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Investigation of the inhibitory effects of chelerythrine chloride on the translocation of the protein kinase C βI, βII, ζ in human neutrophils

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Abstract

The protein kinase C (PKC) is a serine/threonine kinase, consisting of different isoforms, implicated in numerous processes of signal transduction. To understand this enzyme well, different pharmacological tools were developed. To activate PKC specifically, phorbol esters were previously used but recent research has shown that these compounds are able to stimulate other proteins. Our model is the respiratory burst in the polymorphonuclear neutrophils. A decrease in the inflammatory process was measured using chelerythrine chloride. Action on PKC was proved by a binding study and by showing the absence of translocation of this enzyme from the cytoplasm to the plasmic membrane during stimulation.

Keywords: Protein kinase C; Translocation; Respiratory burst; Antioxidant properties; Human neutrophil granulocytes

1. Introduction

In the signal transduction system, there is an enzyme which plays an important part, more particularly by its presence in most cellular types and by the number of pathway networks to which it belongs. This is the protein kinase C (PKC) discovered by Nishizuka [1], a serine/threonine kinase, meaning that it is able to phosphorylate proteins on serine or threonine residue, thus implicating its activation which leads to cellular response. PKC exists in several isoforms divided into three subclasses: classical PKC (α, βI, βII, γ), novel PKC (δ, ε, η, θ) and atypical PKC (ζ, λ), which, added to other proteins, creates a new family: the PKC related kinases (PKC μ, PRK). Classical PKC can be stimulated by calcium, various lipids like diacylglycerol or phosphatidylserine or analogues like phorbol esters. The novel PKC can be activated by diacylglycerol and phosphatidylserine but not by calcium. Stimulation of the atypical PKC is also discussed, as most studies have shown that activation is independent of calcium or diacylglycerol and its analogue [2], whereas some results conclude that phorbol esters can stimulate these enzymes [3]. One PRK, isoform μ, is dependent on the stimulation of phosphatidylserine, diacylglycerol and their analogues [4]. As for other PRKs, their stimulation is independent of calcium or diacylglycerol or phorbol esters; they are sensitive to acid phospholipids like phosphatidyl inositol-4,5-biphosphate, and phosphatidylinositol-3,4,5-triphosphate [5].

There are also other parameters influencing the potential activity of PKC. The first is the presence of phosphorylations or specific sites on the protein that can be phosphorylated. These phosphorylations influence the tridimensional structure of the enzyme, by acting on the affinity of diverse factors such as diacylglycerol, calcium or chemical compounds, the capacity of the enzyme to be activated, its stability and its potential to bind with other proteins [6,7]. Another parameter is binding with anchoring proteins, which can regulate the activity of PKC according to its cellular localisation. When PKC is unstimulated, the enzyme is bound to A Kinase Anchoring Protein (AKAP), and

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is localised in the cytoplasm. As a result of stimulation by factors described before, PKC is translocated by shuttle protein to the plasmic membrane where the enzyme binds with the protein Receptor for Activated C Kinase (RACK); and at this point, PKC attains its enzymatic capacity [8,9].

To activate PKC specifically in in vitro studies, a phorbol ester like phorbol 12-myristic-13-acetate was used, but recent works have shown that this chemical compound family can also stimulate other enzymes such as α and β chimacrin, Ras-GRP and UNC-13 [10]. With these new findings, it was essential to develop a new strategy studying the inhibition of PKC in models using in vitro material as it became unsatisfactory to explain a decrease in cell response after activation by a phorbol ester, by saying that it was caused by an inhibition of PKC. The objective of our study was to confirm the role of a potential inhibitor on PKC.

