Effect of tumour-promoting phorbol ester, thrombin and vasopressin on translocation of three distinct protein kinase C isoforms in human platelets and regulation by calcium

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Protein kinase C (PKC) acts in synergy with Ca^{2+} mobilization for the activation of platelets. Three different PKC subtypes that specifically react with antibodies to α -, β - and ζ -PKC have been detected in human platelets. We have compared the subcellular redistribution of these isoforms in platelets after exposure to the tumour-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) and to two physiological agonists, thrombin and vasopressin. In the presence of PMA, β -PKC is most rapidly translocated to membranes, followed by ζ -PKC and α -PKC [membrane contents of 39 ± 6 , 31 ± 4 and $24 \pm 4\%$ (means $\pm$ S.E.M.) respectively after 2 min incubation]. In contrast, both thrombin and vasopressin induced a biphasic translocation of PKC isoforms. For both agonists, the first phase of translocation occurred within 1 min and was identical for the three isoforms. However, during the second phase, the translocation of ζ -PKC by thrombin and vasopressin differed [membrane contents (mean $\pm$ S.E.M.) of 24 ± 3 and $46 \pm 4\%$ respectively after 10 min]. These results suggest a differential activation of ζ -PKC by vasopressin and thrombin. PMA-induced translocation of α -PKC was decreased from 278 ± 27 to 198 ± 24 (mean $\pm$ S.E.M., $P = 0.02$; percentage increase over control value) in the presence of 1 mM-EDTA, whereas chelation of intracellular Ca^{2+} by Quin2-AM does not influence this response. These results suggest that the PMA-induced translocation of α -PKC depends on the presence of 1 mM concentration of extracellular Ca^{2+} . In addition, the chelation of either extracellular or intracellular Ca^{2+} inhibited both vasopressin- and thrombin-induced translocation of all three isoforms, suggesting that Ca^{2+} is an important requirement for the translocation of α -, β - and ζ -PKC by physiological agonists. In conclusion, the translocation of PKC varies between different isoforms and between different agonists.

INTRODUCTION

In platelets, excitatory agonists involve the activation of phospholipase C and the subsequent generation of the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (DAG), which respectively results in an increase of intracellular Ca^{2+} and activation of protein kinase C (PKC), the major receptor for tumour-promoting phorbol esters [1]. In platelets, bioactive DAGs or phorbol esters act in synergy with the mobilization of intracellular Ca^{2+} to induce a full response of platelets, such as shape change, secretion and aggregation [2–4]. Activation of PKC plays an essential role in both the propagation and termination of cell stimulation [1]. In a previous study we have shown that preincubation of platelets with a tumour-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) decreased the rise in intracellular Ca^{2+} induced by thrombin, and that the addition of PMA to thrombin-preactivated platelets increased the rate of decay of a Ca^{2+} transient [5]. This suggested that activation of PKC can decrease the activation of phospholipase C and stimulate the decrease in internal Ca^{2+} by increasing the reuptake of Ca^{2+} in internal stores, the dense tubular system in platelets or the acceleration of the excretion of Ca^{2+} by stimulation of the Ca^{2+} -ATPase in the plasma membrane. A negative-feedback regulation of the receptor-induced hydrolysis of phosphoinositides, Ca^{2+} mobilization and dense-granule secretion has been confirmed in different studies, as well as stimulation of Ca^{2+} sequestration by phorbol esters [6–10]. These data suggest that PKC accomplishes various functions during

activation of platelets, which may be explained by the presence of several PKC species.

