Convulxin-induced platelet aggregation is accompanied by a powerful activation of the phospholipase C pathway

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Platelet aggregation and stimulation of phosphoinositide-specific phospholipase C (PLC) by thrombin and by convulxin (Cvx), a non-enzymic snake venom glycoprotein, were compared. Cvx-stimulated production of inositol phosphates by washed platelets was independent of the cyclo-oxygenase pathway, formation of platelet-activating factor and ADP release, but prostacyclin (prostaglandin I_2), a stimulator of cyclic AMP formation, suppressed its effects on platelet and PLC activation. Kinetic analysis showed that inositol 1,4,5-trisphosphate formation reached its maximal value 15 s after platelet stimulation with Cvx and persisted for at least 5 min. Neomycin sulphate (10 mM), which complexes phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate, decreased the production of inositol phosphates, partially prevented platelet aggregation induced by a high concentration of Cvx (10 nM) and abolished both platelet aggregation and inositol phosphate formation induced by thrombin (2 units/ml) and by a stable prostaglandin H_2 analogue, U46619 (1 μM). In contrast with neomycin sulphate, Na_2SO_4 had no significant effect against all agonists tested. It is concluded that platelet activation by Cvx is partially mediated by PLC and involves other mechanisms as well.

INTRODUCTION

Convulxin (Cvx), a non-enzymic glycoprotein, first isolated by Prado-Franceschi and Vital Brazil (1981) from the venom of the snakes *Crotalus durissus cascavella* and *Crotalus durissus terrificus*, is a 72 kDa glycoprotein (Marlas et al., 1983; Marlas, 1985) which activates blood platelets (Vargaftig et al., 1980, 1983) through a Ca^{2+}-dependent (Sano-Martins and Daimon, 1992), and fibrinogen-, ADP- and cyclo-oxygenase-independent (Vargaftig et al., 1983) mechanism. The mechanism of action of Cvx on platelets is poorly understood, because it has not been studied in detail.

Since stimulation of platelets leads to the rapid degradation of inositol phospholipids (Billah and Lapetina, 1982; Morrison and Shukla, 1989), which are involved in further cell activation, we examined whether the effect of Cvx on platelets involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide-specific phospholipase C (PLC). The activation of PLC in washed rabbit platelets leads to the breakdown of inositol phospholipids and to the production of inositol 1,4,5-trisphosphate (IP_3), known to mobilize Ca^{2+} from the endoplasmic reticulum to the cytosol (Streb et al., 1983; O'Rourke et al., 1985). IP_3 is rapidly dephosphorylated to inositol bisphosphate (IP_2) and inositol monophosphate (IP_1), which accumulate, particularly when the hydrolysis of IP_1 and further turnover of the phosphatidylinositol cycle are blocked by Li^+ (Berridge et al., 1982). Our results show that aggregation and the breakdown of inositol phospholipids in washed rabbit platelets induced by a high concentration of Cvx is only partially inhibited by neomycin, an aminoglycoside antibiotic which inhibits PLC activity. Under similar conditions, neomycin suppresses the effects of thrombin and U46619, two powerful stimulators of platelet aggregation and of PLC activation (Billah and Lapetina, 1982; Siess et al., 1985).

Our findings indicate that Cvx operates, at least in part, through PLC activation. However, since neomycin decreased, but did not suppress, inositol phosphate formation and platelet activation, additional mechanisms should account for the effects of Cvx.

