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Effects of protein kinase C inhibitors on thromboxane production by thrombin-stimulated platelets

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Abstract

The purpose of these studies was to identify a possible role for protein kinase C in thromboxane production. The effects of four putative protein kinase C inhibitors were studied with platelet stimulation by thrombin (0.5–150 nM), Thrombin Quick I (1.5–500 nM) or a thrombin receptor (protease activated receptor-1) agonist peptide (TRAP) (5–120 μM). Thromboxane production was increased by the bisindolylmaleimide derivative, 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X), unchanged by the inhibitors 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo (2,3-*a*) pyrrolo (3,4-*c*)-carbazole (Gö 6976) and 5,21:12,17-dimetheno-18*H*-dibenzo[*i*,*o*]pyrrolo[3,4-*l*][1,8]diazacyclohexadecine-18,20(19*H*)-dione, 8-[(dimethylamino)methyl]-6,7,8,9,10,11-hexahydro-, monomethanesulfonate (379196), the latter of which is protein kinase C β-selective, and decreased by 1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one (rottlerin), an inhibitor selective for protein kinase C δ. These results indicate complex regulation of thromboxane synthesis in human platelets including a probable role for protein kinase C δ. The results taken together further suggest that GF 109203X may suppress negative feedback resulting from an unidentified kinase and that the classical protein kinase C isoforms α and β do not have a significant role in regulating thromboxane production by platelets.

Keywords: Thrombin; Thromboxane; Protein kinase C; Protein kinase inhibitor; Platelet, human

1. Introduction

A well-recognized immediate consequence of cellular stimulation is the activation of phosphatidylinositol-specific phospholipase C, which results in the formation of inositol trisphosphate and diacylglycerol, with further stimulation of a cytosolic Ca^{2+} flux and activation of protein kinase C, respectively (Nishizuka, 1992; Grand et al., 1996). Despite these observations, determination of the precise role of protein kinase C in cellular stimulation has been somewhat elusive, being further complicated by the presence of multiple isoforms in most cell types. Some specific functions for protein kinase C and its isoforms have been reported (Ettinger et al., 1996; Prekeris et al., 1996; Uberall et al., 1997). Isoform-selective inhibitors have also become available and an inhibitor of protein kinase C β, (*S*)-13-[(dimethylamino)methyl]-10,11,14,15-tetrahydro-4,9:16,21-dimetheno-1*H*,13*H*-dibenzo[*e*,*k*]pyrrolo[3,4-*h*][1,4,13]oxadiazacyclohexadecene-1,3(2*H*)-dione (LY 333531), has been reported to ameliorate several disease processes associated with diabetes mellitus (Ishii et al., 1996; Koya et al., 1997).

Specifically for platelets, stimulation by several agonists results in phosphoinositide hydrolysis, intracellular Ca^{2+} fluxes, and the activation of protein kinase C. With the availability of specific antibodies, several isoforms have been identified in the platelet including the classical isoforms, α and β; the novel isoforms, δ, ε, η′ and θ; and the atypical isoform ζ. There have also been several studies of protein kinase C isoform translocation in response to cellular stimulation by various agonists (Crabos et al., 1991; Cook et al., 1992; Grabarek et al., 1992; Wang et al., 1993). We have investigated the effects of protein kinase C isoform-selective inhibitors on thrombin-, the mutant enzyme Thrombin Quick I-, or thrombin receptor (protease activated receptor-1) agonist peptide (TRAP)-induced thromboxane production. The distinct ef-

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fects observed for the inhibitors provide evidence that at least some protein kinase C isoforms are rapidly activated following platelet stimulation and have differential effects in stimulus–response coupling.

