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# Investigation of the inhibitory effects of HA-1077 and Y-32885 on the translocation of PKCβI, PKCβII and PKCζ in human neutrophils

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**To transmit the information inside the cell, one possibility is the action of an enzyme called kinase that phosphorylates other proteins. To study these enzymes, chemical compound synthesis was needed to know the function and the mechanism of activation. The major difficulty is creating a specific molecule for one kinase. In this study, we test the action of Rho-kinase inhibitors (HA-1077 and Y-32885) on protein kinase C (PKC) in the respiratory burst in the human polymorphonuclear neutrophils. We have shown that these compounds could inhibit the anion superoxide production. To prove their action on PKC, we have shown a decrease of binding of a specific ligand (phorbol-12,13-dibutyrate) with each inhibitor. During its activation, PKC was translocated from the cytoplasm to the plasmic membrane. We have also shown an inhibition of this translocation, proving an inhibition of PKC by HA-1077 and Y-32885.**

**Key words:** Protein kinase C, Translocation, Antioxidant properties

## Aims

In the signal transduction system there is an enzyme that 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 enzyme is protein kinase C (PKC), discovered by Nishizuka *et al.*[1] PKC is a serine/threonine kinase, meaning that it is able to phosphorylate proteins on serine or on threonine residues, thus implicating its activation, which leads to cellular response. PKC exists in several isoforms divided into three subclasses: classical PKC (PKCα, PKCβI, PKCβII, PKCγ), novel PKC (PKCδ, PKCε, PKCη, PKCθ), and atypical PKC (PKCζ, PKCλ) that, added to other proteins, creates a new family known as the PKC-related kinases (PKCμ). Classical PKC can be stimulated by calcium, various lipids like diacylglycerol or phosphatidylserine, or analogues like phorbol esters. Novel PKC can be activated by diacylglycerol and phosphatidylserine but not by calcium. Stimulation of the atypical PKC is also discussed, and 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 PKC-related kinase, the isoform PKCμ, is dependent on the stimulation of phosphatidylserine, diacylglycerol and their analogues.[4] As for other PKC-related kinases, their stimulation is independent of calcium or diacylglycerol or phorbol esters, and 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 on 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 compound, the capacity of the enzyme to be activated, its stability and its potential to bind other proteins.[6,7] Another parameter is binding with anchoring proteins, which can regulate the activity of the PKC according to its cellular localisation. When PKC is unstimulated, the enzyme is bound to a kinase anchoring protein and is localised in the cytoplasm. As a result of the stimulation by factors already described, PKC is translocated by shuttle protein to the plasmic membrane where the enzyme binds with the protein receptor for activated C kinase; at this point, the PKC attains its enzymatic capacity.[8,9]

The kinase family is regularly studied because of its implication in many cellular processes and transduction signals.[10] One aspect of these studies is the research of compounds capable of inhibiting or of regulating the activity of kinases. We test two chemical products. HA-1077, or 1–5-(isoquinoline sulfonyl)-

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homopiperazine HCl, is usually studied as a suppressor of the vascular contraction by action on different enzymes as PKC, myosin light chain kinase or Rho-kinase.[11,12] Y-32885, or (*R*)-(+)-*N*-(4-pyridyl)-4-(–1-aminoethyl)-benzamide, is an inhibitor of Rho-kinase to regulate the phosphorylation of myosin light chain and to inhibit the smooth muscle contraction, or to inhibit the lysophosphatidic acid induced growth cone collapse.[13,14]

In the present study, we chose to take into consideration the implication of the PKC on oxidative stress in human neutrophils. Cells were separated from other blood constituents using a rapid density gradient technique.[15] To check whether the compounds had an action on the stress oxidative, we measured the variation of superoxide anion ($O_2^{\cdot -}$) produced. The activators were *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and the 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,[16] whereas the latter is a chemical compound capable of activating the polymorphonuclear by action on the PKC and capable of inducing a lengthy cell response.[17] To check whether the compounds had an action on PKC, we first purified PKC from the cellular suspension and then measured binding variation between a radioactive phorbol ester and PKC. To confirm this result, we visualised the translocation or inhibition of the PKC during the activation of this enzyme by Western blot.