EXPERIMENTAL

Materials

The following reagents were used: Cvx, purified from the venom of *Crotalus durissus terrificus* (Institut Pasteur, Paris, France), bovine thrombin from Hoffmann–La Roche (Basel, Switzerland); EDTA, EGTA, LiCl, neomycin sulphate, Dowex 1 (Cl^- form; 200–400 mesh), phosphocreatine (CP), creatine kinase (CK) and prostacyclin (prostaglandin I_2; PGI_2) from Sigma (St. Loius, MO, U.S.A.), and Na_2SO_4 from Prolabo (Paris, France). The stable prostaglandin H_2 analogue U46619 [15*S*-hydroxy-11,9-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid] was from Upjohn Co. (Kalamazoo, MI, U.S.A.). Platelet-activating factor (PAF; 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was from Bachem (Bubendorf, Switzerland). The PAF antagonist WEB 2086 ({3-[4-(2-chlorophenyl)-9-methyl-6*H*-thieno(3,2-1)-1,2,4-triazolo(4,3*a*)-1,4-thienodiazepin-2-yl]-1-morpholinyl}-propan-1-one) was from Boehringer (Ingelheim, Germany). *myo*-[2-^{3}H]Inositol (20 Ci/mmol) was obtained from Amersham (U.K.) and [2-^{3}H]inositol 1,4,5-trisphosphate (3–17 Ci/mmol) from Du Pont–New England Nuclear (France).

Preparation and labelling of rabbit platelets

Blood was collected from the central artery of the ear of adult New Zealand rabbits into plastic tubes containing a mixture of disodium and tetrasodium salts of EDTA (5 mM final concn.). Platelet-rich plasma, which was obtained after centrifugation of

Abbreviations used: Cvx, convulxin; PLC, phospholipase C; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; IP_2, inositol bisphosphate; IP_1, inositol monophosphate; PAF, platelet-activating factor; PGI_2, prostaglandin I_2 (prostacyclin); CP, phosphocreatine; CK, creatine kinase.

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blood (375 *g*, 20 min, at room temperature), was centrifuged (1800 *g*, 15 min at 20 °C), and the pellet containing the platelets was washed twice with Tyrode's buffer (137 mM NaCl, 27 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin, supplemented with 0.2 mM EGTA and 1 mM $MgCl_2$), pH 6.4. The platelets were finally resuspended in Tyrode's buffer, pH 7.4, without EGTA and $MgCl_2$. The suspension was adjusted to 4×10^8 platelets/ml.

Platelet aggregation

Platelet aggregation was monitored by measuring the variation in light transmission, as described by Born (1962), with an Icare aggregometer (Marseille, France), of 500 μl samples from washed rabbit platelets that underwent continuous stirring at 1100 rev./min and 37 °C. Ca^{2+} was added to the platelet suspension at a final concentration of 2 mM just before stimulation. The percentage aggregation was determined 3 min after the addition of the stimulating agent.

Measurement of [^{3}H]inositol phosphate bioformation

Inositol phosphates from platelets labelled with [^{3}H]inositol were extracted by chloroform/methanol/HCl and separated by Dowex 1 anion-exchange chromatography as described by Downes et al. (1986). This method separates inositol IP_1, IP_2 and IP_3, but does not distinguish their various isomers.

Concentrated platelet suspensions (2×10^9 cells/ml) were labelled with *myo*-[^{3}H]inositol (50 μCi/ml) for 3 h. In order to inhibit the conversion of inositol phosphates into free inositol (Berridge et al., 1982), platelets were incubated with 15 mM LiCl for 10 min in the aggregometer cuvette before stimulation.

Cvx was added to the platelet suspension alone or in the presence of neomycin, and the reactions were stopped at various times thereafter by transfer of the medium (2×0.5 ml for each sample) with a silicone-treated Pasteur pipette to glass tubes containing 1 ml of chloroform/methanol/1 M HCl (50:50:1, by vol.). After vortex-mixing, 0.5 ml of chloroform was added. Then samples were centrifuged at 800 *g* for 10 min at 4 °C, and the aqueous upper phase containing [^{3}H]inositol phosphates was removed and stored at 4 °C overnight. [^{3}H]Inositol phosphates were separated on 1 ml columns of Dowex anion-exchange resin 1 X 8 (formate form). The following elution protocol was used: 2×5 ml of water (for free inositol), 2×5 ml of 60 mM ammonium formate/5 mM sodium tetraborate (to exclude glycerophosphoinositol), 10×1 ml of 0.2 M ammonium formate/0.1 M formic acid (for inositol monophosphates), 10×1 ml of 0.45 M ammonium formate/0.1 formic acid (for inositol bisphosphates), 10×1 ml of 1 M ammonium formate/0.1 M formic acid for inositol trisphosphate. Columns were calibrated with standard ^{3}H-labelled IP_1, I(1,4)P_2 and I(1,4,5)P_3. Radioactivity in each sample (1 ml) was counted in 5 ml of ACS II, by using a LKB 1212 β-counter for quantification of inositol phosphates.

