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# Effects of L- and D-arginine and some related esters on the cytosolic mechanisms of $\alpha$ -thrombin-induced human platelet activation

<sup>1</sup>Paola Failli, Enrica Cecchi, C. Ruocco, A. Fazzini & A. Giotti

Dipartimento di Farmacologia Preclinica e Clinica 'M. Aiazzi Mancini' and Centro Interuniversitario 'Ipossie', V. le G.B. Morgagni, 65, University of Florence, 50134 Florence, Italy

**1** In Fura-2 preloaded human platelets, the increase in cytosolic calcium induced by  $\alpha$ -thrombin was reduced by some L- and D-arginine ester compounds the  $IC_{50}$  ( $\mu$ M) values of which were 7.4 for TAEE, 56.9 for BAEE, 77.6 for TAME, 560 for T(d)AME, 656.3 for L-ArgOMe and 2206.7 for D-ArgOMe.  $\alpha$ -tosyl-L-Arginine, L- and D-arginine were inactive.

**2** The inhibitory activity of the L-arginine esters was not modified when platelets were pretreated with 100  $\mu$ M N<sup>ω</sup>-monomethyl-L-arginine.

**3** The L-arginine esters did not increase cyclic GMP content in platelets either in the presence or absence of indomethacin and apyrase at rest and after  $\alpha$ -thrombin stimulation.

**4** The kinetic parameters of platelet Na<sup>+</sup>/H<sup>+</sup> antiporter (amiloride-inhibitable, evaluated after cytosolic nigericin-induced acidification) were modified by L- and D-arginine esters, while the native amino acids were ineffective.

**5** The inhibitory effects of the L- and D-arginine esters on platelet activation appear to be mainly due to their inhibitory effect on Na<sup>+</sup>/H<sup>+</sup> antiporter.

**Keywords:** Human platelets;  $\alpha$ -thrombin activation; cytosolic calcium concentration; Na<sup>+</sup>/H<sup>+</sup> antiporter activity; L-arginine; D-arginine; L-arginine esters; D-arginine esters; N<sup>ω</sup>-monomethyl-L-arginine

## Introduction

Recent data from many laboratories point to the role of L-arginine in a wide range of physiological functions (Moncada *et al.*, 1991), including platelet aggregation (Radomski *et al.*, 1987; 1990a,b). L-Arginine has been found to be directly linked to the production of endothelial-derived relaxing factor (EDRF), which has been identified as nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), one of the two nitrogen atoms of the guanidine group of the amino acid being oxidized to produce nitric oxide. The biochemical pathway for synthesizing nitric oxide from L-arginine is competitively inhibited by guanidine-substituted L-arginine derivatives such as N<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) (Hibbs *et al.*, 1987; Moncada *et al.*, 1991). Nitric oxide appears to exert its action through activation of a soluble guanylate cyclase and hence an increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation.

It has been proposed not only that L-arginine might be the substrate for the production of nitric oxide, but also that some L-arginine derivatives may induce endothelium-dependent relaxation in different vascular beds by increasing nitric oxide synthesis (Thomas & Ramwell, 1988; Al-Swayeh & Moore, 1989; Thomas *et al.*, 1990; Busija *et al.*, 1990; Farhat *et al.*, 1990a,b). More recently it has been reported that the vasorelaxation induced by N- $\alpha$ -benzoyl-L-arginine ethyl ester (BAEE) is either not nitric oxide-dependent (Al-Swayeh & Moore, 1989; Fasehun *et al.*, 1990; Schmidt *et al.*, 1990) or is only partially so (Farhat *et al.*, 1990b), while N- $\alpha$ -tosyl-L-arginine methyl ester (TAME) has been described as a nitric-oxide-independent vasorelaxant (Schmidt *et al.*, 1990) or an endothelium-independent inhibitor of contraction induced by several agents in human umbilical arteries (White, 1988).