## Methods

### Chemical products

Buffer compounds and chemical products were purchased from Sigma Chemical Co. (St Louis, MO, USA). The 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. (Amersham, Buckinghamshire, UK).

### Cell separation technique

The 15 ml of fresh drawn human heparinised blood obtained from healthy donor was diluted 1: 2 with a 0.1 M phosphate buffer saline (pH 7.4). Then 10 ml of histopaque®-1077 was placed at the bottom of a conical tube. After centrifugation (400 × *g* for 30 min at 20°C), 10 ml of plasma were 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 conserved at 4°C and gently shaken all the time. The polymorphonuclears were recovered by centrifugation (400 × *g* for 10 min at 4°C), and then the cells were washed twice.[15] Cell viability, determined by blue Trypan exclusion, was over 95%. The cells were kept at 4°C until use.

### 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. Then $5.10^6$ 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 × *g* for 10 min at 4°C), all supernatants were separated, and the pellet of the reference tube was resuspended with 1 ml of water and shaken for 5 min to lyse the cells. This tube was centrifuged (400 × *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 read at 340 nm after adding 0.2 mM NADH and 1.6 mM pyruvate, as described in the manufacturer's protocol.[18] The percentage of lactate dehydrogenase released was estimated in comparison with the maximal activity of this enzyme.

### Anion superoxide assay

The polymorphonuclears ($5.10^6$ GrN/ml) were incubated with the inhibitor, and cells were activated with 160 nM PMA or 1 μM fMLP at 37°C. After incubation, 0.2 mg/ml of ferricytochrome C was added to the cellular suspension, and this compound was reduced, causing a change in colour measured at 550 nm. The concentration of anion superoxide was calculated with the extinction coefficient $E_{550\,nm} = 2.1 \times 10^{-2}\ \mu M^{-1}\ cm^{-1}$ as previously described.[19]

An acellular model was used to determine a scavenger effect. Anion superoxide was produced by a hypoxanthine-xanthine oxidase system.[20] Absorbance was measured at 550 nm.

### Purification of the PKC by chromatography

A total of $25.10^6$ polymorphonuclears at $5.10^6$ GrN/ml were incubated with phenylmethylsulfonyl fluoride (PMSF) at 57 μM for 15 min at 4°C. The suspension was centrifuged (400 × *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 sec, taking

care to cool the tube correctly. The lysate was centrifuged (100,000 × *g* for 30 min at 4°C), the supernatant was mixed volume/volume 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.[21] Then, 57 μM PMSF was added to the purified protein and kept at −80°C until use.

### Binding

Twenty micrograms of purified protein were 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% dimethylsulfoxide (DMSO), 0.2% ethanol; pH 7.5), and 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 was 325 μl. The mixture was put at 30°C for 10 min, and the reaction stopped by adding 1 ml of freezing 0.5% DMSO solution. The tube was emptied on a GF/C filter, before being plunged in a 0.3% polyethylenimine solution. The filter was washed five times with the DMSO solution, and dried by aspiration.[22] Residual radioactivity was measured with a scintillation counter. Non-specific binding was determined by cold phorbol-12,13-dibutyrate.

### Preparation of cells for Western blot

A total of $5.10^6$ cells/ml were incubated for 20 min with the 57 μM PMSF at 4°C and then were centrifuged (400 × *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 of 20 ml of ice-cold Hank's Hepes buffer and centrifuged (400 × *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, 40 μM chymostatin, 3.1 μM aprotinin, 0.25 mM sucrose; pH 7.5) and sonicated six times for 5 sec. The lysate protein was centrifuged (100,000 × *g* for 30 min at 4°C). The supernatant was kept, the pellet was resuspended with 200 μl of the previous buffer, and each sample was mixed with a sodium dodecyl sulf.ate (SDS) sample buffer (32 mM TRIS, 71 mM SDS, 1.35 mM glycerol, 0.76 mM bromophenol blue) and boiled for 10 min.[23] The protein concentration was determined by Folin assay.