Statistical analysis

Statistical significance was analysed by Student's *t* test for unpaired data.

RESULTS

Effects of Cvx on platelet aggregation and inositol phosphate formation

As shown in Figure 1, addition of Cvx (0.5–30 nM) to washed rabbit platelets induced a concentration-dependent aggregation.

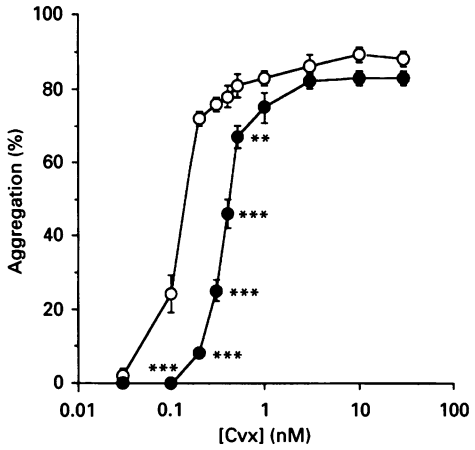

Figure 1 Concentration-dependent platelet aggregation induced by Cvx in the absence and presence of aspirin plus CP/CK

Washed rabbit platelets (500 μl portions; 4×10^8 cells/ml) stirred at 37 °C were incubated with (●) or without (○) aspirin (100 μM) plus CP (300 μM)/CK (25 units/ml) for 15 or 2 min before Cvx addition respectively. Percentage aggregation was determined 5 min after platelet stimulation. Results are expressed as means ± S.E.M. of three separate experiments: significantly different (**$P < 0.01$ and ***$P < 0.001$) compared with control platelets.

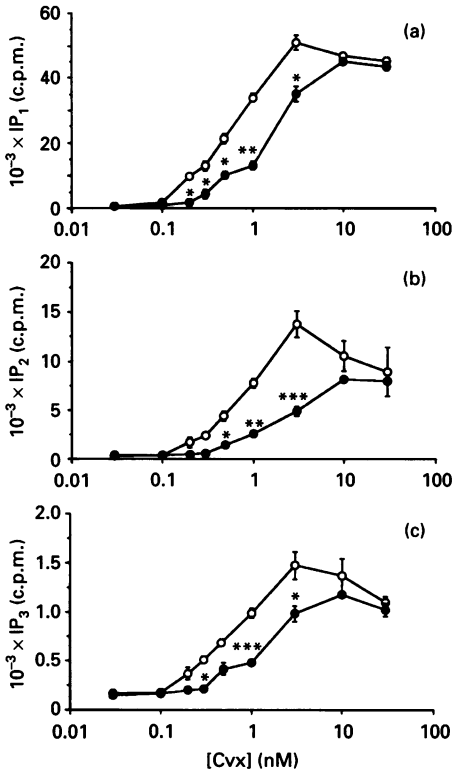

Figure 2 Concentration-dependent formation of inositol phosphates by platelets stimulated with Cvx in the absence and presence of aspirin plus CP/CK

[^{3}H]Inositol-labelled platelets were incubated with 10 mM LiCl for 5 min and then stimulated for 5 min with different concentrations of Cvx under conditions similar to those indicated in the legend of Figure 1. Data are expressed as c.p.m. for IP_1 (**a**), IP_2 (**b**) and IP_3 (**c**). The basal levels of inositol phosphates were 445 ± 119, 318 ± 80 and 164 ± 40 c.p.m., respectively, for IP_1, IP_2 and IP_3. Results are expressed as means ± S.E.M. of three separate experiments: significantly different (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) compared with control platelets.