Molecular cloning has revealed the presence of at least eight subspecies, α -, βI -, βII -, γ -, δ -, ϵ -, ζ - and η -PKC [11–14]. These PKC subspecies differ in their mode of activation, kinetic properties, substrate specificities and cellular or tissue distributions [11,15,16]. They can be subdivided in two groups, a group of Ca^{2+} -dependent forms (α -, β -, γ -PKC) and a group of Ca^{2+} -independent forms (δ -, ϵ -, ζ -, η -PKC) [11]. Only a little information is available on the PKC subtypes present in human platelets. Immunological analysis using subtype-specific monoclonal antibodies and hydroxyapatite column chromatography revealed the presence of type β and type α PKC in human platelets [17,18]. DAG and phorbol esters induce the conversion of the inactive cytosolic PKC into a membrane-associated activated form, which has become a widely recognized mechanism for PKC regulation [19]. However, some studies suggest that PKC activation can occur without translocation of the enzyme [20–23]. Although Fournier *et al.* [24] reported no particulate accumulation or loss of the cytosolic β isoforms when platelets were incubated with PMA, Hagiwara *et al.* [25] demonstrated the translocation of the isoform β from cytosol to membrane.

In a previous study, using immunoblotting analysis with monoclonal antibodies specific for the various subtypes of PKC, we have identified the presence of α -, β - and ζ -PKC in human platelets and have analysed the translocation of these three isoforms after stimulation of platelets by PMA [26]. In that previous work, the specificity of polyclonal antibodies that we

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate.

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had produced was not fully characterized. In the present study, we report the specificity of the PKC-subtype-specific antibodies. Since PMA is not a natural agonist and induces an irreversible translocation of PKC, we compared the time course of redistribution of the different isoforms with two physiological agonists, thrombin and vasopressin. As intracellular Ca^{2+} is known to regulate the kinase activity of PKC subspecies in different ways, we have also looked at the effect of intracellular or extracellular Ca^{2+} on the mechanism of translocation of the distinct PKC isoforms.

EXPERIMENTAL

Materials

Prostacyclin (prostaglandin I_2), apyrase, phenylmethanesulphonyl fluoride, β -mercaptoethanol, SDS, Triton X-100, thrombin and [arginine]vasopressin were purchased from Sigma. PMA was obtained from Calbiochem. Leupeptin and Hepes were from Fluka. Molecular-mass markers for electrophoresis and horse serum were provided by Gibco. The monoclonal antibodies for α -, β - and γ -PKC were purchased from Seikagaku Kogyo, Tokyo, Japan, and ^{125}I -labelled sheep anti-mouse IgG or donkey anti-rabbit IgG ($18 \mu\text{Ci}/\mu\text{g}$) were from Amersham. Sf9 cells and recombinant baculovirus were provided by Silvia Stabel.

Preparation of platelet suspensions

Blood was obtained from healthy volunteers from the blood bank of Basel Kantonsspital and collected in presence of 13 mM-trisodium citrate. Platelet-rich plasma was obtained by centrifugation of the blood at 120 *g* for 15 min at 20 °C, and centrifuged a second time for 15 min at 120 *g* to eliminate the maximum amount of red and white blood cells. The supernatant was centrifuged at 600 *g* for 20 min at 20 °C in the presence of 3 mM-EDTA, 30 μg of apyrase/ml and 300 ng of prostacyclin/ml. The pellet of platelets was resuspended in Hepes/Tyrod's buffer (KCl 2.68 mM, NaCl 136 mM, MgCl_2 0.23 mM, NaH_2PO_4 0.36 mM, D-glucose 5.5 mM, Hepes 20 mM, pH 7.4) at a concentration of $(5-10) \times 10^8$ platelets/ml and divided into 1 ml fractions. Before stimulation with different agonists, platelets were incubated at 37 °C by gentle agitation in Hepes/Tyrod's buffer for 2 h. This step was necessary to obtain the platelets in a resting state and allow them to recover from PGI_2 inhibition. Furthermore, after this period shape change occurred after stimulation and thrombin or vasopressin (results not shown). PMA was prepared from a stock solution (10 mM) in dimethyl sulphoxide. For experiments in the presence or absence of external Ca^{2+} the platelets were preincubated for 10 min with either 1 mM- CaCl_2 or 1 mM-EDTA.