Some of the L-arginine esters also inhibit platelet aggregation (Salzman & Chambers, 1964; Aoki *et al.*, 1978; Failli *et al.*, 1990; Spurej *et al.*, 1990). Preliminary data obtained in our laboratory show that L- and D-arginine esters inhibit aggregation induced by  $\alpha$ -thrombin, while concentrations up

to 500  $\mu$ M are ineffective against collagen-induced aggregation. In order to investigate the antiaggregatory mechanism of arginine esters in human platelets stimulated by  $\alpha$ -thrombin, we have studied their effects against the increases in cytosolic free calcium induced by  $\alpha$ -thrombin: the experimental conditions were chosen to rule out the amplification mechanisms due to either arachidonic acid metabolites (in particular thromboxane A<sub>2</sub>), or ADP by inhibiting arachidonic acid cyclo-oxygenase with indomethacin and by hydrolyzing extraplatelet ADP with apyrase. The possible role of L-arginine esters as nitric oxide precursors has also been investigated, by measuring cyclic GMP platelet content. This set of experiments was performed either in the presence of indomethacin and apyrase or in their absence. Finally, we have monitored Na<sup>+</sup>/H<sup>+</sup> antiporter activity and its modification by the L- and D-arginine esters.

Some of these results were presented at the XIth International Congress of Pharmacology (Failli *et al.*, 1990).

## Methods

### Platelet preparation

Blood was collected by venipuncture from healthy human volunteers and immediately diluted 1/5 with citric acid: trisodium citrate:glucose (1.5%:2.5%:2% w/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 500 g at 25°C for 15 min and incubated with 3  $\mu$ M Fura-2-AM or 2  $\mu$ M BCECF-AM at 37°C for 45 min.

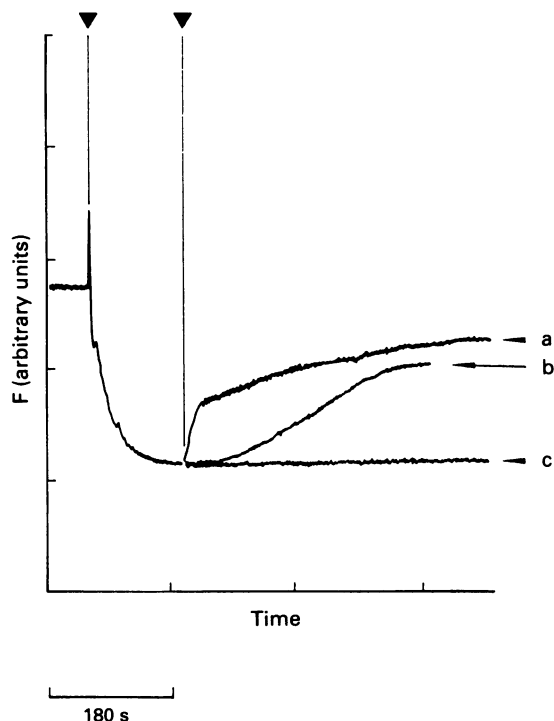
Platelets were then washed twice by centrifugation and resuspended in HEPES buffer of the following composition (mM): NaCl 140, HEPES 10, NaHCO<sub>3</sub> 12, KCl 2.9, MgCl<sub>2</sub> 0.9, NaH<sub>2</sub>PO<sub>4</sub> 0.5 and glucose 10. Apyrase (100 u l<sup>-1</sup>) was added to hydrolyze ATP to ADP and ADP to AMP (Molnar & Lorand, 1961) and indomethacin (10  $\mu$ M) was added to inhibit arachidonic acid cyclo-oxygenase. In experiments to explore the effect of the D- and L-arginine esters on Na<sup>+</sup>/H<sup>+</sup>

<sup>1</sup> Author for correspondence.

antiporter activity, BCECF-loaded platelets were washed twice and suspended in a modified (nominally  $\text{Na}^+$  and  $\text{K}^+$ -free) N-methylglucamine buffer of the following composition (mM): N-methylglucamine 138, HEPES 10, glucose 10,  $\text{MgCl}_2$  0.1, HCl 140 (adjusted to pH 7.4 with choline carbonate) (HEPES-N-methyl-glucamine buffer). D- and L-Arginine esters were dissolved in either HEPES- $\text{NaHCO}_3$  or HEPES-N-methylglucamine buffer; the pH of the solutions was measured and, if necessary, carefully adjusted.