We chose to take into consideration the implication of PKC on oxidative stress in human neutrophils. Cells were separated from other blood constituents using a rapid density gradient technique [11]. To check if the compound had any action on PKC, we first purified this enzyme from the cellular suspension, and then measured binding variation with a radioactive phorbol ester (phorbol-12,13-dibutyrate). To confirm this first result, we visualised the translocation or inhibition of PKC during the activation of this enzyme by Western blot, and a referential inhibitor, chelerythrine chloride, was used to determine optimal conditions. This compound is a benzophenanthridine alkaloid that can inhibit PKC with a 200-fold lower concentration than other kinases. It has two actions on PKC: firstly, binding on the catalytic domain leads to modification of the regulatory domain, preventing a normal binding of the phorbol ester, and secondly chelerythrine chloride is able to methylate the cystein of the protein, decreasing binding with the ligand [12]. The activators were *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13 acetate (PMA). The former is a peptide mimicking a natural ligand with membrane receptors, to obtain stimulation of the cells, proceeding by the classical transduction pathways [13], whereas the latter is a chemical compound, capable of activating the polymorphonuclear by action on the PKC and of inducing considerable and lengthy cell response [14].

2. Materials and methods

2.1. Chemical products

Buffer compounds and chemical products were purchased from Sigma Chemical (St Louis, MO, USA). Chromatography column was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Primary and secondary antibodies were purchased from Tebu (Le Perray en Yvelines, France). ECL Western blotting detection reagents and Hyperfilm ECL were purchased from Amersham International plc (Buckinghamshire, UK).

2.2. Cell separation technique

The 15 ml of freshly drawn human heparinised blood, obtained from healthy donors, was diluted 1:2 with a 0.1 M phosphate buffer saline; pH 7.4; 10 ml of histopaque®-1077 was placed at the bottom of a conical tube. After centrifugation ($400 \times g$ for 30 min at 20 °C), 10 ml of plasma was put aside, the supernatant was eliminated, the pellet was resuspended with the plasma, and the suspension was diluted 1:10 with an ammonium chloride solution (0.15 M ammonium chloride; 10 mM sodium bicarbonate; pH 7.4). After beginning the haemolysis, the tube was kept at 4 °C and gently shaken all the time; the polymorphonuclears were recovered by centrifugation ($400 \times g$ at 4 °C for 10 min) and then the cells were washed twice [11]. Cell viability, determined by blue Trypan exclusion, was over 95%. The cells were kept at 4 °C until use.

2.3. Assessment of the cytotoxicity of the compound

The cytotoxicity of the inhibitor was estimated by dosing lactate dehydrogenase activity in the cellular supernatant of a polymorphonuclear suspension. 5×10^6 GrN were incubated in 1 ml of Hank's Hepes buffer at 37 °C with several concentrations of the compound. The suspension was centrifuged ($400 \times g$ for 10 min at 4 °C), all supernatants were separated, the pellet of the reference tube was resuspended with 1 ml of water and shaken for 5 min to lyse the cells. The tube was centrifuged ($400 \times g$ for 10 min at 4 °C), and the supernatant was also separated. The enzymatic activity of lactate dehydrogenase was measured at 30 °C by enzymatic kinetic reading at 340 nm after adding 0.2 mM NADH and 1.6 mM pyruvate, as described in the manufacturer's protocol [15]. The percentage of lactate dehydrogenase released was estimated in comparison with the maximal activity of this enzyme.

2.4. Anion superoxide assay

The polymorphonuclears (5×10^6 GrN/ml) were incubated with the inhibitor, then cells were activated with 160 nM PMA or 1 μM fMLP at 37 °C. After incubation, ferricytochrome C at 0.2 mg/ml was added to the cellular suspension, and the compound was reduced, causing a change in colour measured at 550 nm [16]. The concentration of anion superoxide was calculated with the extinction coefficient: $E_{550\text{nm}} = 2.1 \times 10^{-2}\ \mu\text{M}^{-1}\ \text{cm}^{-1}$ as previously described.

An acellular model was used to determine the scavenger effect. Anion superoxide was produced by a hypoxanthine–xanthine oxidase system [17]. Absorbance was measured at 550 nm.