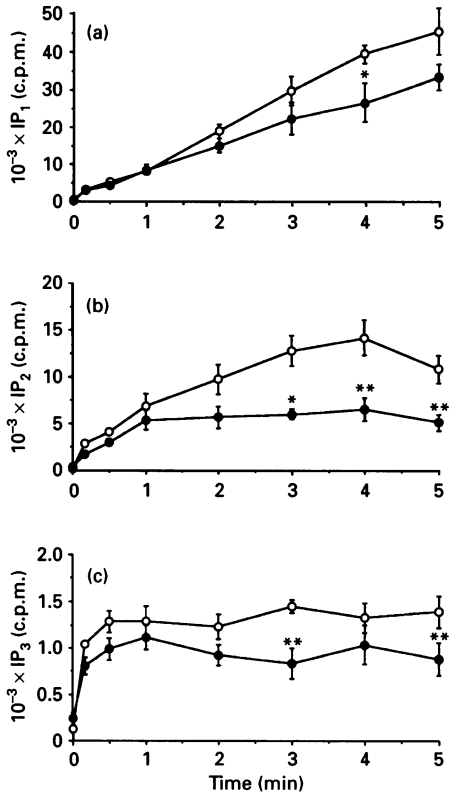

Figure 3 Kinetics of Cvx-induced platelet inositol phosphate formation in the absence and presence of aspirin plus CP/CK

Platelet suspensions, treated as indicated in the legend of Figure 2, were stimulated with Cvx (100 nM) at various time intervals (15 s–5 min). IP_1 (**a**), IP_2 (**b**) and IP_3 (**c**) formed after Cvx addition were measured at the time indicated on the abscissa axis (5 min). Results are expressed as means ± S.E.M. of three separate experiments: significantly different ($^*P < 0.05$ and $^{**}P < 0.01$) compared with control platelets.

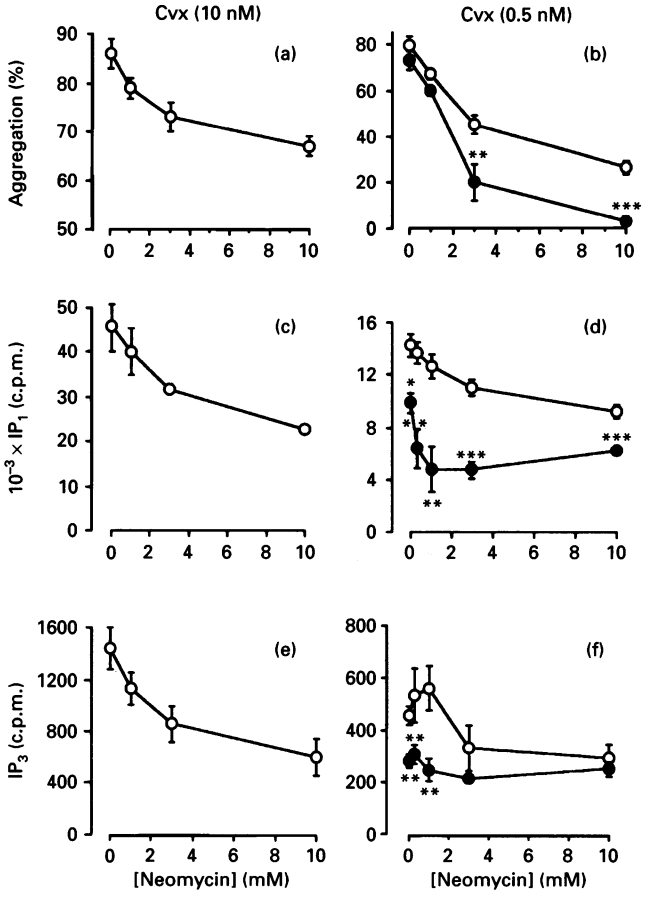

Figure 4 Effect of neomycin on Cvx-induced platelet aggregation and inositol phosphate formation in the absence and presence of aspirin plus CP/CK