Preparation of cytosol and membrane proteins

After the different incubation procedures at 37 °C, platelets suspended in Hepes/Tyrod's buffer were cooled to 4 °C and diluted with the same volume of buffer (0.5 M-sucrose, 0.02 % leupeptin, 2 mM-phenylmethanesulphonyl fluoride, 20 mM- β -mercaptoethanol, 1 mM-EDTA and 1 mM-EGTA, pH 7.5), and the suspension was then sonicated for 4×10 s bursts with a sonifier (Branson Sonic Power Co., Danbury, CT, U.S.A.). The samples were centrifuged at 200 000 *g* for 20 min at 4 °C. The supernatants were collected (cytosol fraction), and the pellet was sonicated as described above in 2 ml of sonication buffer containing 1 % SDS (membrane fraction).

Immunoblotting and quantification of PKC isoforms in platelet fractions

For the characterization of the α , β and γ isoforms of PKC,

the subtype-specific monoclonal antibodies (Seikagaku Kogyo, Tokyo, Japan) were used. Synthetic peptides based on the C-terminal sequence specific for the rat brain δ -PKC (KGFSFVNPKYEQFLE), ϵ -PKC (NQEEFKGFSYFGEDLMP) and ζ -PKC (GFEYINPLLLSAEESV) [12] were coupled to keyhole-limpet haemocyanin by glutaraldehyde [27] and used to immunize rabbits as described previously [28].

Samples (600 μl) of cytosol or membrane fraction were mixed with 300 μl of solution containing 0.0625 M-Tris/HCl, pH 6.8, 10 % glycerol, 2 % SDS, 5 % β -mercaptoethanol and 0.001 % Bromophenol Blue and heated at 96 °C for 3 min. The extracts were stored at -20 °C until required. Portions (100 μl) of these samples were subjected to electrophoresis on SDS/8 %-polyacrylamide gels as described by Laemmli [29]. Proteins were transferred electrophoretically to nitrocellulose membranes and processed for immunoblotting exactly as described [28], by using monoclonal antibody to α -, β - and γ -PKC isoforms (dilution 1:100) or polyclonal antibody to δ -, ϵ - and ζ -PKC isoforms (dilution 1:500). Immunoreactivity was analysed by using ^{125}I -labelled sheep anti-mouse or donkey anti-rabbit antibodies (0.2 $\mu\text{Ci}/\text{ml}$). Detection was achieved by autoradiography, by exposing the nitrocellulose membrane to Kodak X-Omat film overnight at -70 °C. Specificities of anti-PKC antisera towards their respective antigens as well as the other PKC subtypes were tested by using insect cell extracts expressing recombinant bovine α -PKC, human β 1-PKC, human β 2-PKC, human γ -PKC, rat δ -PKC, rat ζ -PKC and mouse η -PKC. Identical amounts of protein kinase activity (10–30 μg) of each individual PKC subtype (determined in crude cellular extracts of Sf9 cells infected with the recombinant baculovirus carrying the coding region of the various subtypes of PKC) were loaded on to SDS/PAGE and transferred to nitrocellulose as described [28].

The radioactive bands identified as PKC were sliced from the nitrocellulose and the radioactivity was determined by a γ -radiation counter. In all experiments the sum of radioactivity (c.p.m.) in the cytosol and membrane was constant. The percentage of PKC in membranes was calculated as follows: $100 \times \text{membrane c.p.m.} / (\text{membrane c.p.m.} + \text{cytosol c.p.m.})$. Statistical analyses were performed by Student's *t* test or by a non-parametric test (Mann-Whitney test).

RESULTS

Identification of PKC isoforms in human platelets

As previously published [26], the immunoblot analysis of PKC isoforms of membrane and cytosol fraction of human platelets in resting state (Fig. 1) shows the presence of a 80 kDa band. The

Fig. 1. Immunoblot analysis of PKC in cytosol and membrane fraction of platelets in resting state

The presence of the distinct PKC isoforms in platelet cytosol (c) and membrane (m) were analysed by immunoblotting as described in the Experimental section. Positions of molecular-mass standards (kDa) are indicated on the left.

