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STIMULUS-DEPENDENT INHIBITION OF PLATELET AGGREGATION BY THE PROTEIN KINASE C INHIBITORS POLYMYXIN B, H-7 AND STAUROSPORINE

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Thrombin, 1-oleoyl-2-acetyl-*rac*-glycerol (OAG), *cis*- or *trans*-octadecadienoic acids (linoleic and linolelaidic acid) and the synergistic combination of octadecadienoic acids plus OAG lead to the activation of gel-filtered human platelets, i.e. aggregation *via* protein kinase C (PKC). Platelet activation by thrombin was only slightly suppressed by polymyxin B, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) or staurosporine, all being potent inhibitors of PKC *in vitro*. The OAG-induced aggregation, however, was strongly inhibited by H-7 or staurosporine but not by polymyxin B. In contrast, octadecadienoic acid-induced aggregation was substantially inhibited only by polymyxin B. Synergistic activation by OAG plus octadecadienoic acids was strongly suppressed by all three PKC inhibitors. Our results indicate (1) that the ability of various compounds to inhibit PKC *in vitro* does not correlate with their inhibitory effects in intact cells and (2) that platelet activation induced by various PKC activators exhibits differential PKC-inhibitor sensitivity.

In platelets, PKC is supposed to be involved in the cellular activation process, which is initiated by the phosphorylation of specific proteins and which results in granule release and aggregation (1-6). In this cellular system, PKC can be activated by several distinct mechanisms which appear to be

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ABBREVIATIONS:
H-7, 1-(5-isoquinolinosulfonyl)-2-methylpiperazine; LA-*cis*, linoleic acid; LA-*trans*, linolelaidic acid; OAG, 1-oleyl-2-acetyl-*rac*-glycerol; PKC, protein kinase C.

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not equivalent: 1) Hormonal agonists, such as thrombin, induce polyphosphoinositide degradation to inositolphosphates and diacylglycerol, the latter activating PKC (1-3). 2) Various synthetic diacylglycerols, such as OAG, and certain phorbol esters may substitute for endogenous diacylglycerol to activate PKC (2,4-6). 3) We recently reported that *cis*- and *trans*-octadecadienoic acids (linoleic and linolelaidic acid) activated purified PKC from rat brain and induced protein phosphorylation and aggregation in human platelets (7-9). 4) In addition, we reported that fatty acids and diacylglycerol synergistically activated PKC *in vitro* and in intact platelets (7-9). Recently, the polypeptide antibiotic, polymyxin B (10), the isoquinolinesulfonamide, H-7 (11) and the fungal alkaloid, staurosporine (12) were found to be potent inhibitors of PKC *in vitro*. In order to compare these inhibitors in an intact cellular system, we studied the effects of these compounds on platelet aggregation induced by different PKC activators and report here on differential, stimulus-dependent inhibition of platelet aggregation by PKC inhibitors.

MATERIALS AND METHODS

Fatty acids (99% pure), OAG (97% pure), polymyxin B and thrombin were obtained from Sigma Chemie (Taufkirchen, F.R.G.). Sepharose 2B-CL was purchased from Pharmacia (Freiburg, F.R.G.). Staurosporine was a kind gift of Prof. S. Omura, Kitasato Institute, Tokyo (Japan). H-7 was synthesized by the Department of Chemical Research of Goedecke AG (Freiburg, F.R.G.). All other chemicals were of analytical grade. Stock solutions of LA-*cis*-and LA-*trans* (20-80 mM) were prepared in absolute ethanol under a nitrogen atmosphere, protected from light and stored at -20°C. OAG (1-6 mM), indomethacin (10 mM) and staurosporine (20 mM) were dissolved in dimethyl sulfoxide. Polymyxin B and H-7 (20 mM) were dissolved in the buffer described below.

Blood (75 ml) was obtained from healthy male volunteers in fasting condition who had not taken any drug for the last 3 weeks. Platelet-rich plasma was obtained by centrifugation of citrated blood for 15 min at 60 x g. Platelets were separated from plasma by gel filtration using a sepharose 2B-CL column equilibrated at room temperature with a buffer consisting of 150 mM NaCl, 5.5 mM glucose, 30 μM bovine serum albumin and 15 mM Tris-HCl, pH 7.4 (13,14). Platelet concentration in pooled peak fractions of the eluate was determined in a Coulter Counter (Coulter Electronics, Krefeld, F.R.G.).

Platelet suspensions were adjusted to a final concentration of 2×10^8 cells/ml and were supplemented with 0.7 mM of $CaCl_2$. Platelet aggregation was measured by the turbidometric method described by Born (15). The presence of dimethyl sulfoxide (1.0%, v/v) or ethanol (0.5%, v/v) plus dimethyl sulfoxide (0.5%, v/v) in the assays did not affect platelet aggregation. Prior to addition of stimuli, platelets were incubated for 2 min in the absence or presence of inhibitors. Aggregation measurements were carried out at 37°C under constant stirring of cells at 10^3 rpm, using a Braun aggregometer (Braun, Melsungen, F.R.G.).

RESULTS

The effects of polymyxin B, H-7 and staurosporine on platelet aggregation induced by various activators of PKC are summarized in Table 1. The concentrations of OAG, LA-*cis*-and LA-*trans* given in the table were found to be the lowest concentrations that induced a maximal extent of aggregation (9). Synergistic aggregation was induced by OAG plus octadecadienoic acids. LA-*cis*-and LA-*trans* did not induce aggregation at concentrations of 250 and 200 μM, respectively (9). The aggregation induced by 5 μM OAG amounted to 16 ± 5% of that induced by thrombin (9). The concentrations of PKC inhibitors present in the aggregation assays were at least 50-fold those required for half-maximal inhibition of PKC *in vitro* (10-12).