### Western blot

The equivalent of 130 μg of protein was analysed by polyacrylamide gel electrophoresis on 10% SDS 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 a rabbit peroxidase-conjugated secondary antibody for 45 min at room temperature. The membrane was abundantly washed with TBS-Tween 20 and then incubated with an ECL mixture of two reagents for 20 min, and the films were developed in a dark room.

### Statistical studies

The data obtained were subjected to statistical analysis using a non-parametric test (Student's *t*-test), where $p < 0.05$ was considered significantly different versus reference.

## Results

### Activation of the PKC

First at all, it was necessary to determine the conditions of time and concentration to activate PKC. This was carried out using the property of this enzyme to be translocated from the cytoplasm to the plasmic membrane during the time of its activation, by Western blot after separation of the cytoplamic and membrane fraction. With both activators (fMLP and PMA), we noted the three isoforms (PKCβI, PKCβII and PKCζ) of the PKC in the cytoplasmic part when the polymorphonuclears were unstimulated and when these isoenzymes were located in the membrane part after the stimulation of the cells by 160 nM PMA for 5 min or 1 μM fMLP for 10 min (Fig. 1).

### Inhibition of $O_2^{\cdot-}$ production in polymorphonuclears

To confirm a possible effect of the compounds on the production of $O_2^{\cdot-}$, it had to be checked that these molecules did not change the biological properties of the polymorphonuclears. The absence of cytotoxicity (< 5%) was controlled in HA-1077 and in Y-32885 for concentrations between $5 \times 10^{-3}$ and $1 \times 10^{-7}$ M 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 compounds did not interact with the $O_2^{\cdot-}$ produced (< 5%). The

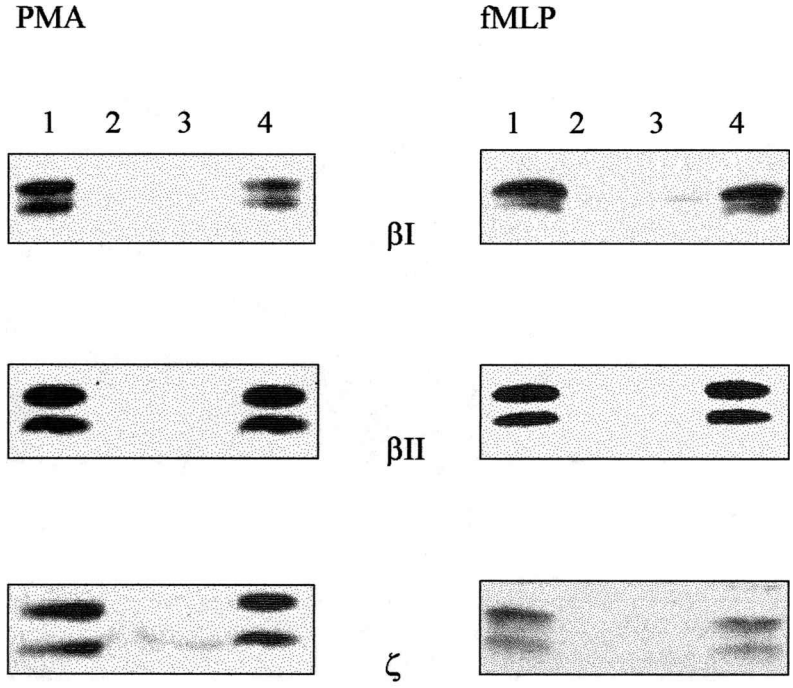


FIG. 1. For the reference, cells were incubated for the same length of time for each activator, then cytoplasm (lane 1) and plasmic membrane (lane 2) were separated by centrifugation. For the activated cells, stimulation was made by 160 nM PMA for 5 min or by 1 μM fMLP for 10 min. After centrifugation,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

acellular system showed that HA-1077 and Y-32885 were not oxygen radical scavengers for concentrations between $5 \times 10^{-3}$ and $1 \times 10^{-7}$ M.