Fig. 2. Specificity of anti- α -PKC, anti- β -PKC and anti- ζ -PKC antibodies

The crude extracts of sf9 cells infected with the recombinant baculovirus carrying the coding region of the various subtypes of PKC were loaded on SDS/PAGE and transferred to nitrocellulose. The different PKC subtypes were analysed by immunoblot using anti- α -, anti- β - and anti- ζ -PKC antisera. Positions of molecular-mass standards (kDa) are indicated on the left.

Fig. 3. Time course of PMA-induced PKC translocation

The platelets were incubated for different times with PMA (100 nM) and the amount of PKC in the membrane and in the cytosol were determined as outlined in the Experimental section. The content of PKC isoforms in the membrane is represented on the ordinate, and the results are expressed as mean percentages of total (cytosol + membrane), $\pm$ S.E.M. from five experiments. The significance of the increase compared with the control was analysed by a non-parametric Mann-Whitney U test (in Figs. 3–5: * P < 0.05, ** P < 0.01, *** P < 0.005, **** P < 0.001).

Fig. 4. Time course of thrombin (0.5 unit/ml)-induced PKC-isoform translocation

The increase in PKC isoforms in the membrane was analysed as in Fig. 3 and is expressed as mean $\pm$ S.E.M. from five experiments.

latter is reactive with antibodies to α -, β - and ζ -PKC, but not with antibodies to γ -, δ - and ϵ -PKC.

In order to demonstrate that the immunoreaction with anti- ζ -PKC antibody was not due to a cross-reaction with α - and β -PKC, we have tested these antibodies against the various PKC subtypes expressed in insect cells obtained from the baculovirus expression system described in the Experimental section. Fig. 2 shows the immunoblot of different isoforms with anti- α -PKC, anti- β -PKC and anti- ζ -PKC antibodies. The monoclonal anti- α -PKC or anti- β -PKC, as well as polyclonal antibody anti- ζ -PKC, show a high specificity for the respective isoform, without any cross-reaction with the other isoforms. The specificity of the monoclonal antibody α -PKC and β -PKC demonstrated by Hidaka *et al.* [30] was confirmed in the present study. It should be noted that the β -PKC-specific monoclonal antibody recognizes both the $\beta 1$ and the $\beta 2$ isoenzyme.

Effect of PMA on PKC-isoform redistribution in human platelets

In physiological conditions with 1 mM external Ca^{2+} there was an increase in the three PKC isoforms in the membrane, from 20% at the resting state to 50% after 1 h incubation with PMA (Fig. 3). Moreover, the time course of PMA-induced trans-

location differed between the three isoforms. In contrast with the ζ - and α -isoforms, β -PKC was more rapidly translocated. After 2 min incubation with PMA, the membrane content was (% of total, mean $\pm$ S.E.M.) $39 \pm 6\%$, $31 \pm 4\%$ and $24 \pm 4\%$ compared with $21 \pm 1\%$, $21 \pm 1\%$ and $20 \pm 1\%$ before stimulation for β -, ζ - and α -PKC respectively. The increase in α -PKC after 2 min incubation with PMA was not significant compared with the control. After 10 min incubation the translocation of β - and ζ -PKC reached a maximum, whereas α -PKC continued to increase and reached a maximum only after 30 min incubation.

Effect of thrombin on PKC isoform translocation

Addition of thrombin (0.5 unit/ml) to platelets induced a rapid translocation of all three isoforms. The amounts of PKC in the membrane after 1 min incubation with thrombin was $36 \pm 5\%$, $36 \pm 6\%$ and $37 \pm 3\%$ (means $\pm$ S.E.M.) for α -, β - and ζ -PKC respectively (Fig. 4). After 2 min there was a slight decrease in all three isoforms, and after 5 min there appeared to be a second phase of translocation of α - and β -PKC which remained sustained over 30 min, whereas the ζ -PKC decreased to basal level.