Internal cytosolic free calcium concentrations ( $[\text{Ca}^{2+}]_i$ ) were estimated in HEPES buffer 1 mM  $\text{CaCl}_2$  according to the method of Pollock & Rink (1986), using a Shimadzu RF-5000 spectrofluorimeter (equipped with a thermostated cuvette holder and magnetic stirrer), wavelength settings being 345 nm for excitation and 500 nm for emission.  $\alpha$ -Thrombin was added directly to the cuvette in the presence or absence of the test compound.

The  $\text{Na}^+/\text{H}^+$  antiporter activity was determined by the spectrofluorimetric technique described by Grinstein *et al.* (1989). Briefly, BCECF-loaded platelets suspended in nominally  $\text{Na}^+$ - and  $\text{K}^+$ -free N-methylglucamine buffer were acidified by addition of nigericin. A typical acid-loading experiment is shown in Figure 1. The decrease in fluorescence (ordinate scale, arbitrary units) indicates the decrease in intracytosolic pH ( $\text{pH}_i$ ). When the fluorescence value had stabilized, the administration of 30 mM NaCl brought about a rapid cytosolic alkalization, indicating restored  $\text{Na}^+/\text{H}^+$  antiporter activity. At the end of each experiment, the pH of the buffer was measured in order to verify that the pH of the medium had remained constant. Intracytosolic pH was then calculated after platelet lysis, and the kinetic parameters of the first phase of alkalization were calculated after measuring specific fluorescence of the BCECF at various pH values and expressed as  $\Delta\text{pH}_i \times \text{min}^{-1}$ .



**Figure 1** Acid-loading and activation of  $\text{Na}^+/\text{H}^+$  antiporter of BCECF-loaded platelets suspended in nominally  $\text{Na}^+$  and  $\text{K}^+$ -free N-methylglucamine buffer. Nigericin,  $2 \mu\text{M}$ , was added to begin acid loading in the absence of  $\text{Na}^+$  (first arrow).  $\text{Na}^+/\text{H}^+$  antiporter was then activated by addition of 30 mM NaCl (second arrow) in the absence (a) or in the presence (b) of 1 mM amiloride. The bottom trace (c) was obtained when, instead of NaCl, 30 mM choline chloride was added at the second arrow (osmotic control). Abscissa scale: time. Ordinate scale: fluorescence (arbitrary units), excitation 505 nm, emission 530 nm.

Test molecules were preincubated with platelet suspensions for 2 min prior to adding  $\alpha$ -thrombin.

### Cyclic GMP measurement

Measurements of cyclic GMP were performed by radioimmunoassay, using kits supplied by Amersham International. In brief, twice-washed platelets were suspended at a density of  $10^8$ – $10^9$  platelets per  $100 \mu\text{l}$  in indomethacin/apyrase HEPES/ $\text{NaHCO}_3$  buffer, 1 mM  $\text{CaCl}_2$ , containing isobutylmethylxanthine ( $30 \mu\text{M}$ ). In some experiments, indomethacin and apyrase were not added. Test molecules were preincubated with platelets at  $37^\circ\text{C}$  for 20 min and then  $0.03 \text{ u ml}^{-1}$   $\alpha$ -thrombin (final concentration) or the same volume of buffer was added. Incubation was continued for an additional 5 min and then the reaction stopped by addition of  $50 \mu\text{l}$  cold 20%  $\text{HClO}_4$  and transfer of the samples onto ice for 5 min. The reaction mixture was neutralized with  $110 \mu\text{l}$   $1.08 \text{ M K}_3\text{PO}_4$ , tubes were centrifuged and 3 aliquots of supernatant ( $100 \mu\text{l}$ ) were used for cyclic GMP determination.