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 $O_2^{\cdot -}$. After incubation with the inhibitors, the $O_2^{\cdot -}$ production decreased in a concentration-dependent manner, to be totally inhibited. The $IC_{50}$ values calculated for the HA-1077 were $1.18 \times 10^{-4} \pm 0.21 \times 10^{-4}$ M for PMA activation and $4.36 \times 10^{-4} \pm 0.16 \times 10^{-4}$ M for fMLP activation (Fig. 2A). The $IC_{50}$ values calculated for the Y-32885 were $0.79 \times 10^{-4} \pm 0.14 \times 10^{-4}$ M for PMA activation and $1.99 \times 10^{-4} \pm 0.08 \times 10^{-4}$ M for fMLP activation (Fig. 2B).

## Binding

To demonstrate that HA-1077 and Y-32885 were implicated in the inhibition of the oxidative burst in the human polymorphonuclear, we studied the binding of these compounds with the purified PKC. For this binding, a phorbol ester was used: phorbol-12,13-dibutyrate, which is a specific ligand for PKC. After incubation of 15 min incubation with the protein, phorbol ester was added to the sample and the binding variation was measured and compared with the sample without inhibitor. The $IC_{50}$ values of the binding were $0.789 \times 10^{-6} \pm 0.024 \times 10^{-6}$ M with Y-32885 and $1.317 \times 10^{-6} \pm 0.057 \times 10^{-6}$ M with HA-1077, and the decreases in binding were dependent on concentration (Fig. 3).

## Inhibition of the translocation of the PKC

Visualisation of the presence or absence of translocation was followed by Western blot. The stimulation was carried out by 160 nM PMA for 5 min or 1 μM fMLP for 10 min. Two concentrations of HA-1077 and Y-32885 were used, 0.5 and 10 mM, to show the influence of these compounds on PKC.

For the three isoforms of PKC (PKCβI, PKCβII and PKCζ), its translocation was confirmed during stimulation whatever the activators used, either fMLP or PMA. For a weak concentration (0.5 mM) of HA-1077 or Y-32885, translocation from the cytoplasm to the plasmic membrane was also shown but, on the contrary, with a high concentration of inhibitors (10 mM), no difference was noted in localisation of the three isoenzymes during the same time of incubation (Fig. 4).

## Conclusions

PKC is involved in activating other proteins in the pathway network, by phosphorylating to facilitate their cellular response.[24] This system of control is diffused in the organism, and explains the broad distribution of this enzyme and its biological importance.[25] We have shown a cellular action of HA-1077

