Comparison of the modes of action of Ca^{2+} ionophore A23187 and thrombin in protein kinase C activation in human platelets

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In human platelets, the Ca^{2+} ionophore A23187 stimulated the phosphorylation of a 40 kDa protein and myosin light chain (MLC) to the same extents as those induced by thrombin, but the doses of A23187 for 40 kDa protein phosphorylation were higher than those for MLC phosphorylation, although the doses of thrombin for both reactions were nearly the same. Moreover, A23187 produced much less diacylglycerol than thrombin. However, the sites of the 40 kDa protein phosphorylated by the action of A23187 and thrombin were identical, and the 40 kDa protein phosphorylation induced by A23187 and thrombin was inhibited by tetracaine, an inhibitor for protein kinase C. Neither A23187 nor thrombin induced the production of a catalytic fragment of protein kinase C which might be generated by limited proteolysis with Ca^{2+}-dependent protease. These results indicate that A23187 induces protein kinase C activation which phosphorylates the 40 kDa protein, but higher doses of A23187 are required for the activation of this enzyme than for the activation of MLC kinase.

Platelet *Ca^{2+} ionophore* *Protein kinase C* *Protein phosphorylation* *Diacylglycerol* *Cytoplasmic Ca^{2+}*

1. INTRODUCTION

Platelet aggregation and release reaction are elicited in response to many agonists including thrombin, collagen and PAF (review [1]). The modes of action of these agonists have been investigated extensively, and protein phosphorylation, particularly the phosphorylation of a 40 kDa protein and MLC, has been demonstrated to be involved in release reaction from platelets [2,3]. MLC and the 40 kDa protein are phosphorylated by MLC kinase and protein kinase C, respectively, during the action of thrombin, collagen and PAF [4–7]. MLC kinase is activated by Ca^{2+} in a calmodulin-dependent manner [8]. Protein kinase C is activated in 2 different manners as far as tested in a cell-free system: proteolytic activation by Ca^{2+}-dependent protease [9,10] and non-proteolytic activation by diacylglycerol and Ca^{2+} in the presence of membrane phospholipid [11–13]. The diacylglycerol is derived from phosphoinositide turnover which is induced by the agonists described above in a receptor-linked manner [5–7].

The Ca^{2+} ionophore A23187 is also known to induce platelet aggregation and release reaction [14]. This agonist stimulates the phosphorylation of the 40 kDa protein as well as MLC to the same extent as that induced by other agonists described above [3]. However, it has not been clarified which protein kinase is involved in A23187-induced phosphorylation of the 40 kDa protein. Furthermore, Rittenhouse-Simmons [15] has reported that

Abbreviations: PAF, platelet-activating factor; MLC, myosin light chain

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A23187 produces much less diacylglycerol than thrombin. It has also been reported that Ca^{2+}-dependent proteolysis occurs during stimulation of platelets with A23187 [16]. Therefore, we attempted to identify the enzyme which is responsible for A23187-induced 40 kDa protein phosphorylation and to compare the mode of action of A23187 with that of thrombin.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Washed human platelets were prepared by the method of Baenziger and Majerus [17]. Carrier-free $^{32}P_i$ and [^{3}H]arachidonic acid (78.2 Ci/mmol) were purchased from Japan Radioisotope Association and New England Nuclear, respectively. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [18]. Bovine thrombin and A23187 were the products of Mochida Pharmaceutical and Calbiochem, respectively. Tetracaine hydrochloride was donated by Kyorin Pharmaceutical. Calf thymus H1 histone was prepared as described [9]. A mixture of phospholipids was extracted from bovine brain and fractionated on a silicic acid column as in [11]. Other materials and chemicals were obtained from commercial sources.

2.2. *Preparation and stimulation of platelets*

The platelets were prelabelled with either $^{32}P_i$ or [^{3}H]arachidonic acid according to Lyons et al. [2] and Rittenhouse-Simmons [19], respectively. The radioactive platelets were then stimulated by either thrombin or A23187 as indicated in each experiment. The radioactive lipids were directly extracted from the platelets by the method of Bligh and Dyer [20], and subjected to thin-layer chromatography on a silica gel G plate using a solvent system of benzene/diethyl ether/ethanol/NH_4OH (50:40:2:0.1) for separating diacylglycerol [19], or chloroform/methanol/acetic acid/water (25:15:4:2) for separating phospholipids [21]. Radioactive platelet proteins were directly subjected to SDS-slab gel electrophoresis, stained, dried on filter paper, and then exposed to an X-ray film to prepare an autoradiograph as described in [5]. Electrophoresis was carried out by the method of Laemmli [22]. The relative intensity of each band was quantitated by densitometric tracing of the autoradiograph using a Shimadzu model CS-910 dual-wavelength chromatogram scanner. Two-dimensional mapping of tryptic peptides from the radioactive 40 kDa protein recovered from SDS-polyacrylamide gel was performed as in [6].