### Reagents

Amiloride HCl, N-methyl-D-glucamine, digitonin, D-arginine (D-Arg), N- $\alpha$ -p-tosyl-L-arginine ( $\alpha$ -tosyl-L-arginine), N- $\alpha$ -p-tosyl-L-arginine methyl ester HCl (TAME), isobutylmethylxanthine (IBMX), N- $\alpha$ -benzoyl-L-arginine ethyl ester HCl (BAEE) and N $^\omega$ -monomethyl-L-arginine acetate salt (L-NMMA) were obtained from Sigma; L-arginine (L-Arg) and sodium nitroprusside (NP) from Merck; L-arginine methyl ester  $2\text{HCl}$  (L-ArgOMe) from Fluka; nigericin (sodium salt), Fura-2 and BCECF were from Calbiochem; Fura-2-AM, BCECF-AM from Molecular Probes;  $\alpha$ -thrombin from Boehringer-Mannheim. All other reagents were of analytical grade. D-Arginine methyl ester  $2\text{HCl}$  (D-ArgOMe), N- $\alpha$ -p-tosyl-D-arginine methyl ester HCl (T(d)AME), N- $\alpha$ -benzoyl-D-arginine methyl ester HCl (B(d)AME) and N- $\alpha$ -p-tosyl-L-arginine ethyl ester HCl (TAEE) were the kind gift of Drs Buzzetti and Sala of Italfarmaco (Milano, Italy).

### Statistical analysis

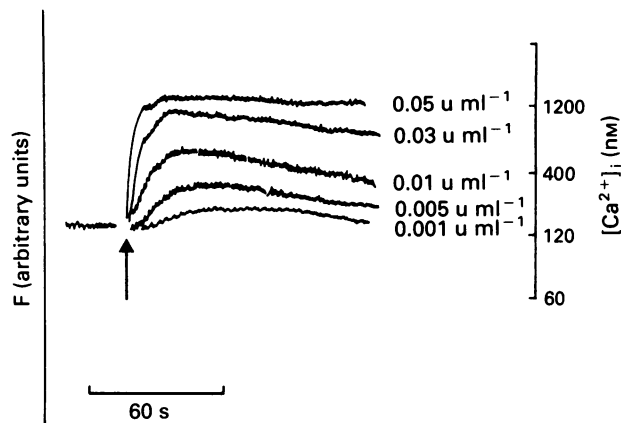
All values are expressed as mean  $\pm$  s.e. mean of the number of experiments indicated and were compared by Student's *t* test for paired data, with  $P < 0.05$  considered as statistically significant.  $\text{IC}_{50}$  values were estimated from at least 4 separate dose-effect curves by computerized linear regression analysis.

## Results

### Internal calcium

The average value for basal  $[\text{Ca}^{2+}]_i$ , measured within the 5 h duration of experiments, was  $145 \pm 4 \text{ nM}$  ( $n = 33$ ). Addition of  $\alpha$ -thrombin induced a rapid, sustained and dose-dependent increase in  $[\text{Ca}^{2+}]_i$  (Figure 2). A concentration-response curve for  $\alpha$ -thrombin was performed for each separate sample of platelets in order to choose a suitable agonist concentration for studying potential antagonists at equivalent activation levels, i.e. around the half-maximal response. The concentration selected was between  $0.03 \text{ u ml}^{-1}$  and  $0.01 \text{ u ml}^{-1}$  (final).

L- or D-Arginine esters, added 2 min before stimulation, did not influence basal  $[\text{Ca}^{2+}]_i$  values, but reduced the  $[\text{Ca}^{2+}]_i$  response induced by  $\alpha$ -thrombin in a concentration-dependent way;  $\text{IC}_{50}$  values are shown in Table 1. Esterification of the parent amino acids engendered an antithrombin activity in the compounds, with L-arginine esters being more active than D-arginine esters. Sodium nitroprusside also concentration-dependently inhibits the  $[\text{Ca}^{2+}]_i$  response induced by  $\alpha$ -thrombin, dose-dependently ( $\text{IC}_{50} = 497.4 \mu\text{M}$  – Table



**Figure 2** Increases in internal free calcium ( $[Ca^{2+}]_i$ ) induced by  $\alpha$ -thrombin on Fura-2 loaded platelets.  $\alpha$ -Thrombin ( $0.05 \text{ u ml}^{-1}$ – $0.001 \text{ u ml}^{-1}$ ) was added at the arrow. Abscissa scale: time. Left ordinate scale: fluorescence (arbitrary units), excitation 345 nm, emission 500 nm; right ordinate scale:  $[Ca^{2+}]_i$  calibration scale.