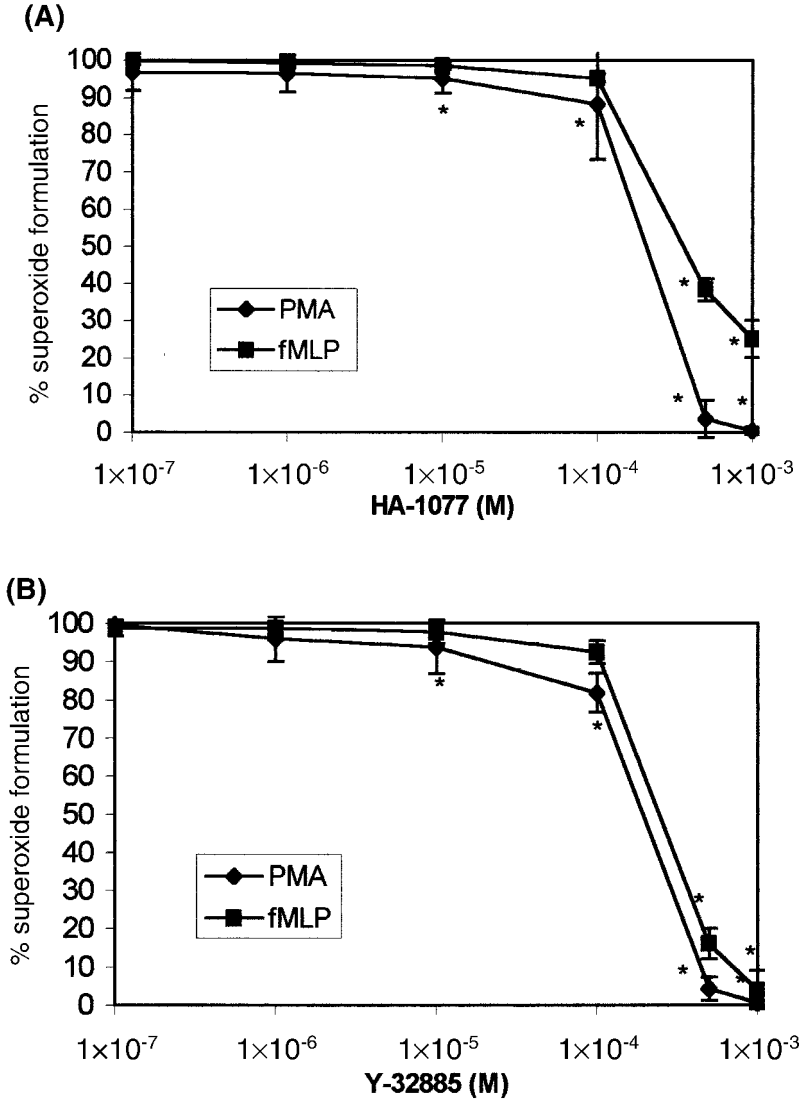


FIG. 2. Action of HA-1077 (A) and Y-32885 (B) on the superoxide production by the human polymorphonuclear. The cells were incubated with various concentrations of the compound for 15 min at 37°C. Stimulation was then started by 160 nM PMA for 5 min or 1 μM fMLP for 10 min at 37°C. Cytochrome C was added to the samples to measure the superoxide produced. Results are the mean ± SEM of six experiments. Significant difference versus the reference was evaluated by Student's *t*-test, $p < 0.05$.

and Y-32885 on the superoxide anion production in the human polymorphonuclear neutrophils. This production was inhibited, and the $IC_{50}$ values calculated for the HA-1077 were $1.18 \times 10^{-4} \pm 0.21 \times 10^{-4}$ M for PMA activation and $4.36 \times 10^{-4} \pm 0.16 \times 10^{-4}$ M for activation by the fMLP. For the Y-32885, the $IC_{50}$ values calculated were $0.79 \times 10^{-4} \pm 0.14 \times 10^{-4}$ M for PMA activation and $1.99 \times 10^{-4} \pm 0.08 \times 10^{-4}$ M for fMLP activation. These results prove that the two compounds can have an action on the inflammatory processes implicating the reactive oxygen species, but it is important to know how HA-1077 and Y-32885 decrease the quantity of superoxide anion. The reactive oxygen species were produced by an enzyme: the NADPH oxidase.[26] Recent studies have shown that PKC has an important role for the activation of the NADPH oxidase.[27] So, we have verified that HA-1077 and Y-32885 can inhibit the superoxide production by acting towards PKC.