Effect of vasopressin on PKC isoform translocation (Fig. 5)

In the presence of vasopressin ($1 \mu\text{M}$) the translocation of α - and β -PKC was biphasic. There was a first increase, with a maximum at 1 min. The content of PKC isoform in the membrane after 1 min incubation with vasopressin was $32 \pm 4\%$ and $35 \pm 7\%$ (means $\pm$ S.E.M.) for α - and β -PKC respectively, which decreased at 2 min to basal level. After 5 min there was a second phase of translocation of α - and β -PKC that remained elevated for 30 min.

The translocation of ζ -PKC showed a different pattern of translocation compared with α - and β -PKC. The translocation gradually increased from 30 s to 5 min, without returning to basal level after 2 min, and then remained sustained over 30 min.

Effect of Ca^{2+} on PKC isoform redistribution induced by different agonists

Ca^{2+} has been shown to be an activator of the kinase activity of PKC only for α -, β - and γ -PKC *in vitro* [1]. Since Ca^{2+} is known to be a modulator of the interaction of different proteins with the plasma membrane, we have studied the effect of external or internal Ca^{2+} on the mechanism of translocation of different

Fig. 5. Time course of vasopressin ($1 \mu\text{M}$)-induced PKC-isoform translocation

The increase in PKC isoforms in the membrane was analysed as in Fig. 3 and is expressed as mean $\pm$ S.E.M. from five experiments.

Table 1. Effect of Ca^{2+} on the translocation of PKC isoforms by different agonists

The increase of PKC isoforms in the membrane was analysed after 30 min incubation with 100 mM-PMA or after 1 min incubation with 0.5 unit of thrombin/ml or $10 \mu\text{M}$ -vasopressin, in the absence of external Ca^{2+} by replacing Ca^{2+} by 1 mM-EDTA and in the absence of internal Ca^{2+} by incubating the platelets for 10 min in presence of 100 mM-Quin2-AM. The results are expressed as percentages of the control value without stimulation (100%), and are means $\pm$ S.E.M. (n). The significance (P value) of the decrease in translocation by EDTA (P^a) or Quin2 (P^b) compared with Ca^{2+} is analysed by a non-parametric Mann-Whitney U test (NS, not significant).

PKC	Agonist	Increase in PKC isoform in membrane				
		Ca^{2+}	EDTA	P^a	Ca^{2+} Quin	P^b
α	PMA	278 ± 27 (9)	198 ± 24 (8)	0.02	281 ± 6 (5)	NS
	Thrombin	142 ± 27 (9)	104 ± 9 (8)	0.007	111 ± 8 (6)	0.02
	Vasopressin	151 ± 13 (15)	106 ± 7 (6)	0.01	109 ± 9 (9)	0.02
β	PMA	210 ± 20 (10)	229 ± 23 (9)	NS	216 ± 35 (5)	NS
	Thrombin	118 ± 5 (13)	108 ± 8 (9)	NS	100 ± 5 (5)	0.02
	Vasopressin	139 ± 6 (14)	98 ± 2 (5)	0.003	102 ± 4 (8)	0.002
ζ	PMA	244 ± 31 (9)	238 ± 29 (9)	NS	175 ± 20 (3)	NS
	Thrombin	137 ± 7 (11)	106 ± 5 (10)	0.0006	116 ± 7 (5)	0.03
	Vasopressin	138 ± 7 (12)	115 ± 4 (9)	0.01	106 ± 5 (5)	0.007

PKC isoforms by the different agonists PMA, thrombin and vasopressin.