**Table 1**  $IC_{50}$  values ( $\mu\text{M}$ ) of L- and D-arginine compounds and sodium nitroprusside (NP) on the  $\alpha$ -thrombin-induced increase of  $[Ca^{2+}]_i$  in human platelets

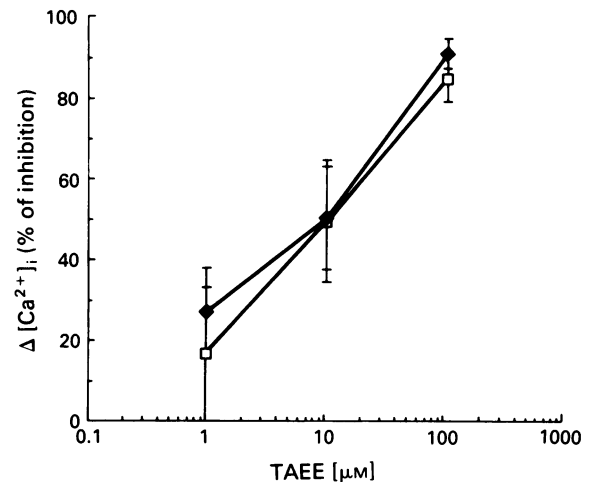
Compounds	$IC_{50}$ ( $\mu\text{M}$ )
L-Arg	No effect
D-Arg	No effect
L-ArgOMe	656.3 (608–712)
D-ArgOMe	2206.7 (1834–2769)
TAME	77.6 (50–138)
T(d)AME	560.0 (430–810)
TAE	7.4 (5–12)
BAEE	56.9 (47–71)
B(d)AME	No effect
$\alpha$ -tosyl-L-arginine	No effect
NP	497.4 (339–932)

Confidence limits in parentheses.  
For abbreviations, see text.

1). Unesterified L-arginine was ineffective, even when preincubated at a high concentration (10 mM) with platelets for 30 min at  $37^\circ\text{C}$ . The increase in  $[Ca^{2+}]_i$  induced by  $\alpha$ -thrombin was not modified by preincubating platelets (30 min at  $37^\circ\text{C}$ ) with  $100 \mu\text{M}$  L-NMMA, the increase being  $701 \pm 51 \text{ nm}$  and  $692 \pm 51 \text{ nm}$  with and without L-NMMA respectively (mean of at least 17 different determinations). N<sup>ω</sup>-monomethyl-L-arginine ( $100 \mu\text{M}$ ) did not diminish the inhibitory effect of L-arginine esters TAE, BAEE, TAME tested in the ratios 1:1, 1:10, 1:100. Figure 3 shows results for the most effective compound, TAE. Inhibition by one of the least active esters, L-ArgOMe (1 mM) was also not modified by L-NMMA ( $36.3 \pm 12\%$  inhibition in the absence and  $45.3 \pm 10\%$  inhibition in the presence of  $100 \mu\text{M}$  L-NMMA; mean of 4 experiments).

### Cyclic GMP content

**Experiments in the presence of indomethacin and apyrase**  
Cyclic GMP content of human platelets was  $0.67 \pm 0.14 \text{ pmol per } 10^8 \text{ platelets}$  in basal conditions and  $0.73 \pm 0.1 \text{ pmol per } 10^8 \text{ platelets}$  after  $\alpha$ -thrombin stimulation in indomethacin/apyrase HEPES/NaHCO<sub>3</sub> buffer, 1 mM CaCl<sub>2</sub>. These values were increased in a dose-dependent manner by 1, 10 and  $100 \mu\text{M}$  sodium nitroprusside. At the maximal concentration tested ( $100 \mu\text{M}$ ), sodium nitroprusside increased the basal content of cyclic GMP to  $1.5 \pm 0.25 \text{ pmol per } 10^8 \text{ platelets}$  ( $P < 0.05$  vs no drugs at rest) and  $1.9 \pm 0.45 \text{ pmol per } 10^8 \text{ platelets}$  ( $P < 0.05$  vs no drugs after  $\alpha$ -thrombin



**Figure 3** Effect of N- $\alpha$ -p-tosyl-L-arginine ethyl ester (TAE) in the presence (◆) or absence (□) of  $100 \mu\text{M}$  N<sup>ω</sup>-monomethyl-L-arginine (L-NMMA). Each point is the mean ( $\pm$  s.e.mean) of at least 3 experiments.

administration), respectively, at rest and after  $\alpha$ -thrombin stimulation (Figure 4a). Neither L-arginine esters (at concentrations near to their  $IC_{50}$  values for inhibition of  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase) nor  $100 \mu\text{M}$  L-arginine increased platelet cyclic GMP content (Figure 4a).