Thrombin-induced aggregation was largely unaffected by H-7 and was only slightly inhibited by polymyxin B and staurosporine. The OAG-mediated platelet aggregation was almost completely suppressed by staurosporine. H-7 also had a substantial inhibitory effect, whereas polymyxin B showed only a marginal effect. In contrast to the OAG-induced aggregation, that induced by LA-*cis* and LA-*trans* was substantially inhibited by polymyxin B, whereas staurosporine and H-7 showed only weak inhibitory effects. Finally, platelet aggregation synergistically induced by OAG plus LA-*cis* or LA-*trans* was strongly suppressed by all three PKC inhibitors examined.

DISCUSSION

In this study, we demonstrate differential inhibition of platelet aggregation, using various activators and inhibitors of PKC. This inhibition pattern is probably not due to insufficient permeation of the compounds into the platelets, as each inhibitor substantially inhibited platelet activation induced by at least one of the three different classes of individual PKC-activators. One possibility to explain this differential inhibitor profile is that there may exist multiple isoenzymes in human platelets. Thus, different stimuli could activate distinct subsets of PKC molecules which may vary with respect to allosteric regulation and substrate specificity. It has been reported that isoenzymes of PKC exhibit quantitative differences in the ac-

Table 1: Platelet aggregation induced by various protein kinase C activators and its differential inhibition by polymyxin B, H-7 or staurosporine

Stimulus	Control aggregation (Light transmission in %)	Platelet aggregation: Inhibition of control aggregation (%)		
		Polymyxin B (100 μM)	H-7 (300 μM)	Staurosporine (1 μM)
Thrombin (0.25 U/ml)	100 (8)	28 ± 7 (3)	6 ± 3 (3)	22 ± 8 (4)
OAG (30 μM)	94 ± 3 (5)	13 ± 3 (3)	67 ± 4 (3)	97 ± 6 (4)
LA-*cis* (400 μM)	95 ± 6 (6)	78 ± 18 (5)	28 ± 10 (4)	11 ± 3 (3)
LA-*trans* (400 μM)	93 ± 6 (8)	76 ± 8 (4)	20 ± 18 (4)	7 ± 3 (4)
OAG (5 μM) + LA-*cis* (250 μM)	78 ± 8 (3)	81 ± 26 (3)	100 (2)	100 (2)
OAG (5 μM) + LA-*trans* (200 μM)	74 ± 26 (4)	67 (2)	82 ± 20 (3)	100 (2)

Platelet aggregation was measured as described in MATERIALS AND METHODS. The extent of aggregation induced by different stimuli is referred to that induced by thrombin (0.25 U/ml), which amounted to 100%. Inhibition of aggregation is referred to that induced by the specific stimulus in the absence of inhibitors. The numbers in parentheses indicate the number of individual donors investigated. Each experiment depicted in the table was repeated at least twice with each donor and showed an intraindividual variation of less than 10%. Data represent the mean of two independent experiments with different donors or the mean ± S.D. of three to eight independent experiments.

tivation by fatty acids, diacylglycerol and phorbol esters (16-18). It remains to be clarified, however, whether the isoenzymes may also show differential inhibitor sensitivities. Another reason for differential inhibitory effects may be seen in the fact that the substrates which are phosphorylated by PKC depend on the stimulus used to activate the enzyme (19). In addition, it has been shown recently that different activators of PKC lead to the phosphorylation of one and the same substrate molecule at various sites, also pointing to stimulus-specific interaction of the enzyme with substrate proteins (4).

In neutropils, polymyxin B but not H-7 inhibited phorbol ester-mediated cell activation (20,21), whereas in basophils H-7 effectively blocked phorbol ester- and diacylglycerol-induced histamine release (22). In HL-60 leukemic cells, polymyxin B did not inhibit protein phosphorylation induced by phorbol esters (23). We found that in platelets H-7 but not polymyxin B substantially inhibited OAG-induced platelet aggregation. These results clearly indicate that the effectiveness of PKC-inhibitors to suppress cellular responses elicited by a specific class of PKC activators exhibits striking cell type differences.

All three PKC inhibitors investigated failed to inhibit thrombin-induced platelet aggregation, although there is ample evidence that PKC is activated upon stimulation with thrombin (1-6). It has recently been shown that this kinase indeed plays a crucial role in the activation process (24). These results indicate that activation of an alternative signalling pathway, i.e. activation of Ca^{2+}/calmodulin-dependent enzymes, may compensate for PKC inhibition (1,2, 3,21). Thus, the failure of PKC inhibitors to suppress hormone-induced platelet aggregation does not at all rule out the possibility that PKC is involved in the physiological activation process.

In conclusion, our results point to differential interactions of PKC or different isoenzymes of PKC with various activators, inhibitors and presumably with substrate proteins in intact cells which appear to be of much greater complexicity than *in vitro*. As there is no apparent correlation between the *in vitro* and *in vivo* effects of PKC inhibitors, studies using these compounds to

evaluate the role of this enzyme in cellular activation processes have to be interpreted with great caution. Our results suggest that inhibitor studies of this kind should include different activators and chemically unrelated inhibitors of PKC. Therefore, the development of isoenzyme-specific or stimulus-specific PKC inhibitors will be an important task to explore the physiological roles of PKC as well as to interfere pharmacologically with its activation.

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