Platelet suspensions pretreated with various concentrations of neomycin for 2 min in the absence (○) and the presence (●) of aspirin plus CP/CK at the concentrations indicated in the legend of Figure 2 were stimulated by Cvx (0.5 or 10 nM). Platelet aggregation (**a** and **b**) and inositol phosphate formation (IP_1, **c** and **d**; IP_3, **e** and **f**). Results are expressed as means ± S.E.M. of three separate experiments: significantly different ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) compared with control platelets.

The concentration of Cvx required for half-maximal response (EC_{50}) was about 0.2 nM. Aspirin, a cyclo-oxygenase inhibitor, in combination with the ADP-scavenging association of CP and PK, caused only a moderate inhibition of platelet aggregation; the dose/response curve was slightly shifted to the right, with an EC_{50} of about 0.6 nM.

We next examined the influence of various concentrations of Cvx on the formation of inositol phosphate derivatives, particularly IP_3, after platelet labelling with *myo*-[^{3}H]inositol. As shown in Figure 2, addition of Cvx to platelets also increased gradually the levels of IP_1 (Figure 2a), IP_2 (Figure 2b) and IP_3 (Figure 2c). As for aggregation, the exposure of platelets to aspirin and CP/CK together caused a slight inhibition of inositol phosphate formation for concentrations of Cvx up to 3 nM. Inhibition was overcome by higher concentrations of Cvx (Figure 2). Further, Cvx (10 nM) stimulated inositol phosphate formation in a time-dependent manner. Thus a maximal effect was obtained after 15–20 s and 4–5 min respectively for IP_3 and IP_2 (Figure 3).

As expected, thrombin also increased in a concentration-dependent manner platelet aggregation and inositol phosphate formation (results not shown). The exposure of platelets to aspirin plus CP/CK induced only a weak inhibition of aggregation (Figure 1a) and of inositol phosphate formation (results not shown).

Effect of neomycin on Cvx-induced platelet aggregation and inositol phosphate formation

Aggregation and inositol phosphate formation by washed rabbit platelets exposed to Cvx (10 nM) were attenuated by neomycin in a concentration-dependent manner, with a maximal inhibition of 30% (Figures 4a, 4c and 4e). When a threshold concentration of Cvx was used (0.5 nM) in the presence of aspirin plus CP/CK, platelet aggregation was abolished and inositol phosphate formation was strongly inhibited, particularly by a higher concentration of neomycin (10 mM) (Figures 4b, 4d and 4f).

In experiments comparing aggregation and inositol phosphate formation, using Cvx (3 nM) and thrombin (1 unit/ml), neomycin (10 mM) slightly inhibited Cvx-induced platelet aggregation and inositol phosphate formation. By contrast, under similar conditions, neomycin abolished the platelet responses to thrombin (Figure 5) and to compound U46619 (Figure 6c).

In order to examine whether the inhibition seen with neomycin was due to sulphate groups, platelets were pretreated with Na_2SO_4 before being stimulated with different agonists. As shown in Figure 6, both aggregation and inositol phosphate formation were not modified by Na_2SO_4 up to 50 mM. However,

