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Leupeptin does not affect the normal signal transduction mechanism in platelets

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Calpains are Ca^{2+}-dependent serine proteases that can regulate protein kinase C-mediated cellular events by cleaving the membrane-bound native enzyme to yield an activated cytosolic fragment. Inhibition of calpain by leupeptin may cause enhancement or inhibition of cellular functions depending on the nature of the protein kinase C reaction involved. We have studied the effects of leupeptin on platelet responses (aggregation, secretion, thromboxane B_2 formation and intracellular Ca^{2+} and pH changes) induced by either thrombin, collagen or phorbol 12-myristate 13-acetate (TPA), which are known to activate protein kinase C by different mechanisms. Only thrombin-induced responses were inhibited by leupeptin. This suggests that the inhibitory effect of leupeptin is not due to antagonism of calpain in this system, but to direct interference with the proteolytic effect of thrombin.

Leupeptin; Ca^{2+}, intracellular; pH, intracellular; Thromboxane B_2; (Platelet)

1. INTRODUCTION

It has been reported that the calpain inhibitor leupeptin [1] inhibits several thrombin-induced platelet responses, including aggregation, secretion, PIP_2 hydrolysis, protein phosphorylation and fibrinogen receptor expression [2,3]. In neutrophils leupeptin enhances oxygen radical production and reduces exocytosis through a mechanism involving the inhibition of proteolysis of the membrane-attached protein kinase C [4]. The aim of the present work is to test whether or not a similar mechanism is responsible for the effects of leupeptin in platelets. Three different aspects of platelet activation, changes of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), intracellular pH (pH_i), and thromboxane A_2 (TXA_2) generation, were studied. Cellular responses such as aggregation and secretion were also followed. Three different agonists which differ in their action mechanism, thrombin, collagen and phorbol 12-myristate 13-acetate (TPA), were used. Thrombin is a universal agonist, capable of inducing changes in all the parameters studied [5–7], collagen acts mainly through TXA_2 generation [5], and TPA promotes direct activation of protein kinase C without modifying $[Ca^{2+}]_i$ [8].

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Abbreviations: TPA, phorbol 12-myristate 13-acetate; BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; BSA, bovine serum albumin; ACD, acid-citrate dextrose; DG, diacylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PLA_2, phospholipase A_2; TXB_2, thromboxane B_2

2. EXPERIMENTAL

Platelet-rich plasma was obtained from freshly drawn citrated blood and centrifuged for 20 min at 350 × *g*. Platelets were resuspended at a concentration of 3×10^8 cells/ml in nominally Ca^{2+}-free standard medium with the following composition (mM): 145 NaCl; 5 KCl; 1 $MgSO_4$; 10 glucose; 10 Na-Hepes, pH 7.4. This suspension was divided in two halves and cells were loaded with either 2 μM BCECF or 2 μM fura-2 by incubation for 45 min at 37°C with their respective esters (Molecular Probes, Junction City, OR, USA). The cell suspen-

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sions were then diluted with two volumes of standard medium containing 1 mg/ml BSA and 2% ACD. The cells were sedimented by centrifugation and resuspended in fresh standard medium. Immediately before the corresponding experiment 1 mM of either $CaCl_2$ or EGTA was added to each sample. Specific fluorescence was measured at 10^8 cells/ml at 37°C with continuous stirring. Calibration of the fura-2 signal was performed as described before [9] and that of BCECF was performed in a high-K medium containing 2 μM nigericin (a gift of Lilly Indiana de España) as described previously [10]. Aggregation and ATP secretion was followed simultaneously in a Coulter lumi-aggregometer. Secretion of ^{14}C-serotonin [11] was also studied in parallel in some experiments to check the adequacy of the lumi-aggregometer signal. Thromboxane B_2 formation was assayed by radioimmunoassay using a specific antibody obtained from Diagnostics Pasteur.