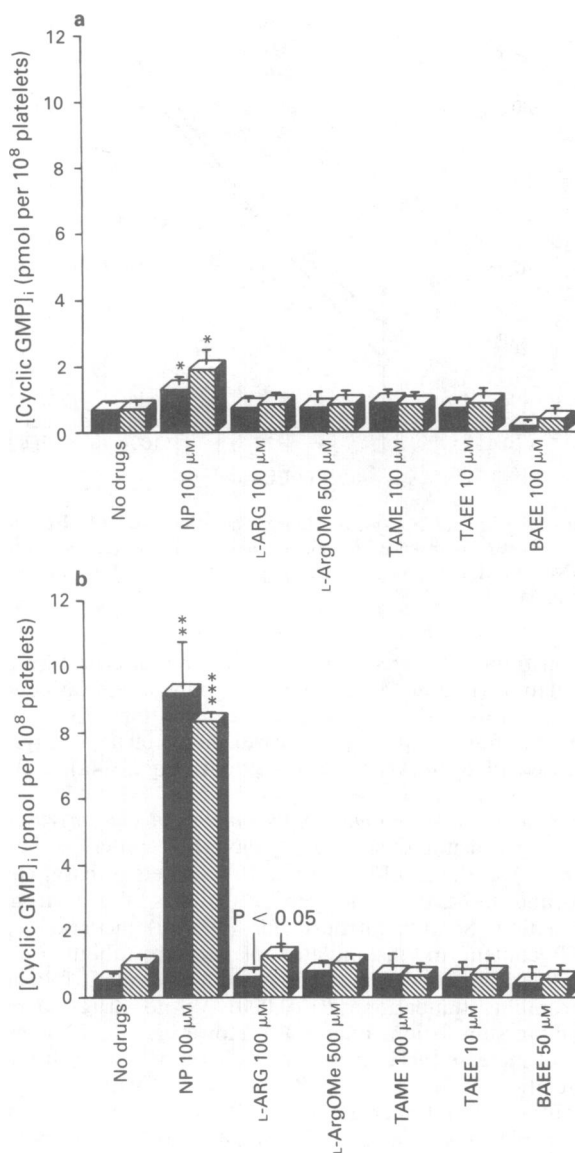
**Experiments in the absence of indomethacin and apyrase**  
In these experimental conditions, cyclic GMP content of human platelets was  $0.5 \pm 0.13 \text{ pmol per } 10^8 \text{ platelets}$  in basal conditions and  $0.97 \pm 0.18 \text{ pmol per } 10^8 \text{ platelets}$  after  $\alpha$ -thrombin stimulation. Sodium nitroprusside ( $100 \mu\text{M}$ ) increased cyclic GMP content in unstimulated platelets by about 18 fold ( $P < 0.001$  vs no drugs at rest) and by about 9 fold after  $\alpha$ -thrombin stimulation ( $P < 0.0001$  vs no drugs after  $\alpha$ -thrombin stimulation, Figure 4b). However, L-arginine esters did not increase this content either at rest or after  $\alpha$ -thrombin stimulation (Figure 4b). Preincubation with L-arginine ( $100 \mu\text{M}$ ) did not increase cyclic GMP content in unstimulated platelets ( $0.63 \pm 0.13$  and  $0.5 \pm 0.13 \text{ pmol per } 10^8 \text{ platelets}$  L-arginine and no drugs at rest respectively,  $P < 0.5$ , not significant), while  $\alpha$ -thrombin (administered after 20 min preincubation with  $100 \mu\text{M}$  L-arginine) increased it ( $0.63 \pm 0.13 \text{ pmol per } 10^8 \text{ platelets}$  to  $1.25 \pm 0.14 \text{ pmol per } 10^8 \text{ platelets}$ ,  $P < 0.05$  vs L-arginine at rest, Figure 4b). However, platelet cyclic GMP content after L-arginine ( $100 \mu\text{M}$ ) and  $\alpha$ -thrombin did not differ from that in  $\alpha$ -thrombin-treated platelets, being  $1.25 \pm 0.14 \text{ pmol per } 10^8 \text{ platelets}$  and  $0.97 \pm 0.18 \text{ pmol per } 10^8 \text{ platelets}$ , respectively ( $P < 0.295$ , not significant).