2.3. *Assay for protein kinase C*

Protein kinase C was assayed in a reaction mixture (0.25 ml) containing 5 μmol Tris-HCl at pH 7.5, 1.25 μmol magnesium acetate, 50 μg H1 histone, 2.5 nmol [γ-^{32}P]ATP (1×10^5 cpm/nmol), 225 nmol $CaCl_2$, 10 μg phospholipid, 0.2 μg diolein and an enzyme preparation. Where indicated, 0.5 μmol EGTA was added instead of diolein, Ca^{2+} and phospholipid. After the reaction was carried out for 3 min at 30°C, the acid-precipitable radioactivity was determined as described [9].

3. RESULTS

When platelets were incubated with various doses of either thrombin or A23187, the 40 kDa

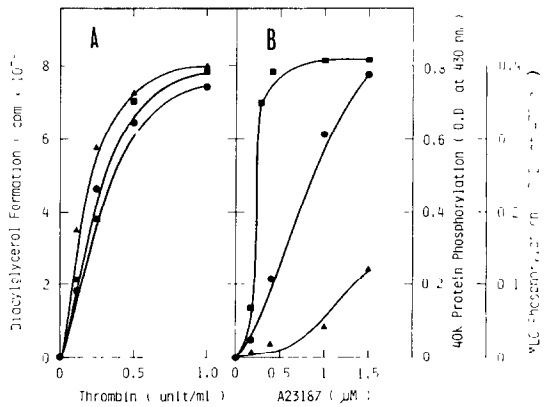

Fig.1. Dose-response curves for A23187- and thrombin-induced diacylglycerol formation, 40 kDa protein phosphorylation and MLC phosphorylation. The platelets, which were labelled with [^{3}H]arachidonic acid or $^{32}P_i$, were stimulated by various doses of either A23187 or thrombin. The incubation was carried out for 15 s at 37°C to measure diacylglycerol formation, and for 1 min to measure protein phosphorylation. Other details are described in section 2. (A) Stimulated by thrombin, (B) stimulated by A23187. (▲—▲) Diacylglycerol formation, (●—●) 40 kDa protein phosphorylation, (■—■) MLC phosphorylation. Each value is the mean of triplicate determinations. 40K protein, 40 kDa protein.

protein and MLC were phosphorylated in a dose-dependent manner as shown in fig.1. The maximum levels of the 40 kDa protein and MLC phosphorylation induced by thrombin and A23187 were nearly the same. However, the doses of A23187 for 40 kDa protein phosphorylation were higher than those for MLC phosphorylation, whereas the doses of thrombin for both reactions were roughly equal. Moreover, A23187 produced much less diacylglycerol than thrombin. The doses of A23187 for diacylglycerol formation were again higher than those for MLC phosphorylation, whereas the doses of thrombin for both reactions were nearly the same. This small formation of diacylglycerol during the action of A23187 was not simply due to its rapid conversion to phosphatidic acid via the pathway of phosphoinositide turnover, nor to its degradation to monoacylglycerol and free fatty acid by the action of diacylglycerol lipase, since A23187 did not stimulate ^{32}P incorporation into phosphatidic acid nor inhibit the thrombin-induced reaction (not shown).

In spite of these differences in the actions of thrombin and A23187, the sites of the 40 kDa protein phosphorylated by the action of these 2 agonists were identical as judged by fingerprint analysis as shown in fig.2. Moreover, A23187- as well as thrombin-induced 40 kDa protein phosphorylation was markedly inhibited by tetracaine, an inhibitor for protein kinase C [23], as shown in table 1. Under similar conditions, this drug did not inhibit A23187- and thrombin-induced diacylglycerol formation. These results strongly suggest that the same protein kinase is involved in both A23187- and thrombin-induced 40 kDa protein phosphorylation. Since protein kinase C has been demonstrated to be responsible for thrombin-induced 40 kDa protein phosphorylation [5,6], it is most likely that the same enzyme is also involved in A23187-induced phosphorylation of this protein.

In the last set of experiments, we investigated whether protein kinase C is activated in a proteolytic or non-proteolytic manner during the action of A23187 and thrombin. After stimulation with A23187 platelets were disrupted and centrifuged. The enzymatic activities of protein kinase C in the soluble and particulate fractions were then assayed. Most of the protein kinase C activity was found in the soluble fraction and the enzymatic activity in the soluble fraction was dependent on diacylglycerol, Ca^{2+} and phospholipid as shown in table 2. The catalytic fragment of protein kinase C, which was fully active even without these activators and might be produced by limited proteolysis with Ca^{2+}-dependent protease, could not be detected. Similar results were obtained for the platelets stimulated by thrombin. These results indicate that protein kinase C is activated in a non-proteolytic manner during the action of A23187 as well as of thrombin.