2.5. Purification of PKC by chromatography

25×10^6 polymorphonuclears at 5×10^6 GrN/ml were incubated along with phenylmethylsulfonyl fluoride (PMSF) at 57 μM for 15 min at 4 °C. The suspension was centrifuged ($400 \times g$ for 10 min at 4 °C) and the pellet was resuspended by a lysate buffer (20 mM Tris; 0.33 mM EGTA; 2 mM EDTA; 0.33 mM sucrose; 0.5 μg/ml leupeptin; 0.30 μg/ml PMSF; 50 mM β-mercaptoethanol; pH 7.5) at 50×10^6 GrN/ml. The cells were lysed by sonication six times for 5 s, taking care to cool the tube correctly. The lysate was centrifuged ($100\,000 \times g$ at 4 °C for 30 min), the supernatant was mixed v/v with DEAE-Sephacel, and the suspension was shaken for 1 h at 4 °C. The mixture was then put into the column and washed with 10 ml of lysate buffer, and the elution of PKC was obtained by adding 150 mM sodium chloride [18]. PMSF (57 μM) was added to the purified protein and kept at − 80 °C until use.

2.6. Binding

Purified protein (20 μg) was used per tube, the compound was incubated with the enzyme for 15 min at 30 °C in a specific buffer (20 mM Tris; 100 mM potassium chloride; 0.5 mM calcium chloride; 50 μg/ml phosphatidylserine; 0.17% DMSO; 0.2% ethanol; pH 7.5), then 30 mM [^{3}H]phorbol-12,13-dibutyrate (activity 18.6 Ci/mmol) was placed in each tube to start the reaction, the final volume being 325 μl. The mixture was kept at 30 °C for 10 min, and the reaction was stopped by adding 1 ml of freezing DMSO 0.5% solution. The tube was emptied on a GF/C filter (Whatman), before being plunged in a 0.3% polyethylenimine solution. The filter was washed five times with the DMSO solution, and dried by aspiration [19]. Residual radioactivity was measured with a scintillation counter. Non-specific binding was determined by cold phorbol-12,13-dibutyrate.

2.7. Preparation of cells for Western blot

5×10^6 cells/ml were incubated for 20 min with the 57 μM PMSF at 4 °C, then were centrifuged ($400 \times g$ for 10 min at 4 °C) to eliminate the PMSF solution. They were resuspended in a Hank's Hepes buffer pH 7.4, then incubated with the inhibitor at 37 °C and activation was started by 160 nM phorbol-12-myrystate-13-acetate at 37 °C for 5 min or 1 μM fMLP at 37 °C for 10 min. The final volume was 5 ml. Stimulation was stopped by adding 20 ml of ice cold Hank's Hepes buffer and centrifuged ($400 \times g$ for 10 min at 4 °C). The pellet was resuspended in 500 μl of buffer (20 mM Tris; 5 mM EGTA; 2 mM EDTA, 50 mM β-mercaptoethanol; 1 mM PMSF; 330 μM leupeptin; 350 μM antipain; 350 μM pepsatin A; 40 μM chymostatin; 3.1 μM aprotinin; 0.25 mM sucrose; pH 7.5) and sonicated six times for 5 s. The lysate protein was centrifuged ($100\,000 \times g$ for 30 min at 4 °C). The supernatant was kept, the pellet was resuspended with 200 μl of the previous buffer, each sample was mixed with a sodium dodecyl sulfate buffer (32 mM Tris; 71 mM SDS; 1.35 mM glycerol; 0.76 mM bromophenol blue) and boiled for 10 min [20]. The protein concentration was determined by Folin assay.