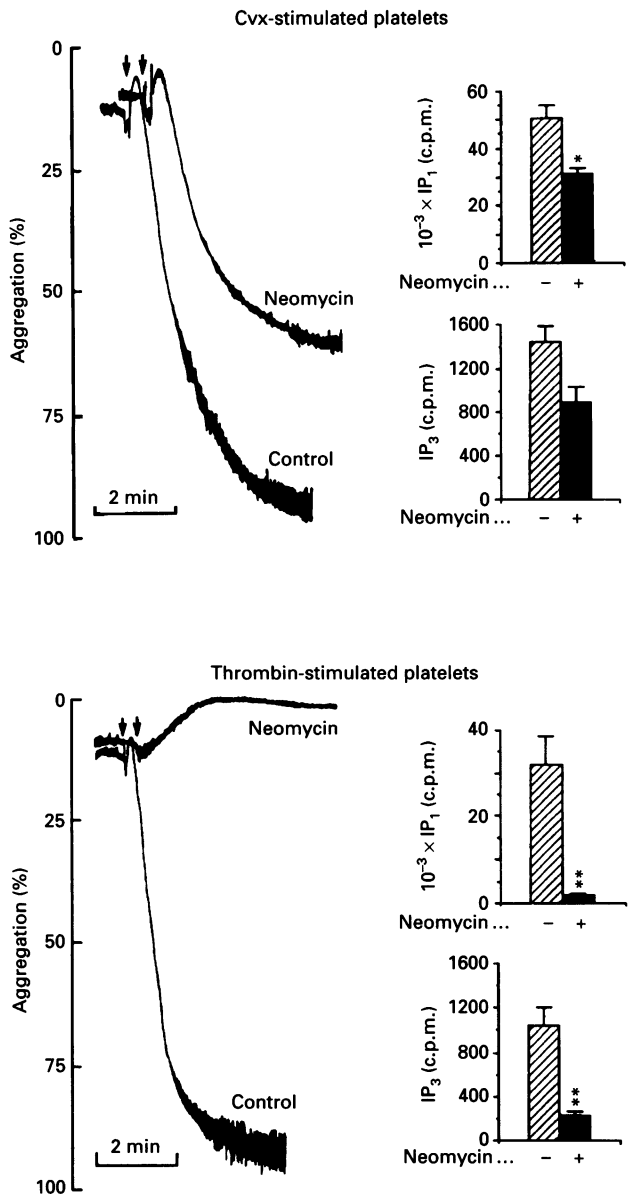

Figure 5 Effect of neomycin on Cvx-induced platelet aggregation and inositol phosphate formation: comparison with thrombin

[^{3}H]Inositol-labelled platelets and unlabelled platelets were incubated with neomycin (10 mM) for 2 min and stimulated with 3 nM Cvx (upper panel) or 1 unit/ml thrombin (lower panel). Aggregation (tracings) and inositol phosphate formation (columns; IP_1 and IP_3) were determined 3 and 5 min, respectively, after Cvx addition. Results are expressed as means ± S.E.M. of three separate experiments: significantly different ($^*P < 0.05$ and $^{**}P < 0.01$) compared with control platelets.