In this study we measured the translocation of PKC isoforms in the absence of external Ca^{2+} by replacing Ca^{2+} by 1 mM-EDTA, and in the absence of internal Ca^{2+} by incubating the platelets for 10 min in the presence of 10 μM -Quin2-AM as described [31]. The translocation is measured by the increase in PKC isoforms in the membrane compared with the resting state and is expressed as a percentage (mean value) of the respective control value without stimulation (100%). The suppression of external Ca^{2+} in the presence of 1 mM-EDTA (Table 1) results in a significant decrease in the PMA-induced translocation of α -PKC, from $278 \pm 27\%$ in the presence of 1 mM external Ca^{2+} to $198 \pm 24\%$ (mean $\pm$ S.E.M.) in the presence of 1 mM-EDTA ($P < 0.05$ by both Student's *t* test and Mann-Whitney U-test). In contrast, there was no effect of EDTA on the PMA-induced translocation of β - and ζ -PKC. Incubation in the presence of Quin2-AM has no effect on PMA-induced translocation of all the three isoforms. Moreover, incubation of human platelets with EDTA or Quin2-AM inhibited the thrombin- and vasopressin-induced translocation of all three PKC subtypes.

DISCUSSION

According to our data, human platelets express α -, β - and ζ -PKC, as determined by using specific antibodies. Since ζ -PKC belongs to a family of Ca^{2+} -independent PKCs and Nishizuka and colleagues [18] have reported the presence of an isoform in platelets which was independent of Ca^{2+} , our results suggest that this Ca^{2+} -independent PKC may be attributed to ζ -PKC. Moreover, the molecular mass of the PKC subtype recognized by our anti- ζ -PKC antibody is approx. 80 kDa, comparable with that of the rat ζ -PKC expressed in baculovirus. This apparent molecular mass contrasts with the value of 64 kDa reported for ζ -PKC partially purified from COS cells [32]. In that purification, proteolytic degradation of ζ -PKC cannot be ruled out. Proteolytic degradation during partial purification of ϵ -PKC expressed from COS cells has been reported [33]. Since the open reading frame of the ζ -PKC cDNA encodes a protein with 592 amino acids, with a calculated molecular mass of 68 kDa, this apparent molecular mass of 80 kDa can only be explained by an anomalous migration of this particular PKC subtype in SDS gels. In addition, it has been demonstrated that ζ -PKC is unable to bind to and to be activated by phorbol ester, presumably owing to the fact that this subspecies of PKC only contains one cysteine-rich repeat region [32]. Our results are in contrast with this finding, since PMA is able to induce the translocation of ζ -PKC in human platelets. We can conclude that in human platelets exists a PKC isoform recognized by ζ -PKC-specific antibodies and which interacts with phorbol ester. This finding would be comparable with the results published by Cazaubond *et al.* [34] and Ono *et al.* [35], which indicate that only one of the two cysteine-rich sequences is sufficient for the phorbol ester binding.

The time course of the translocation of PKC isoforms varies between the three isoforms and between different agonists. The β -PKC is translocated faster, followed by ζ -PKC and α -PKC, after incubation with 100 nM-PMA. These results confirm our previous results [26], as well as the data obtained by Hagiwara *et al.* [25]. They are in contradiction with the study by Fournier *et al.* [24], who claimed the absence of translocation of β -PKC in the presence of PMA. This discrepancy between the results could be explained by the use of different antibodies.

Addition of physiological agonists (thrombin or vasopressin) to platelets induced a biphasic translocation of α - and β -PKC isoforms. In contrast, ζ -PKC was differently translocated in the

presence of thrombin or vasopressin. In the presence of thrombin, there was a rapid translocation within 1 min, which returned to basal level after 5 min, whereas in the presence of vasopressin the translocation of ζ -PKC was slower than with thrombin, but remained sustained for 30 min. The difference between thrombin and vasopressin as far as the translocation of the three PKC isoforms is concerned is not likely to be due to the secretion of thromboxane B₂, which can activate phospholipase C in a later phase, since this secretion is induced with thrombin as well with vasopressin [3]. Thrombin and vasopressin are known to stimulate phospholipase C [3] and to produce DAG, which activates PKC. It has been shown that thrombin induced a biphasic DAG accumulation in the platelets [36]. The first DAG peak was rapid and transient after stimulation, paralleled a transient increase in InsP_3 , suggesting that the DAG source was PtdInsP_2 . The second phase of DAG accumulation was sustained and observed after $\text{Ins}(1,4,5)\text{P}_3$ returned nearly to basal level. Moreover, the source has been demonstrated to be PtdIns and not phosphatidylcholine, suggesting a phospholipase D activity on PtdIns [36]. According to our results, the first phase of translocation of all the three isoforms may be attributed to the first phase of production of DAG, and the sustained translocation of α - and β -PKC may be attributed to the second sustained phase of DAG production. In contrast with thrombin, vasopressin induced a sustained and pronounced translocation of ζ -PKC over 30 min. This difference may be explained by other sources of DAG than the metabolism of phosphatidylcholine, which contains 1-alkyl-2-acyl molecular species in addition to the diacyl form. This would indicate that the activation profiles of PKC isoenzymes would be different among different types of molecular species of DAGs, resulting in a multiplicity of biochemical pathways, which can be selectively activated after agonist stimulation [37–39].