2.8. Western blot

The equivalent of 130 μg of protein was analysed by polyacrylamide gel electrophoresis on 10% SDS-PAGE for 15 h at 40 mA. The proteins were electrophoretically transferred to the nitrocellulose membrane for 2 h at 36 v. The membrane was saturated with a solution of 10% fat-free dried milk in Tris buffer saline 0.05% Tween 20 (TBS-Tween 20) for 30 min, followed by incubation with rabbit polyclonal antibodies—anti-PKCβI, anti-PKCβII or anti-PKCζ (1/1000 dilution)—for 1.5 h at room temperature. The membrane was washed twice for 10 min with TBS-Tween 20, followed by incubation with (1/10 000) dilution of rabbit peroxidase conjugated secondary antibody for 45 min at room temperature. The membrane was abundantly washed with TBS-Tween 20, then incubated with an ECL mixture of two reagents for 20 min and the films were developed in a dark room.

2.9. Statistical studies

The data obtained were subjected to statistical analysis using a non-parametric test (Student), where P values < 0.05 were considered significant.

3. Results

3.1. Activation of the PKC

First of all, it was necessary to determine the conditions of time and concentration to activate PKC. This was done using the property of the enzyme to be translocated from the cytoplasm to the plasmic membrane during the time of its activation, by Western blot after separation of the cytoplasmic and membrane fraction. With both activators, fMLP and PMA, we noted the three isoforms (βI, βII and ζ) of PKC in the cytoplasmic part when the polymorphonuclears were

unstimulated and these isoenzymes were located in the membrane part after stimulation of the cells by 160 nM PMA for 5 min or 1 μM fMLP for 10 min (Fig. 1).

3.2. Inhibition of $O_2^{\bullet -}$ production in polymorphonuclears by chelerythrine chloride

To confirm a possible effect of the compound on $O_2^{\bullet -}$ production, it had to be checked that this

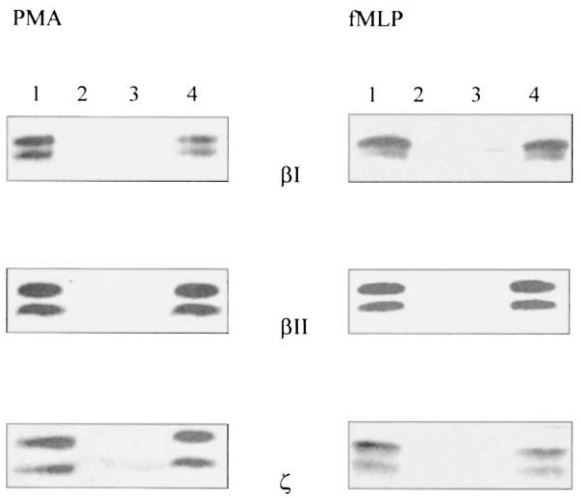

Fig. 1. For the reference, cells were incubated for the same length of time for each activator, then the cytoplasm (lane 1) and plasmic membrane (lane 2) were separated by centrifugation. For the activated cells, stimulation was made by PMA 160 nM for 5 min or by fMLP 1 μM for 10 min; after centrifugation, the cytoplasm (lane 3) was separated from the plasmic membrane (lane 4). Results are representative of three experiments.

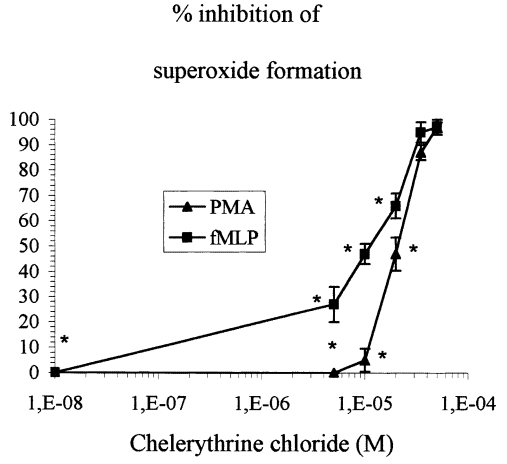

Fig. 2. Action of chelerythrine chloride on superoxide production by the human polymorphonuclear. The cells were incubated with various concentrations of the compound for 15 min at 37 °C. Stimulation was then started by PMA 160 nM for 5 min or fMLP 1 μM for 10 min at 37 °C; cytochrome C was added to the samples to measure the superoxide produced. Results are mean ± SEM of six experiments. Significant differences were evaluated by Student's test $P < 0.05$.