2. Materials and methods

2.1. Materials

Acetylsalicylic acid (aspirin) and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). 2-[1-(3-Dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X), 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*) pyrrolo (3,4-*c*)-carbazole (Gö 6976) and 1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one (rottlerin) were purchased from Calbiochem (San Diego, CA, USA). The protein kinase C β-selective inhibitor, 5,21:12,17-dimetheno-18*H*-dibenzo[*i*,*o*]pyrrolo[3,4-*l*][1,8]diazacyclohexadecine-18,20(19*H*)-dione, 8-[(dimethylamino)methyl]-6,7,8,9,10,11-hexahydro-, monomethanesulfonate (379196, Fig. 1) was synthesized and supplied by Lilly Research Laboratories (Indianapolis, IN, USA). The inhibitory properties of this compound were determined as described (Jirousek et al., 1996). A 0.5 M stock solution of aspirin was prepared in 95% ethanol. Stock solutions of other inhibitors (1–10 mM) were prepared in dimethylsulfoxide (DMSO) and stored at −20°C. The highest concentration of DMSO present in any platelet activation mixture was 1%. A lyophilized preparation of the peptide, SFLLRNPND-KYEPF (TRAP), corresponding to residues 42–55 of the human thrombin receptor (Vu et al., 1991) was a generous gift from William R. Church and Laurie Ouellette (Department of Biochemistry, University of Vermont, Burlington, VT, USA). A stock solution (1.46 mM) of peptide was prepared in water and stored at −80°C. The peptide concentration in the stock solution was determined by amino acid analysis. Human α-thrombin (thrombin) was prepared as described (Henriksen et al., 1980) and stored at −80°C in 250 mM NaCl, 50 mM Tris–HCl, pH 7.5. Thrombin Quick I was prepared and stored at −80° as described (Leong et al., 1992). Thrombin and Thrombin Quick I concentrations in stock solutions were determined from the absorbance at 280 nm, corrected for light scattering determined at 320 nm, with an extinction coefficient of 1.74 ml mg^{-1} cm^{-1} (Fenton et al., 1977), M.W. = 36,000. When indicated, residues of thrombin are numbered according to the prothrombin sequence (Degen et al., 1983) with the chymotrypsinogen numbering (Bode et al., 1989) in parentheses.

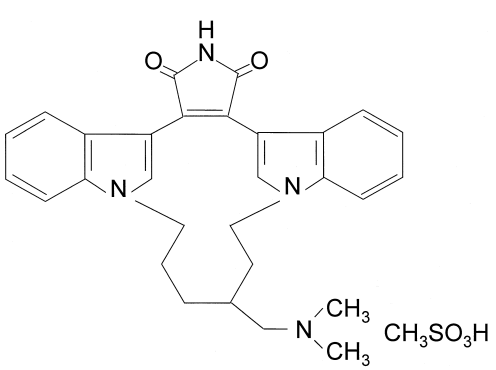

Fig. 1. Structure of 379196.

2.2. Experimental procedure

These studies were approved by the East Carolina University Policy and Review Committee on Human Research, and all procedures were in accordance with institutional guidelines. After obtaining informed consent, blood was obtained by the two-syringe technique from healthy, nonsmoking adults denying use of antiplatelet medication for 10 days before phlebotomy. Washed human platelets were prepared and experiments were performed as described (Henriksen et al., 1997) at a final platelet count of $3.6–4.0 \times 10^8$ platelets/ml in the presence of 1 mM Ca^{2+}. Platelets were stirred with inhibitor or vehicle, containing DMSO at the appropriate concentration, for 2.0 min prior to addition of 1/10 volume agonist solution after which stirring was continued for another 1.0 min. The reaction was stopped by centrifugation for 1.0 min at $16,000 \times g$ to remove platelets and the thromboxane concentration in the supernatant was determined by competitive enzyme-linked immunosorbent assay (ELISA) using a kit obtained from Neogen (Lexington, KY, USA). Assay of samples treated with inhibitors but without agonist indicated that the inhibitors did not cross-react in this assay. Data are presented without correction for spontaneous thromboxane production which was approximately 1% of the maximum response. Experiments were performed three or more times with different platelet donors.

For determination of [^{3}H]arachidonic acid release, platelets were prepared as above, then resuspended in wash buffer at one-tenth the original blood volume and counted. Aspirin, to a final concentration of 0.5 mM, was added to platelets and incubated for 20 min at 37°C. Then 50 μl of [^{3}H]arachidonic acid, 210 Ci/mmol, 0.1 mCi/ml, obtained from DuPont NEN (Boston MA, USA), in ethanol was added and the platelets were incubated for another 2 h. Unincorporated arachidonic acid was removed by centrifugation at $2000 \times g$ for 10 min. Platelets were subjected to one additional wash, resuspended as described (Henriksen et al., 1997) and used immediately. Incorporation of [^{3}H]arachidonic acid was about 80%, as determined from the radioactivity of aliquots withdrawn from the labeling mixture before and after removal of platelets as well as

Table 1
Concentrations of protein kinase C (PKC) inhibitors producing 50% inhibition (IC_{50}) of isoforms present in platelets[a]