The study of effects of Ca^{2+} on the translocation of PKC isoforms by PMA has shown that chelation of external Ca^{2+} , but not internal Ca^{2+} , decreases the translocation of α -PKC in the presence of PMA. The PMA-induced translocation of β - or ζ -PKC was not affected by either EDTA or Quin2. This result suggests that a Ca^{2+} influx, or an increase in Ca^{2+} in a subcellular membrane domain dependent on external Ca^{2+} , stimulates the translocation of α -PKC. In adrenal chromaffin cells [40] as well as in rat pinealocytes [41] it has been shown that Ca^{2+} influx, after either hormones or an increase in extracellular K^+ or addition of the Ca^{2+} ionophore A23187, causes a rapid translocation of PKC to the membrane. However, isoenzymes of PKC were not determined in this instance. Siess & Lapetina [42] have proposed that Ca^{2+} mobilization primes PKC in human platelets by placing the enzyme in a strategic position for activation by phorbol ester. Thus the coupling of the translocation of PKC to a Ca^{2+} influx could be specific to α -PKC, since β - and ζ -PKC did not demonstrate a Ca^{2+} -dependence. This is in accordance with the observation by Sekiguchi *et al.* [15], who demonstrated a lower Ca^{2+} requirement of the β -PKC for the binding and anchoring of PKC to the membrane than for α -PKC. In addition, the ζ -PKC, which lacks the C2 fragment, is not dependent on Ca^{2+} for its membrane binding and kinase activity [32].

In the case of thrombin- or vasopressin-induced translocation, the presence of EDTA or Quin2 inhibits the translocation of the three isoforms. This result can be explained by a decrease in the activity of PLC, and therefore a decrease of production of DAG, by chelation of external or internal Ca^{2+} . The production of DAG occurring after 1 min in thrombin-stimulated human platelets has been demonstrated to be abolished in the presence of EGTA [36]. de Chaffoy de Courcelles *et al.* [31] have shown that incubation of platelets in the absence of external Ca^{2+} decreases the activity of phospholipase C, but the 5-hydroxytryptamine-receptor-coupled phospholipase C activity

was not inhibited in the presence of Quin2-AM, and Quin2-AM loading did not affect phosphatidic acid formation or protein phosphorylation. If these results can be generalized to all physiological agonists, our results suggest that Quin2 loading affects the mechanism of translocation of the three PKC isoforms induced by physiological agonists.

From this study, we conclude, that platelets indeed contain at least three different isoforms, recognized by antibodies specific for α -, β - and ζ -PKC. The pattern of the kinetics of translocation from cytosol to membrane varied in the presence of the different agonists, PMA, thrombin and vasopressin. These results suggest that the activation profile of PKC isoenzymes is dependent on the physiological agonist, which can produce different forms of DAGs at different times after activation. In addition, the translocation of PKC by physiological agonists is dependent on intra- and extra-cellular Ca^{2+} . In the presence of PMA, only the translocation of α -PKC is dependent on extracellular Ca^{2+} . This study was the first attempt to demonstrate a different translocation of PKC isoforms with different physiological agonists in human platelets.

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