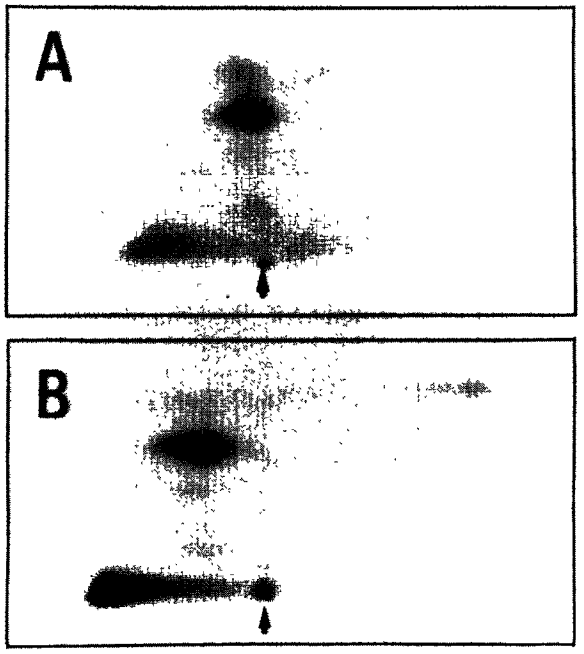

Fig.2. Autoradiographs of tryptic digests of the 40 kDa protein phosphorylated in platelets during the action of A23187 and thrombin. The phosphorylation of the 40 kDa protein was carried out as described in the legend to fig.1. The radioactive 40 kDa protein was extracted from the gel, digested with trypsin, and subjected to cellulose coated thin-layer plate electrophoresis in the horizontal dimension (negative pole left, positive pole right) followed by ascending chromatography in the vertical dimension as described [6]. Arrowheads indicate the origin. (A) Stimulated by thrombin, (B) stimulated by A23187.

Table 1

Effects of tetracaine on A23187- and thrombin-induced diacylglycerol formation and 40 kDa protein phosphorylation

Treatment	Diacylglycerol formation (cpm)	40 kDa protein phosphorylation (A_{430nm})
None	80	0.18
A23187	380	0.60
A23187 + tetracaine	350	0.39
Thrombin	1320	0.72
Thrombin + tetracaine	1360	0.38
Tetracaine	70	0.18

The platelets, which were labelled with [^{3}H]arachidonic acid or $^{32}P_i$, were incubated with tetracaine (2 mM) for 2 min at 37°C, and then stimulated by either A23187 (2 μM) or thrombin (1 unit/ml) under the conditions described in the legend to fig.1. Each value is the mean of triplicate determinations

Table 2

Non-proteolytic activation of protein kinase C during the action of A23187 and thrombin

Treatment	Protein kinase activity	
	With diolein, $CaCl_2$ and phospholipid	With EGTA
None	8720	1110
A23187	9050	1220
Thrombin	8860	1180

Platelets (5×10^8 cells) were stimulated by either A23187 (2 μM) or thrombin (1 unit/ml) for 3 min at 37°C, cooled on ice and then centrifuged at 8750 × g for 1 min. The platelets were suspended in 1 ml of 20 mM Tris-HCl at pH 7.5 containing 0.25 M sucrose, 10 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA and 2 mM phenylmethylsulfonyl fluoride, and then disrupted by sonication using a Kontes Micro-ultrasonic cell disrupter. The homogenate was centrifuged for 60 min at 100000 × g. The supernatant (20 μl) was assayed for protein kinase C activity under the conditions described in section 2. Each value (in cpm) is the mean of triplicate determinations

4. DISCUSSION

It is well established that protein kinase C is activated by Ca^{2+}, diacylglycerol and phospholipid [11–13]. Kinetic studies in a cell-free system have revealed that Ca^{2+} and phospholipid are essential for the activation of the enzyme and that diacylglycerol increases the affinity of the enzyme for Ca^{2+} [12,13]. Therefore, it is conceivable that protein kinase C can be activated by either diacylglycerol formation, Ca^{2+} increase or both in intact cells. The present results indicate that A23187 elicits the full activation of protein kinase C but does not induce diacylglycerol formation to a great extent compared with thrombin. Moreover, the doses of A23187 for protein kinase C activation are higher than those for MLC kinase activation, whereas the doses of thrombin for the activation of both enzymes are nearly the same. Since MLC kinase is activated in proportion to an increase of cytoplasmic free Ca^{2+} concentrations [24], our results strongly suggest that, during the action of A23187, protein kinase C is activated at Ca^{2+} concentrations higher than those necessary for the activation of MLC kinase, whereas, during the action of thrombin, protein kinase C is activated at the Ca^{2+} concentrations nearly the same as those necessary for the activation of MLC kinase. It is likely that diacylglycerol produced by the action of A23187 is not enough to decrease the Ca^{2+} concentrations to the levels at which MLC kinase is fully activated, and that protein kinase C may be activated by relatively higher concentrations of Ca^{2+} in the presence of a small amount of diacylglycerol.

In conclusion, A23187 as well as thrombin induces protein kinase C activation which then phosphorylates the 40 kDa protein, but the modes of activation of this enzyme during the action of these agonists are slightly different.

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