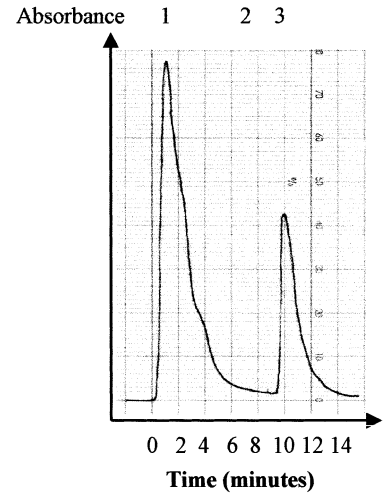

Fig. 3. Chromatogram obtained at 280 nm to elute the three isoforms of PKC. Lane 1: wash of the column in 10 ml of buffer; lane 2: adding of NaCl 150 mM; lane 3: elution of PKC. Results are representative of three experiments.

molecule did not change the biological properties of the polymorphonuclears. The absence of cytotoxicity ($< 5\%$) was controlled in chelerythrine chloride for concentrations between 1 and 200 μM under the conditions previously described: 1 μM fMLP for 10 min and 160 nM PMA for 5 min.

It was also necessary to check that the compound did not interact with the $O_2^{\bullet -}$ produced ($< 5\%$). The acellular system showed that chelerythrine chloride was not an oxygen radical scavenger for concentrations between 1 and 200 μM.

Human polymorphonuclears stimulated with 160 nM PMA for 5 min or 1 μM fMLP for 10 min were able to release a large quantity of $O_2^{\bullet -}$. After incubation with the inhibitor, $O_2^{\bullet -}$ production decreased in a concentration-dependent manner to be totally inhibited. The IC_{50} calculated for PMA activation was 11.6 ± 1.6 μM and the IC_{50} for fMLP activation was 21.1 ± 2.1 μM (Fig. 2).

3.3. Purification of PKC by chromatography

To check the presence of PKC after chromatography, several extracts were collected and the different parts analysed by Western blot.

Chromatography elution was followed by a UV–visible detector at 280 nm (Fig. 2). For each peak observed on the chromatogram, Western blot determined the presence of PKC. When 150 mM sodium chloride was added to the buffer, we could detect the isoforms βI, βII and ζ of the PKC in this eluted fraction (Figs. 3 and 4).

3.4. Binding

To demonstrate that chelerythrine chloride was implicated in the inhibition of oxidative burst in the human polymorphonuclear, we studied the binding of this compound with purified PKC. For this binding a phorbol ester was used: phorbol-12,13-dibutyrate, which is a specific ligand for PKC. After 15 min incubation with the protein, phorbol ester was added to the sample, and binding variation was measured and compared to the sample without inhibitor. The IC_{50} of the binding was 0.71 ± 0.016 nM, and the decrease in binding was dependent on concentration (Fig. 5).

3.5. Inhibition of the translocation of the PKC

Visualisation of the presence or absence of translocation was followed by Western blot. Stimulation was carried out by 160 nM PMA for 5 min or 1 μM fMLP for 10 min. Two concentrations of chelerythrine chloride were used: 4 and 40 μM to show the influence of this compound on PKC.

For the three isoforms of PKC (βI, βII and ζ), its translocation was confirmed during stimulation whatever the activator used, either fMLP or PMA. For a weak concentration (4 μM) of chelerythrine chloride, translocation from cytoplasm to plasmic membrane was also shown, but on the contrary, with a high concentration of inhibitor (40 μM), no difference was noted in localisation of the three isoenzymes during the same incubation time (Fig. 6).