PKC isoform	IC_{50} (μM)			
	GF 109203X[b]	Gö 6976[b]	379196	Rottlerin[c]
α	0.0084	0.0023	0.6	30
β	0.018 (β1)	0.0062 (β1)	0.0062 (β1), 0.03(β2)	42
δ	0.21	No inhibition	0.7	3 (porcine spleen), 6
ε	0.13	No inhibition	5	100
ζ	5.8	No inhibition	4.8	100
η	No data	No data	3	82
θ	No data	No data	No data	No data

[a]Assays were performed with recombinant protein kinase C isoforms in the presence of 10–30 μM ATP.
[b]Martiny-Baron et al. (1993).
[c]Gschwendt et al. (1994).

from the final suspension of platelets. Experiments were performed as described for thromboxane production except that 50 μl of the final platelet supernatant was collected and the radioactivity was determined by scintillation counting.

2.3. Statistical analysis

Experiments were performed three times with different platelet donors. Results are reported as mean ± SEM.

3. Results

3.1. Effect of GF 109203X on thromboxane production

To investigate a possible contribution of protein kinase C to phospholipase A_2 activation and thromboxane synthesis, the effects of the protein kinase C inhibitor, GF 109203X, were determined. GF 109203X is reported to be selective for inhibition of the α and β isoforms and at higher concentrations for the novel isoforms, δ and ε (IC_{50} values shown in Table 1). Platelets were treated with 5 nM thrombin in the presence of varied concentrations of GF 109203X and thromboxane levels were determined. The results, shown in Fig. 2, indicate that this inhibitor enhances thromboxane production in response to thrombin. For 5 nM thrombin, this effect is maximal at approximately 2 μM GF 109203X. To further characterize this response, the thrombin, Thrombin Quick I and TRAP concentration dependence of thromboxane production at 1.0 μM GF 109203X was determined. In a separate study (data not shown), 1.0 μM GF 109203X completely inhibited phorbol-ester-induced platelet aggregation and this concentration is also equivalent to the 50% inhibitory concentration reported for the phosphorylation of P47 in human platelets (Toullec et al., 1991). Thrombin Quick I is a site-specific mutant of thrombin in which the substitution, $Arg^{382(67)} \rightarrow Cys$, occurs within anion binding exosite I (the fibrinogen binding exosite) of thrombin (Henriksen and Mann, 1988; Bode et al., 1989). As a consequence of this mutation, the activity of Thrombin Quick I in releasing fibrinopeptide A from fibrinogen or in stimulating prostacyclin production by human umbilical vein endothelial cells is less than 2% of that observed for normal thrombin. However, Thrombin Quick I is 30% as effective as thrombin in stimulating thromboxane production by platelets (Henriksen and Brotherton, 1983; Henriksen and Owen, 1987). Because of its distinct enzymatic properties, this mutant form was included in our studies to determine possible differential effects of GF 109203X on thromboxane production among the three agonists, thrombin, Thrombin Quick I and TRAP. The results, shown in Fig. 3, indicate that the dose–response curves for the three agonists, thrombin (0.5–150 nM), Thrombin Quick I (1.5–500 nM) and TRAP (5–120 μM) are shifted to the left by GF 109203X. The maximal response to thrombin and Thrombin Quick I is not increased, but in response to TRAP maximal thromboxane production does increase, although

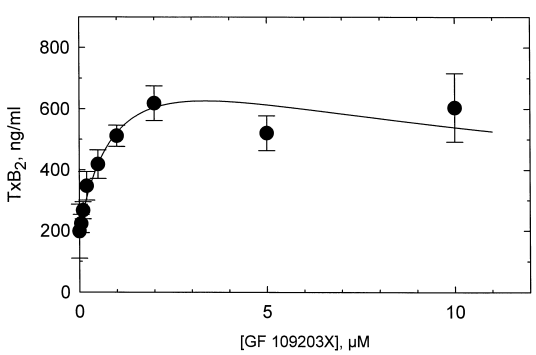

Fig. 2. Concentration dependence for GF 109203X in thrombin-induced thromboxane production. Washed platelets (4×10^8/ml) were stirred with GF 109203X for 2 min at 37°C. Then thrombin was added to a final concentration of 5 nM and platelets were stirred for 1 min, after which platelets were removed by centrifugation and the thromboxane B_2 concentration in the supernatant was determined by ELISA. Results shown are mean ± SEM for three experiments with platelets obtained from different donors.