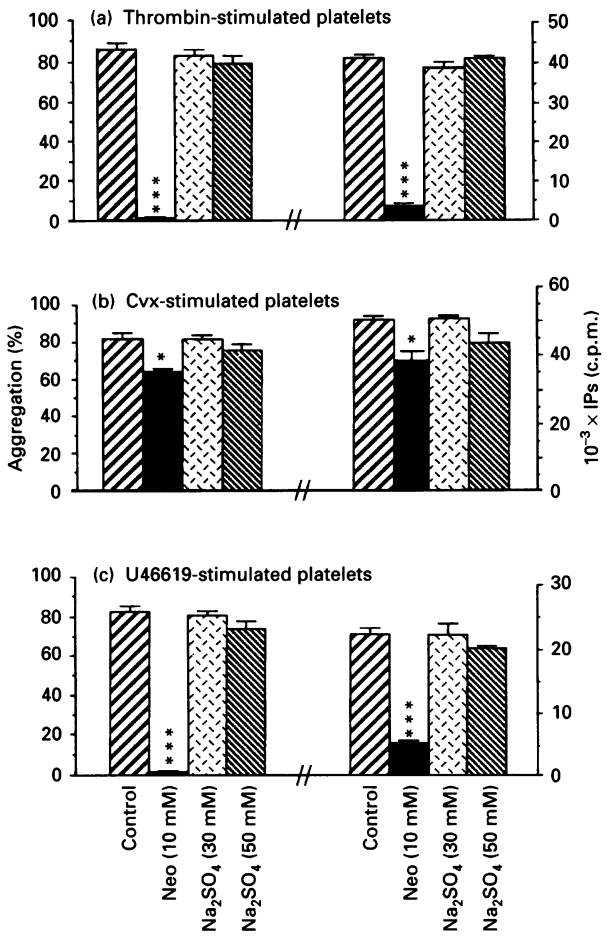

Figure 6 Effect of Na_2SO_4 on platelet aggregation and inositol phosphate formation induced by thrombin, U46619 or Cvx

Platelet suspensions pretreated with Na_2SO_4 or neomycin (Neo), as indicated on the abscissa, were stimulated with thrombin (1 unit/ml), Cvx (3 nM) or U46619 (1 μM). Aggregation and inositol phosphate (IPs: $IP_2 + IP_3$) formation were determined as indicated in the Experimental section. Results are expressed as means ± S.E.M. of four separate experiments: significantly different ($^*P < 0.05$ and $^{***}P < 0.001$) compared with appropriate control.

for a higher concentration of Na_2SO_4 (100 mM) a partial inhibition was obtained for each agonist (results not shown).

Interference of PGI_2, Ca^{2+} and WEB 2086 with Cvx-induced platelet aggregation and inositol phosphate formation

To investigate whether endogenous PAF mediates the effects of Cvx, platelets were pretreated with the PAF antagonist compound WEB 2086. Aggregation by Cvx (0.5 nM) was not affected by 0.1 mM WEB 2086 (70 ± 4% for control platelets, 73 ± 5% for neomycin-pretreated platelets) under conditions where aggregation by PAF (1 μM) was suppressed. When Ca^{2+} was omitted or PGI_2 was added to the platelet suspension, aggregation by Cvx was suppressed (results not shown). In addition, the levels of inositol phosphate formation evaluated in parallel were strongly decreased, but not suppressed (Table 1).

Table 1 Effects of PGI_2 and Ca^{2+} on Cvx-induced IP_1 and IP_3 formation

[^{3}H]Inositol-labelled platelets pretreated without or with Ca^{2+} (2 mM) or PGI_2 (0.1 μM) were stimulated with Cvx (10 nM). Formation of IP_1 and IP_3 was determined 5 min after platelet stimulation. Results are expressed as means ± S.E.M. of three separate experiments: significantly different ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) compared with respective controls (left column).

	Inositol phosphate formation (c.p.m.)		
	With Ca^{2+}		
	Without PGI_2	With PGI_2	Without Ca^{2+}
IP_1	44306 ± 609	4340 ± 384**	15370 ± 3118*
IP_3	1373 ± 172	192 ± 18***	289 ± 12***

DISCUSSION

Activation of platelets involves the production of stimulatory second messengers such as IP_3 and diacylglycerol and a rise in cytosolic Ca^{2+} concentrations (Billah and Lapetina, 1982; Berridge, 1984; Nishizuka, 1984; Watson et al., 1985). Results presented here demonstrate that Cvx causes the aggregation of platelets and PLC activation to an extent similar to those with high concentrations of thrombin. Neomycin, which complexes PIP and PIP_2 (Schacht, 1978; Reid and Gajjar, 1987), decreased the production of inositol phosphates and partially prevented platelet aggregation induced by a high concentration of Cvx. However, as reported by others (Polascik et al., 1987; Siess and Lapetina, 1986), neomycin completely inhibited platelet aggregation and inositol phosphate formation induced by thrombin. This dissociation between Cvx and thrombin, as well as the absence of cross-desensitization (Vargaftig et al., 1983), reinforces the concept of distinct sites of action for both agonists. In addition, the fact that neomycin, even at a concentration of 10 mM, inhibits, but does not suppress, platelet activation by Cvx, indicates that it is not cytotoxic. This partial inhibitory effect of neomycin on Cvx-induced platelet aggregation and inositol phosphate formation is not due to the sulphate groups contained in neomycin molecules, since Na_2SO_4 had no significant effect on Cvx responses up to concentrations above those formed from neomycin (Figure 6b).