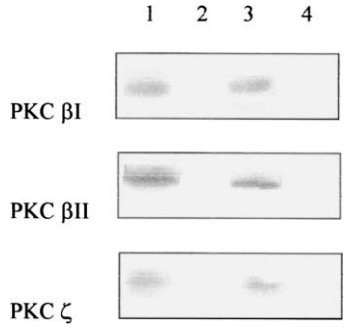

Fig. 4. Western blot corresponding to each fraction obtained according to chromatography spectrum. Different samples were compared, a first consisting of the cellular extract (lane 1), a second corresponding to the first wash of the column (lane 2), a third with the eluate where PKC would be present (lane 3) and a fourth after a wash of the column with NaCl 2 M. Results are representative of three experiments.

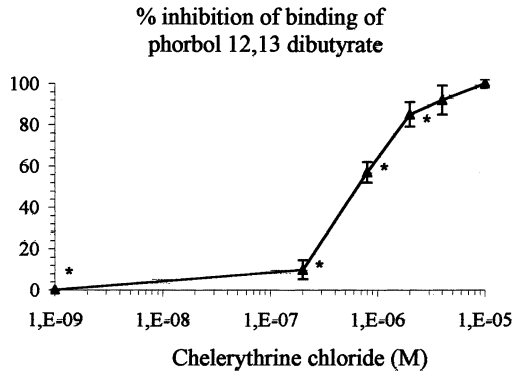

Fig. 5. Action of chelerythrine chloride on the binding of phorbol-12,13-dibutyrate with PKC. The cells were incubated with the compound at 30 °C for 10 min. Phorbol ester was then added to the samples for 15 min at 30 °C, and residual radioactivity was measured by a scintillation counter. Non-specific binding was determined by cold phorbol-12,13-dibutyrate. Results are mean ± SEM of six experiments. Significant differences were evaluated by Student's test $P < 0.05$.

4. Discussion

The PKC is involved in activating other proteins in the pathway network, by phosphorylating them to facilitate cellular response [21]. This system of control is diffused in the organism, and explains the broad distribution of this enzyme and its biological importance [22]. As PKC has a follow-on role with other enzymes, it is difficult to prove whether it has a specific action on PKC, especially because the activators described as specific activators of PKC (phorbol esters) can stimulate other proteins like α and β chimacrin, RAS-GRP and UNC-13 [23–25]. These facts imply that a new strategy had to be developed to test potential inhibitors of PKC in a cellular model. Our study concerns respiratory burst in the granulocyte neutrophil, the involvement of PKC and the possibility to visualise the action of a compound on this enzyme.

The PKC has above all to be translocated from the cytoplasm to the plasmic membrane during its activation [26]. This was confirmed using fMLP and PMA for activators; the three isoforms (βI, βII and ζ) of PKC were translocated with 1 μM fMLP for 10 min and 160 nM PMA for 5 min. As for the isoenzymes βI and βII, first results confirm other studies on classical PKC [27], but activation of the isoform ζ by PMA is a controversial point. Several publications have shown that the isoform ζ cannot be translocated to the plasmic membrane, but a lot of research has reached the same conclusions as ours [3,28]. This result can be explained by the presence of a cystein rich domain on this isoform which could be implicated in binding with concentrated phorbol esters. Another explanation may be the involvement of other pathways. A recent study shows that one isoform of PKC can phosphorylate, and therefore activate, another PKC in unchanged cells, and as our model uses unchanged blood cells, we have preserved the transduction pathway, contrary to other studies using changed cells [10]. So as to show the action of chelerythrine chloride on the polymorphonuclear, we measured the antioxidant activity of this