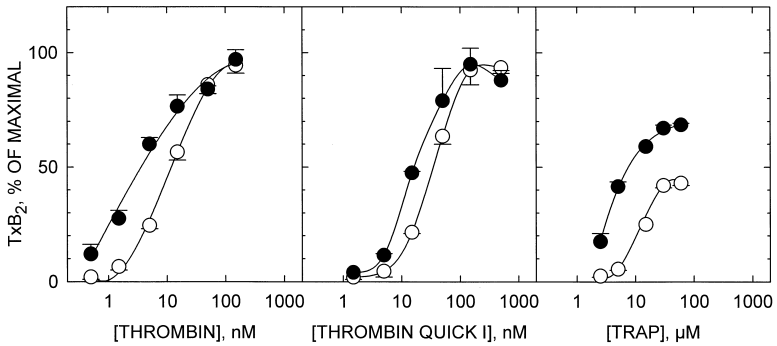

Fig. 3. Effect of 1.0 μM GF 109203X on thromboxane production induced by thrombin-related agonists. Experiments were performed as described for Fig. 2. Platelets were stirred with 1.0 μM GF 109203X (●) or with buffer (○), prior to addition of the indicated concentration of agonist. Maximum thromboxane production in each experiment was defined as 100%.

it remains less than the maximal response obtained for thrombin. When aspirin-treated platelets, prelabeled with [^{3}H]arachidonic acid, were treated with thrombin or TRAP in the presence or absence of GF 109203X, the pattern of release of radiolabeled products was similar to that observed when thromboxane production was monitored, indicating that GF 109203X acts at a step prior to that catalyzed by cyclooxygenase (results not shown).

3.2. Effect of the protein kinase C β-selective inhibitor, 379196

To further characterize the response to GF 109203X, reported to inhibit primarily protein kinase C isoforms α and β, the effects of an inhibitor selective for protein kinase C β, 379196 (Fig. 1), were determined. In contrast to the results obtained with GF 109203X, thromboxane production was not increased by 150 nM 379196 (Fig. 4). This concentration of 379196 is well above the reported IC_{50} for protein kinase C β yet below the IC_{50} for the other protein kinase C isoforms (Table 1) and is also similar to the 50% effective concentration for a related protein kinase C β-selective inhibitor, LY 333531, in the inhibition of plasminogen activator release from cultured endothelial cells (Jirousek et al., 1996). These results suggest that protein kinase C β does not have a significant role in regulating thromboxane production, even though it is known to be present in platelets.

3.3. Effect of Gö 6976 on thromboxane production

Like GF 109203X, the inhibitor Gö 6976 also inhibits α and β protein kinase C isoforms, but with increased selectivity and a decreased IC_{50}. The results for thromboxane production by platelets in response to varied concentrations of thrombin, Thrombin Quick I and TRAP in the presence and absence of 1 μM Gö 6976, shown in Fig. 5, indicate that thromboxane production is minimally affected under these conditions. Studies in which platelets were stimulated with 5 nM thrombin or 10 μM TRAP in the presence of concentrations of Gö 6976 increasing to 10 μM did not show enhancement of thromboxane production over the value obtained in the absence of the inhibitor (data not shown). These data, together with the results obtained for 379196, suggest that neither the α or β protein kinase C isoform contributes to the regulation of thromboxane B_2 production.

3.4. Effect of rottlerin on thromboxane production

The above findings, suggesting that enhanced thromboxane production observed in the presence of GF 109203X does not result from inhibition of protein kinase C α or β, raised the possibility that protein kinase C δ or ε might contribute to this effect since the IC_{50} of GF 109203X with respect to these isoforms (Table 1) is below the 1 μM concentration used in the experiments shown in Fig. 3. Therefore, thromboxane production was determined in the presence and absence of rottlerin, an inhibitor reported to

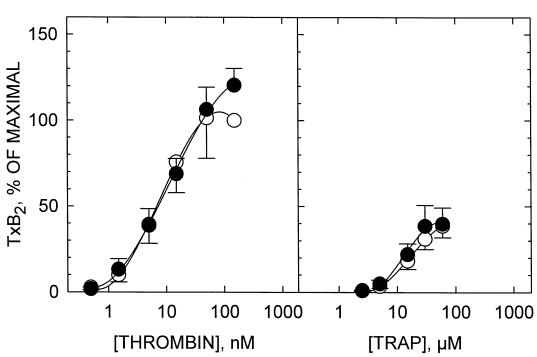

Fig. 4. Effect of 150 nM 379196 on thromboxane production induced by thrombin or TRAP. Experimental conditions were as described for Fig. 2. Results are shown for treatment of platelets with 379196 (●) or with buffer (○) and the indicated concentration of agonist. For these experiments, 100% thromboxane is defined as the response to 150 nM thrombin in the absence of the inhibitor.