The first element proving that the inhibition of the oxidative burst was due to an action of chelerythrine chloride on PKC was decreased binding with a compound having a highly affinity for this enzyme.[28] The chosen ligand was the 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 to phorbol ester. The $IC_{50}$ values calculated were $0.789 \times 10^{-6} \pm 0.024 \times 10^{-6}$ M for Y-32885 and $1.317 \times 10^{-6} \pm 0.057 \times 10^{-6}$ M for HA-1077, but are insufficient to prove that PKC is inhibited because phorbol esters can bind with other proteins leading to superoxide anion

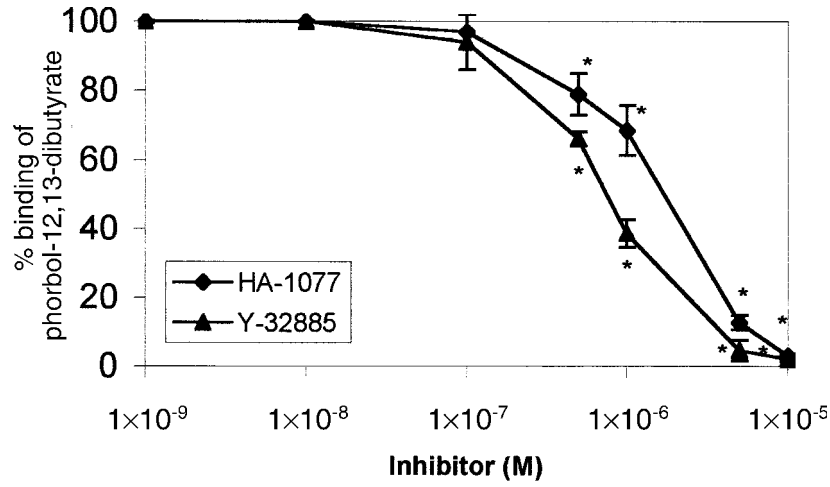


FIG. 3. Action of HA-1077 and Y-32885 on the binding of phorbol-12,13-dibutyrate with PKC. The proteins were incubated with the compounds at 30°C for 10 min. Phorbol ester was then added to the samples for 15 min at 30°C, and the residual radioactivity was measured by a scintillation counter. Non-specific binding was determined by cold phorbol-12,13-dibutyrate. Results are the mean ± SEM of six experiments. Significant difference versus the reference was evaluated by Student's *t*-test, $p < 0.05$.

production as Ras-GRP and UNC-13.[29] Moreover, the difference between the values for the inhibition of the superoxide anion production and the values for the inhibition binding is easily explainable. The former takes the crossing of membranes into account whereas the latter measures directly an action of compounds on PKC, hence lower values.

It was necessary to develop a further strategy to assign the decrease in respiratory burst to inhibition of PKC. The PKC has to be translocated from the cytoplasm to the plasmic membrane during its activation.[30] This was confirmed using fMLP and PMA as activators; the three isoforms (PKCβI, PKCβII and PKCζ) were translocated with 1 μM fMLP for 10 min and 160 nM PMA for 5 min. For the isoenzymes PKCβI and PKCβII, first results confirm other studies about classical PKC,[31] but activation of the isoform PKCζ by PMA is a controversial point. Several publications had shown that the isoform PKCζ cannot be translocated to the plasmic membrane, but a lot of research has reached the same conclusions as us.[3,32] This result can be explained by the presence of a cystein-rich domain on this isoform that could be implicated in binding with concentrated phorbol esters. Another explana-