Washed platelets were incubated with 10–500 μM leupeptin (Sigma) for 10 min prior to agonist addition. It has been postulated that this 10 min incubation of washed platelets with leupeptin was needed to achieve intracellular permeation of this compound [12]. We find, however, that reducing this period to 1 min did not modify the inhibitory pattern of leupeptin (results not shown). The final concentrations of agonists were 0.1–5 U/ml thrombin (Calbiochem), 10 μg/ml collagen (Horm) and 100 nM TPA (Sigma). Stocks were prepared in buffer (thrombin and collagen) or dimethylsulphoxide (TPA). The amount of the latter solvent in the cell suspension was always below 0.2%.

3. RESULTS AND DISCUSSION

Fig.1A shows the changes in $[Ca^{2+}]_i$ induced by thrombin and collagen under control conditions (left) and after a 10 min treatment with 100 μM leupeptin (right). Fig.1B illustrates pH_i changes induced by thrombin and TPA in control (left) and leupeptin-treated cells (right). Only in thrombin-treated cells did leupeptin modify agonist-evoked changes in $[Ca]_i$ and pH_i. Similar results were also obtained when aggregation and secretion were followed (data not shown). These results discount possible inhibition of protein kinase C by leupeptin in platelets since TPA-induced alkalinization is mediated by protein kinase C [6,7]. The increase in $[Ca^{2+}]_i$ induced by collagen is mainly due to thromboxane generation through the cyclooxygenase pathway [5]. The inability of leupeptin to affect $[Ca^{2+}]_i$ responses induced by collagen would therefore suggest that it does not interfere with thromboxane formation. This was confirmed by quantitating the increase in TXB_2 induced by collagen and thrombin in the presence and absence of

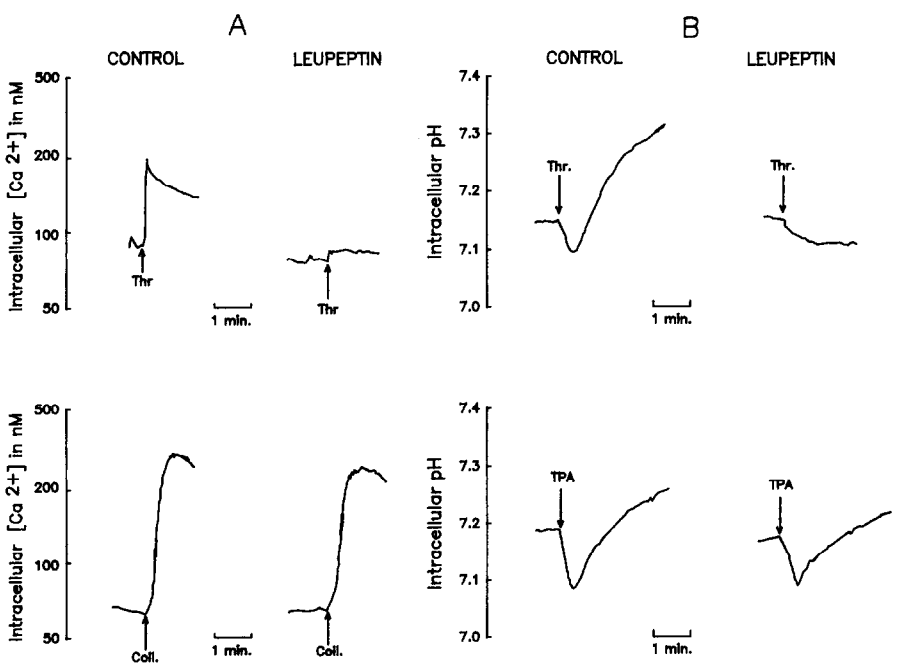

Fig.1. Effects of leupeptin on agonist-induced changes in intracellular Ca^{2+} (A) and pH (B) in human platelets. Leupeptin (100 μM) was added 10 min before agonist addition. Experiments with collagen (10 μg/ml) were performed in medium containing 1 mM $CaCl_2$ and those with thrombin (0.1 U/ml) or TPA (100 nM) in Ca^{2+}-free medium.

