhibitors on phorbol-induced stimulation of oxygen consumption and superoxide production by rat pulmonary macrophages. *Biochem. Pharmacol.* **31**, 775–780
- 15 Duque, R. E., Phan, S. H., Sulavik, M. C. and Ward, P. A. (1983) Inhibition by TPCK of membrane potential changes in rat neutrophils. Correlation with the inhibition of biological activity. *J. Biol. Chem.* **258**, 8123–8128
- 16 Mark, D. E., Lazzari, K. G. and Simons, E. R. (1988) Are serine proteases involved in immune-complex activation of neutrophils? *J. Leukocyte Biol.* **44**, 441–447
- 17 Curnutte, J. T., Badwey, J. A., Robinson, J. M., Karnovsky, M. J. and Karnovsky, M. L. (1984) Studies on the mechanism of superoxide release from human neutrophils stimulated with arachidonate. *J. Biol. Chem.* **259**, 11851–11857
- 18 Tsan, M. F. (1983) Inhibition of neutrophil sulfhydryl groups by chloromethylketones. A mechanism for their inhibition of superoxide anion production. *Biochem. Biophys. Res. Commun.* **112**, 671–677
- 19 Suter, S., Lew, P. D. and Waldvogel, F. A. (1986) Effect of the synthetic inhibitor tosylamino-phenylethyl-chloromethylketone on chemotactic peptide receptor activation and superoxide production in human neutrophils. *Pediatr. Res.* **20**, 848–852
- 20 Gervais, A., Kessels, G. C. R., Suter, S., Lew, P. D. and Verhoeven, A. J. (1991) The chymotrypsin inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone interferes with the neutrophil respiratory burst mediated by a signaling pathway independent of PtdInsP₂ breakdown and cytosolic free calcium. *J. Immunol.* **147**, 1912–1919
- 21 Conseiller, E. C. and Lederer, F. (1989) Inhibition of NADPH oxidase by aminoacyl chloromethane protease inhibitors in phorbol-ester-stimulated human neutrophils: a reinvestigation. Are proteases really involved in the activation process? *Eur. J. Biochem.* **183**, 107–114
- 22 Conseiller, E. C., Schott, D. and Lederer, F. (1990) Inhibition by aminoacyl-chloromethane protease inhibitors of superoxide anion production by phorbol-ester-stimulated human neutrophils. The labeled target is a membrane protein. *Eur. J. Biochem.* **193**, 345–350
- 23 Chollet-Przednowed, E. and Lederer, F. (1993) Aminoacyl chloromethanes as tools to study the requirements of NADPH oxidase activation in human neutrophils. *Eur. J. Biochem.* **218**, 83–93
- 24 Solomon, D. H., O'Brian, C. A. and Weinstein, I. B. (1985) TLCK and TPCK inhibit protein kinase C. *FEBS Lett.* **190**, 342–344
- 25 Ambruso, D. R., Bolscher, B. Q. J. M., Stokman, P. M., Verhoeven, A. J. and Roos, D. (1990) Assembly and activation of the NADPH:O₂ oxidoreductase in human neutrophils after stimulation with phorbol myristate acetate. *J. Biol. Chem.* **265**, 924–930
- 26 Böyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest. Suppl.* **97**, 77–89
- 27 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 28 Pilloud, M. C., Doussière, J. and Vignais, P. V. (1989) Parameters of activation of the membrane-bound superoxide generating oxidase from bovine neutrophils in a cell-free system. *Biochem. Biophys. Res. Commun.* **159**, 783–790
- 29 Grzeskowiak, M., Della Bianca, V., Cassatella, M. A. and Rossi, F. (1986) Complete dissociation between the activation of phosphoinositide turnover and of NADPH oxidase by formyl-methionyl-leucyl-phenylalanine in human neutrophils depleted of Ca²⁺ and primed by subthreshold doses of phorbol 12-myristate 13-acetate. *Biochem. Biophys. Res. Commun.* **135**, 785–794
- 30 Steinbeck, M. J., Hegg, G. G. and Karnovsky, M. J. (1991) Arachidonate activation of the neutrophil NADPH oxidase. Synergistic effects of protein phosphatase inhibitors compared with kinase activators. *J. Biol. Chem.* **266**, 16336–16342
- 31 Tauber, A. I., Brettler, D. B., Kennington, E. A. and Blumberg, P. M. (1982) Relation of human neutrophil phorbol ester receptor occupancy and NADPH oxidase activity. *Blood* **60**, 333–339
- 32 Erikson, R. W., Malawista, S. E., Garrett, M. C., Blaricom, G. V., Leto, T. L. and Curnutte, J. T. (1992) Identification of a thermolabile component of the human neutrophil NADPH oxidase. A model for chronic granulomatous disease caused by deficiency of the p67^{phox} cytosolic component. *J. Clin. Invest.* **89**, 1587–1595
- 33 Clark, R. A., Volpp, B. D., Leidel, K. G. and Nauseef, W. M. (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J. Clin. Invest.* **85**, 714–721
- 34 Quinn, M. T., Evans, T., Loetterle, L. R., Jesaitis, A. J. and Bokoch, G. M. (1993) Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. *J. Biol. Chem.* **268**, 20983–20987