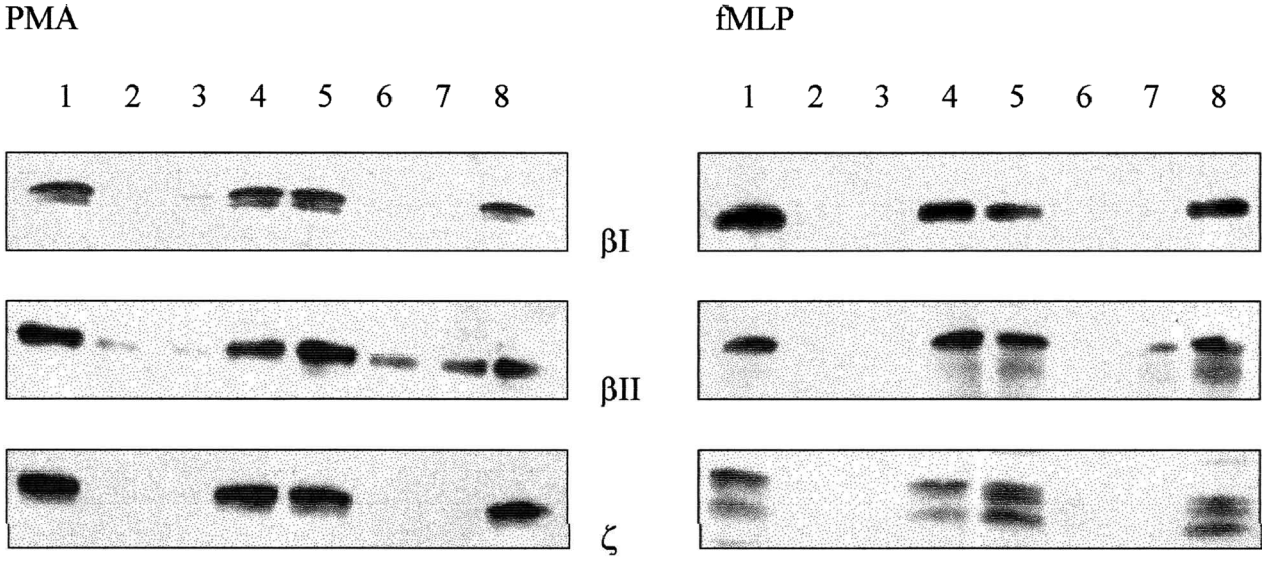


FIG. 4. Action of HA-1077 and Y-32885 on the translocation of PKC. The cells were incubated with the compounds for 15 min with different concentrations (0.5 and 10 mM), and the polymorphonuclears were activated by 160 nM PMA for 5 min at 37°C or by 1 μM fMLP for 10 min at 37°C. Lane 1, Cytoplasm of incubated cells with 10 mM HA-1077; lane 2, membrane of incubated cells with 10 mM HA-1077; lane 3, cytoplasm of incubated cells with 0.5 mM HA-1077; lane 4, membrane of incubated cells with 0.5 mM HA-1077; lane 5, cytoplasm of incubated cells with 10 mM Y-32885; lane 6, membrane of incubated cells with 10 mM Y-32885; lane 7, cytoplasm of incubated cells with 0.5 mM Y-32885; lane 8, membrane of incubated cells with 0.5 mM Y-32885. Results are representative of three experiments.

tion 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. As our model uses unchanged blood cells, we have conserved the transduction pathway, contrary to other studies using changed cells.[29] If inhibition of this translocation could be shown, it would also prove the inhibition of PKC, which is correlated with the antioxidant properties of HA-1077 and Y-32885. 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 μ fMLP for 10 min. For a weak concentration (0.5 mM) in inhibitors, translocation to the plasmic membrane was maintained; whereas for a high concentration in compounds (10 mM), translocation to the plasmic membrane was totally inhibited. In addition, these experiments may involve some specific action of HA-1077 and Y-32885 towards one PKC isoform.

In this study, we have proved the inhibition action of HA-1077 and Y-32885 on respiratory burst in human polymorphonuclears. Usually used to research about Rho-kinase, they can also inhibit PKC. Another molecule of the same family of HA-1077, the H7, has a low selectivity for one kinase. The $IC_{50}$ value, in a model using isolated enzyme, for PKC is 1.8 μM, for protein kinase A it is 1.6 μM and for the phosphorylase kinase it is 6.5 μM.[33] This can be explained by the action mode of these family compounds: they bind the ATP site of the enzyme but this part of the protein is highly conserving,[34] making it difficult to have good selectivity for one kinase. It must be important to develop compounds with better specificity for one kinase family to study them and to have lead compounds for therapeutic application.

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