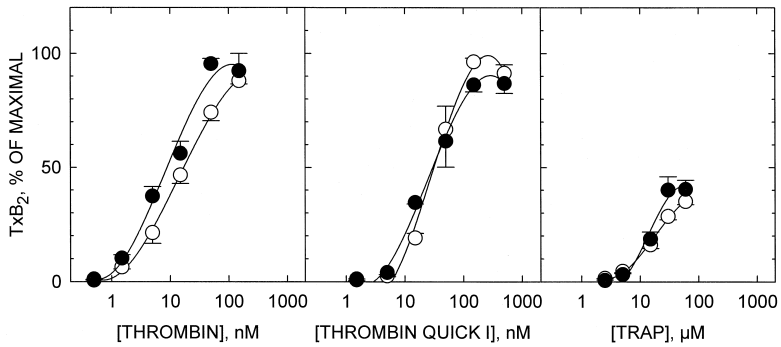

Fig. 5. Effect of 1.0 μM Gö 6976 on thromboxane production induced by thrombin-related agonists. Experimental conditions were as described for Fig. 3. Results are shown for treatment of platelets with 1.0 μM Gö 6976 (●) or with buffer (○) and the indicated concentration of agonist.

be selective for protein kinase C δ (Gschwendt et al., 1994). Fig. 6A shows the rottlerin concentration dependence for thromboxane production in response to 5 nM thrombin. Additional experiments were performed in the presence of 10 μM rottlerin which produced > 80% inhibition of thromboxane production by 5 nM thrombin. Thromboxane production as a function of thrombin or TRAP concentration is shown in Fig. 6B and C, respectively. In contrast to the results above with other protein kinase C inhibitors, rottlerin inhibited thromboxane production. There is similar, marked inhibition of thromboxane production at all thrombin and TRAP concentrations. At 10 μM, the rottlerin concentration exceeds the reported IC_{50} only for protein kinase C δ, among the kinases for which data is reported (Table 1). These results suggest that protein kinase C δ might have a role in signal transduction for at least one step leading to thromboxane production. To demonstrate that the effect of this inhibitor was not directly on platelet cyclooxygenase or thromboxane synthetase, release of radioactivity from platelets labeled with [^{3}H]arachidonic acid prior to stimulation with thrombin or TRAP in the presence and absence of rottlerin was determined. The results obtained (not shown) were similar to those presented in Fig. 6B and C for thromboxane production.

4. Discussion

With identification of multiple protein kinase C isoforms, questions regarding their roles are even more intriguing as the differential effects of these isoforms are investigated. Although protein kinase C is rapidly activated in platelets, specific roles for protein kinase C isoforms have not been fully elucidated. These studies were undertaken to characterize a possible role for protein kinase C in thromboxane production, an early event in platelet activation. We have examined the effect of GF 109203X and other selective protein kinase C inhibitors on thromboxane production by human platelets. The results suggest a complex pattern of both positive and negative regulation of thromboxane production by kinases in response to thrombin-related agonists.

In the presence of 1 μM GF 109203X, thromboxane production by washed human platelets was enhanced by the three agonists, thrombin, Thrombin Quick I and TRAP,

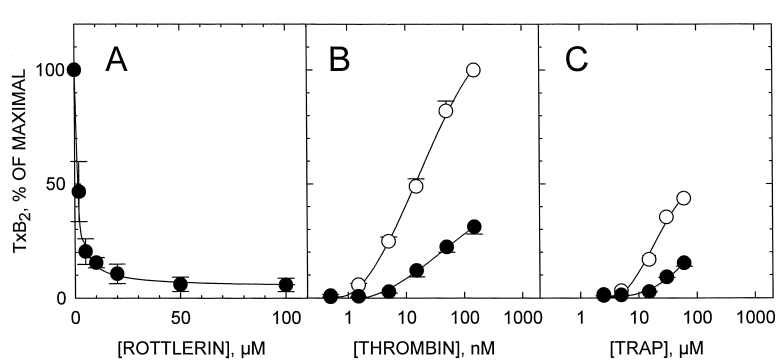

Fig. 6. Effect of 10 μM rottlerin on thromboxane production induced by thrombin and TRAP. Experimental conditions were as described for Fig. 3. Panel A shows the rottlerin concentration dependence for thromboxane production induced by 5 nM thrombin. Panels B and C show the concentration dependence for thrombin and TRAP, respectively, for the production of thromboxane in the presence of 10 μM rottlerin (●) or with buffer (○).