leupeptin (table 1). Leupeptin inhibited the generation of TXB_2 in thrombin-stimulated cells but did not have significant effect on the response to collagen. The lack of effect of leupeptin on collagen-induced TXB_2 generation excluded possible interactions of calpain with any of the enzymes involved in the process. This includes PLA_2, DG lipase, cyclooxygenase or thromboxane synthetase. Furthermore these results indicate that leupeptin does not interfere with platelet receptors for TXA_2 [13].

Fig.2 shows the effects of increasing leupeptin concentrations on the secretion induced by either thrombin or TPA. TPA-induced secretion was not significantly affected at any of the concentrations of leupeptin tested, reinforcing the idea that leupeptin does not inhibit a protein kinase C-mediated event. In contrast, leupeptin inhibited thrombin-induced secretion in a dose-dependent manner. The inhibition by leupeptin could be relieved by increasing the concentration of thrombin suggesting a 'competitive' interaction. Submaximal inhibitions of secretion correlated well with the effect of leupeptin on thrombin-evoked $[Ca^{2+}]_i$ and pH_i changes (data not shown).

The insensitivity of TPA- and collagen-induced platelet responses to leupeptin suggests that leupeptin does not interfere with intracellular calpains in platelets, in contrast with proposals in other cell systems [4]. It should then affect an early step in the interaction of thrombin with platelets. The 'competitive' nature of the thrombin-leupeptin interaction and the observation that preincubation with leupeptin is not necessary for inhibition of thrombin effects suggest that the interaction takes place in the extracellular medium. Since it has been shown previously that leupeptin inhibits fibrinogen cleavage without interfering with the ability of thrombin to bind to its specific receptor [14], the most likely explanation for our results is that leupeptin produces a loss of proteolytic activity of thrombin which is crucial for the activation of the receptor. This mechanism would also explain the correlation among $[Ca^{2+}]_i$, pH_i and secretion at submaximal inhibition values.

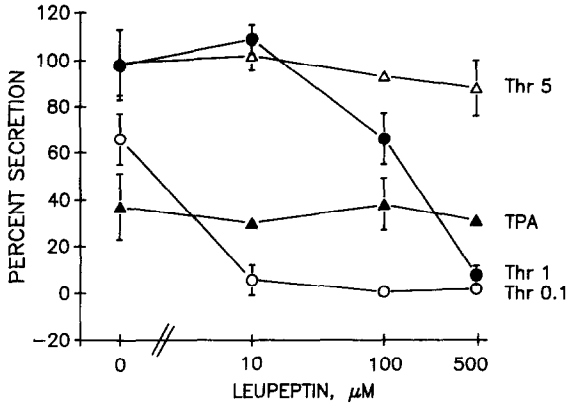

Fig.2. Effects of leupeptin on ATP secretion induced by thrombin or TPA in human platelets. Thrombin concentration is indicated in the figure in U/ml. TPA concentration was 100 nM. Note that the scale for leupeptin concentration is logarithmic. ATP secretion is expressed as percent of the maximal response obtained. Values are means ± SD of at least three different experiments. Other details as in fig.1.

Table 1

Effects of leupeptin on the rise of thromboxane B_2 formation induced by collagen or thrombin in human platelets

Agonist	Thromboxane B_2 (ng/10^8 cells)	
	Control	+ Leupeptin
Collagen	877 ± 72	734 ± 104
Thrombin	161 ± 46	27 ± 5.5

Data represent the increments in TXB_2 levels from the resting level of 46.5 ± 8 ng/10^8 cells. Platelets were incubated for 2.5 min with the agonist and then the cells were sedimented by centrifugation. Thromboxane B_2 was determined in the supernatants. Other details as for fig.1. Values represent mean ± SD of three experiments

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