It has been suggested that stimulation of Cvx membrane sites may mediate platelet activation by stimulating effectors other than PLC, such as protein kinase C or a membrane Ca^{2+} channel (Gerrard et al., 1989; O'Rourke et al., 1985). In fact, it cannot be excluded that neomycin, in addition to its ability to bind to phosphatidylinositol phosphates (Herrmann and Jakobs, 1988), regulates the activity state of membrane proteins such as G-proteins.

In the absence of Ca^{2+}, platelets did not aggregate in response to Cvx, although inositol phosphate formation persisted, even thought less intensively than in controls. On the other hand, when platelets were pretreated with PGI_2, an agent known to inhibit platelet aggregation by elevating the intracellular cyclic AMP content (Haslam et al., 1978; Mills and Smith, 1972), the platelet response to Cvx was also completely blocked.

PAF formation by Cvx-stimulated platelets does not account for inositol phosphate generation and aggregation, since the PAF antagonist WEB 2086 (Casals-Stenzel et al., 1987) was ineffective. In addition, the scavenging of released ADP by CP/CK and the inhibition of cyclo-oxygenase by aspirin only inhibited partially the platelet response to Cvx, ruling out a major role of ADP and of pro-aggregatory arachidonic acid derivatives on Cvx-induced platelet activation. However, since neomycin potentiated the inhibitory effects of CP/CK and aspirin, endogenous ADP and arachidonic acid derivatives play an amplificater role in Cvx-induced platelet activation. Siess and Lapetina (1986) and Polascik et al. (1987) reported that neomycin blocks full indexes of thrombin-induced platelet activation, including the shape change. At the low concentration of 0.3 mM, neomycin abolished inositol phosphate formation, shape change and platelet aggregation induced by the pro-inflammatory polysaccharide carrageenan (Hatmi et al., 1993). In contrast, over the same range of concentrations, neomycin inhibited aggregation, but not the shape change, induced by ADP, collagen and the Ca^{2+} ionophore A23187 (Polascik et al., 1987). Neomycin has been shown to bind tightly to phosphatidylinositol phosphates (Schacht, 1978; Reid and Gajjar, 1987) and to prevent their enzymic degradation (Downes and Michell, 1981). However, the selective inhibition of thrombin-induced shape change indicates the presence of a second site of action of neomycin in platelets (Polascik et al., 1987). Tysnes et al. (1991) reported that the inhibition by neomycin of human platelet responses to thrombin is not due to interference with the inositol phospholipid metabolism, but is due to blockade by the agonist of its receptor.

It is tempting to speculate that neomycin binds directly to a G-protein which interacts with PLC via thrombin receptors. Cvx, which interacts with a membrane site distinct from that of thrombin (I. M. B. Francischetti, M. Hatmi, J. Randon, A. Faili and C. Bon, unpublished work), cannot interact with such a G-protein and, as a consequence, both PLC and platelet activation are not suppressed by neomycin. The inhibition of platelet responses to U46619 by neomycin (Figure 6c) reinforces this hypothesis. Studies on the regulation of the signal transduction in platelet membranes are needed to understand the underlying coupling mechanisms.

In conclusion, Cvx, after interacting with specific membrane sites, causes platelet activation by at least two mechanisms: first, through the stimulation of PLC, leading to the production of the second messengers IP_3 and diacylglycerol, and secondly, by stimulating distal effectors, such as protein kinase C or a membrane Ca^{2+} channel.

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