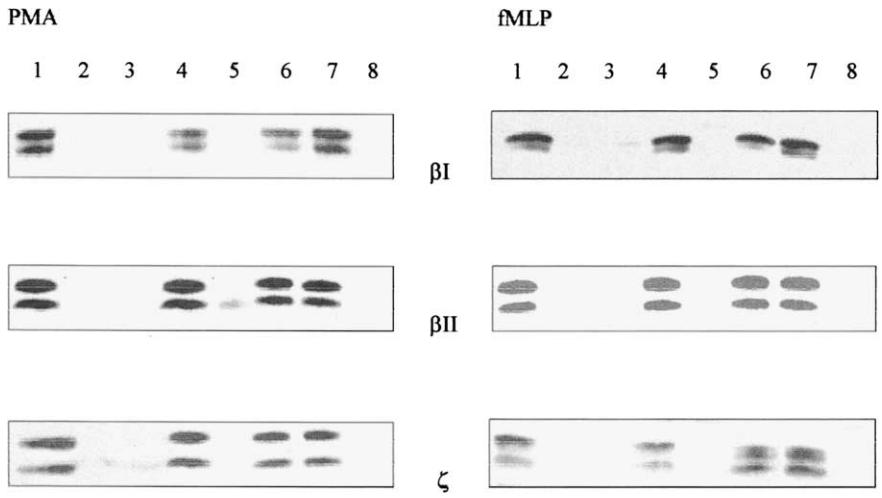

Fig. 6. Action of chelerythrine chloride on the translocation of PKC. For the reference, the cells were treated in the same conditions as the other samples, a centrifugation separated the cytoplasm (lane 1) and the plasmic membrane (lane 2). To activate the cells, the PMA 160 nM was used for 5 min or fMLP 1 μM for 10 min; the cytoplasm (lane 3) was separated from the plasmic membrane (lane 4) by centrifugation. For the sample with the inhibitor, a weak concentration (4 μM) was used, then the cells were stimulated as before; the cytoplasm (lane 5) and the plasmic membrane (lane 6) were analysed. Another sample was made with a concentration in compound of 40 μM and following the same method; the presence or absence of the isoforms βI, βII and ζ of the PKC was visualised in the cytoplasm (lane 7) and in the plasmic membrane (lane 8). Results are representative of three experiments.

compound on cells with a superoxide anion assay after stimulation of the cells by fMLP or PMA. The IC_{50} was 11.6 ± 1.6 μM when fMLP was used and 21.1 ± 2.1 μM for activation by PMA. The margin between the two IC_{50} values is easily accountable as fMLP used the natural pathway to stimulate PKC, whereas PMA directly and mainly activated the enzyme, causing strong activation with no control over the superoxide anion production of the cell [12].

The first element proving that the inhibition of oxidative burst was due to an action of chelerythrine chloride on PKC, was decreased binding with a compound having high affinity with this enzyme [29]. The chosen ligand was phorbol-12,13-dibutyrate. The first step was to partially purify PKC to avoid binding with other proteins. The study revealed decreased recognition of the protein by phorbol ester. The IC_{50} calculated was 710 ± 16 nM, which is comparable to values obtained in different studies [30], but insufficient to prove that PKC is inhibited because phorbol esters can bind with other proteins leading to superoxide anion production.

It was necessary to develop a further strategy to assign the decrease in respiratory burst to inhibition of PKC. PKC has to be translocated to acquire its enzymatic activity. If inhibition of this translocation could be shown, it would also prove the inhibition of PKC, which is correlated with the antioxidant properties of chelerythrine chloride. Having already defined the conditions to visualise the translocation of PKC from cytoplasm to plasmic membrane, they were again applied i.e. 160 nM PMA for 5 min or 1 μM fMLP for 10 min. For a weak concentration (4 μM chelerythrine chloride), translocation was maintained whereas for a high concentration (40 μM chelerythrine chloride) translocation to the plasmic membrane was totally inhibited proving that it was obtained in a concentration-dependent manner. In addition, this experiment may involve some specific action towards one PKC isoform.

In this study, we have proved the inhibition action of chelerythrine chloride on respiratory burst in human polymorphonuclears, by showing its action on the PKC. This was carried out by measuring binding with a phorbol ester and by showing in particular the inhibition of the activation mechanism of PKC, and its translocation from the cytoplasm to the plasmic membrane. This latest strategy has the advantage of showing the specific action of a compound on one isoform of PKC and can be applied to different cellular types with other isoenzymes.

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