- 35 Abo, A., Webb, M. R., Grogan, A. and Segal, A. W. (1994) Activation of NADPH-oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rho GDI) followed by its translocation to the plasma membrane. *Biochem. J.* **298**, 585–591
- 36 Tsunawaki, S. and Yoshikawa, K. (2000) Relationships of p40^{phox} with p67^{phox} in the activation and expression of the human respiratory burst NADPH oxidase. *J. Biochem. (Tokyo)* **128**, 777–783
- 37 Curnutte, J. T., Erickson, R. W., Ding, J. and Badwey, J. A. (1994) Reciprocal interactions between protein kinase C and components of the NADPH oxidase complex may regulate superoxide production by neutrophils stimulated with a phorbol ester. *J. Biol. Chem.* **269**, 10813–10819
- 38 Lu, D. J., Takai, A., Leto, T. L. and Grinstein, S. (1992) Modulation of neutrophil activation by okadaic acid, a protein phosphatase inhibitor. *Am. J. Physiol.* **262**, C39–C49
- 39 Garcia, R. C., Whitaker, M., Heyworth, P. G. and Segal, A. W. (1992) Okadaic-acid produces changes in phosphorylation and translocation of proteins and in intracellular calcium in human neutrophils. Relationship with the activation of the NADPH oxidase by different stimuli. *Biochem. J.* **286**, 687–692
- 40 Djerdjouri, B., Combadière, C., Pedrucci, E., Hakim, J. and Perianin, A. (1995) Contrasting effects of calyculin A and okadaic acid on the respiratory burst of human neutrophils. *Eur. J. Pharmacol.* **288**, 193–200
- 41 Ballif, B. A., Shimamura, A., Pae, E. and Blenis, J. (2001) Disruption of 3-phosphoinositide-dependent kinase 1 (PDK1) signaling by the anti-tumorigenic and anti-proliferative agent N- α -tosyl-L-phenylalanyl chloromethyl ketone. *J. Biol. Chem.* **276**, 12466–12475
- 42 Schoellmann, G. and Shaw, E. (1962) A new method for labelling the active center of chymotrypsin. *Biochem. Biophys. Res. Commun.* **7**, 36–40
- 43 Kreutter, K., Steinmetz, A. C., Liang, T. C., Prorok, M., Abeles, R. H. and Ringe, D. (1994) Three-dimensional structure of chymotrypsin inactivated with (2S)-N-acetyl-L-alanyl-L-phenylalanyl α -chloroethane: implications for the mechanism of inactivation of serine proteases by chloroketones. *Biochemistry* **33**, 13792–13800
- 44 Kupfer, A., Gani, V., Jimenez, J. S. and Shaltiel, S. (1979) Affinity labeling of the catalytic subunit of cyclic AMP-dependent protein kinase by N- α -tosyl-L-lysine chloromethyl ketone. *Proc. Natl Acad. Sci. U.S.A.* **76**, 3073–3077
- 45 Dryjanski, M., Kosley, L. L. and Pietruszko, R. (1998) N-tosyl-L-phenylalanine chloromethyl ketone, a serine protease inhibitor, identifies glutamate 398 at the coenzyme-binding site of human aldehyde dehydrogenase. Evidence for a second 'naked anion' at the active site. *Biochemistry* **37**, 14151–14156
- 46 McCray, J. W. and Weil, R. (1982) Inactivation of interferons: halomethyl ketone derivatives of phenylalanine as affinity labels. *Proc. Natl Acad. Sci. U.S.A.* **79**, 4829–4833
- 47 Cromlish, J. A. and Roeder, R. G. (1989) Human transcription factor IIIC (TFIIIC). Purification, polypeptide structure, and the involvement of thiol groups in specific DNA binding. *J. Biol. Chem.* **264**, 18100–18109
- 48 Peter, M. E., Brockmüller, J., Jonak, J. and Sprinzl, M. (1989) Identification of the N-tosyl-L-phenylalanine chloromethylketone modification site in *Thermus thermophilus* elongation factor Tu. *FEBS Lett.* **257**, 219–222
- 49 Lee, R. and McElroy, W. D. (1969) Role and reactivity of sulfhydryl groups in firefly luciferase. *Biochemistry* **8**, 130–136

- 50 Stoppler, H., Stoppler, M. C., Adduci, A., Koval, D. and Schlegel, R. (1996) The serine protease inhibitors TLCK and TPCK react with the RB-binding core of HPV-18 E7 protein and abolish its RB-binding capability. *Virology* **217**, 542–553
- 51 Guesdon, F., Ikebe, T., Stylianou, E., Warwik-Davies, J., Haskill, S. and Saklatvala, J. (1995) Interleukin 1 induced phosphorylation of MAD3, the major inhibitor of nuclear factor κ B of HeLa cells. *Biochem. J.* **307**, 287–295
- 52 Huang, Y., Sheikh, M. S., Fornace, A. J. and Holbrook, N. J. (1999) Serine protease inhibitor TPCK prevents taxol-induced cell death and blocks c-Raf-1 and Bcl-2 phosphorylation in human breast carcinoma cells. *Oncogene* **18**, 3431–3439
- 53 Kim, K., Choi, K. H., Fu, Y. M., Meadows, G. G. and Joe, C. O. (2003) Dephosphorylation of p53 during cell death by *N*- α -tosyl-L-phenylalanyl chloromethyl ketone. *Biochem. Biophys. Res. Commun.* **306**, 954–958

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