compared to the same agonist in the absence of GF 109203X. In contrast to earlier studies in which we observed differential effects of prostaglandin E_1 and genistein on thromboxane production in response to thrombin and TRAP (Henriksen et al., 1997), GF 109203X has similar effects for all three agonists at submaximal levels of thromboxane production. Enhancement of both thromboxane and leukotriene C_4 production by GF 109203X has been observed previously in human eosinophils stimulated with platelet-activating factor (PAF) (Dent et al., 1998). Our results obtained in the presence of inhibitors selective for protein kinase C α, β, and δ indicate that the enhanced thromboxane production seen in the presence of GF 109203X is most probably attributable to another protein kinase C isoform or an unidentified kinase. For the protein kinase C β-selective inhibitors, Gö 6976 (1 μM) or 379196 (150 nM), no effect on thromboxane production was observed. This observation is distinct from an earlier report indicating that protein kinase C β is a specific negative regulator of thrombin-stimulated extracellular Ca^{2+} entry into cultured human erythroleukemia cells (Xu and Ware, 1995). It may, therefore, be inferred that extracellular Ca^{2+} does not contribute significantly to thrombin-induced thromboxane production, consistent with our observation that thromboxane production is only minimally altered in the absence of added Ca^{2+} (Samokhin and Henriksen, unpublished observation).

In the presence of rottlerin, reported to be a selective inhibitor of protein kinase C δ (Table 1), thromboxane production was inhibited. Protein kinase C δ, a novel isoform, lacks the Ca^{2+} binding domain of the classical isoforms, but is subject to activation by phospholipid. There are also other reports suggesting a specific role for protein kinase C δ in regulating cellular activation. Recently, it has been reported that protein kinase C δ is co-immunoprecipitated by antibodies to PI 3-kinase following stimulation of human erythroleukemia cells or rabbit platelets (Ettinger et al., 1996). It has also been reported that activation of the epidermal growth factor receptor in epidermal keratinocytes or the treatment of these cells with phorbol ester results in tyrosine phosphorylation of protein kinase C δ and the inhibition of enzymatic activity (Denning et al., 1996).

Early appearance of a signal transduction intermediate would be necessary for it to participate in the regulation of thromboxane production. It has been shown that the level of phosphatidylinositol(3,4,5)trisphosphate increases transiently in platelets at less than 1 min after stimulation by 10 nM thrombin (Kucera and Rittenhouse, 1990) and further that this metabolite activates protein kinase C δ, ε, and η in vitro (Toker et al., 1994), suggesting that activation of these protein kinase C isoforms could occur rapidly following platelet stimulation.

The reported IC_{50} for GF 109203X acting on protein kinase C ζ is greater than the concentration used in our experiments, suggesting that this atypical isoform does not contribute to the observed effect of GF 109203X on thromboxane production. The reported IC_{50} value for protein kinase C ε would make it a possible candidate for the effects of GF109203X observed in our studies. Data for the specificity of GF 109203X with respect to the protein kinase C isoforms η and θ both present in platelets have not been reported.

Except for rottlerin, the protein kinase C inhibitors used in these studies are structurally related to bisindolylmaleimide. All of these inhibitors act by interfering with ATP binding to the enzyme. Therefore, it is also possible that kinases other than the protein kinase C isoforms may be involved in the effects that have been observed.

Because rottlerin similarly inhibits both thromboxane production and the release of labeled arachidonic acid metabolites, our results suggest that protein kinase C δ may participate in regulating thromboxane production at some point between the cell surface and phospholipase A_2. The results obtained for GF 109203X indicate that at least one other protein kinase C isoform or kinase must participate in the attenuation of thromboxane production in response to thrombin. Because thromboxane may contribute to thrombotic events and also to inflammatory diseases such as allergic asthma, our results suggest that isoform-selective inhibitors of protein kinase C might have therapeutic potential as antithrombotic or anti-inflammatory agents.

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