### Na<sup>+</sup>/H<sup>+</sup> antiporter activity

After the administration of 30 mM Na<sup>+</sup> ions in acid-loaded platelets, the pH<sub>i</sub> began to increase rapidly ( $1.20 \pm 0.12 \text{ pH units min}^{-1}$ , Figure 1 and Table 2, control). Amiloride (1 mM) strongly modified this response (Figure 1 and Table 2), while choline chloride did not induce any changes in pH<sub>i</sub> (Figure 1 and Table 2). The effects of L- and D-arginine esters on Na<sup>+</sup>/H<sup>+</sup> antiporter activity are summarized in Table 2. The most active compound was TAE, its effect being concentration-dependent in the range 0.5–0.01 mM. L-Arginine and D-arginine, although tested at 20 mM, were inactive (Table 2).

### Discussion

Our data show that some methyl- and ethyl esters of both D- and L-arginine inhibit the  $[Ca^{2+}]_i$  increase induced in Fura-2



**Figure 4** (a) Effect of L-arginine esters (tested at concentrations near their IC<sub>50</sub> values for inhibition of  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase) in comparison with 100  $\mu$ M sodium nitroprusside (NP) and 100  $\mu$ M L-arginine on cyclic GMP concentration measured in human platelets suspended in HEPES/NaHCO<sub>3</sub> buffer containing 1 mM CaCl<sub>2</sub>, 10  $\mu$ M indomethacin, 100  $\mu$ M apyrase and 30  $\mu$ M isobutyl methyl xanthine (IBMX), estimated either at rest (solid columns) or after stimulation by  $\alpha$ -thrombin (0.03  $\mu$ M) (hatched columns). Values are the mean ( $\pm$ s.e.mean) of at least 3 experiments. \* $P < 0.05$  vs no drugs. (b) Effect of L-arginine esters (tested at concentrations near their IC<sub>50</sub> values for inhibition of  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase) in comparison with 100  $\mu$ M sodium nitroprusside (NP) and 100  $\mu$ M L-arginine on cyclic GMP concentration measured in human platelets suspended in HEPES/NaHCO<sub>3</sub> buffer containing 1 mM CaCl<sub>2</sub> and 30  $\mu$ M isobutyl methyl xanthine (IBMX), estimated either at rest (solid columns) or after stimulation by  $\alpha$ -thrombin (0.03  $\mu$ M) (hatched columns). Values are the mean ( $\pm$ s.e.mean) of at least 3 experiments. \*\* $P < 0.001$  vs no drugs; \*\*\* $P < 0.0001$  vs no drugs.

loaded human platelets by  $\alpha$ -thrombin. The inhibitory activities of these esters are increased by substitutions on the primary  $\alpha$ -amino group, tosyl substitution being more effective than benzoyl. It is also interesting to note that ethyl esters are more effective than methyl esters (TAME < TAE).

In the same experimental conditions, sodium nitroprusside, which increases cyclic GMP content through a direct activation of soluble NO-sensitive guanylate cyclase, reduced

**Table 2** Effects of amiloride, L- and D-arginine and L- and D-arginine esters on Na<sup>+</sup>/H<sup>+</sup> antiporter activity in human platelets

No drugs	$\Delta pH \text{ min}^{-1}$
Control (30 mM NaCl)	$1.20 \pm 0.12$
Choline chloride (30 mM)	0.00
<i>Compounds</i>	
Amiloride (1 mM)	$0.37 \pm 0.08^*$
Amiloride (0.1 mM)	$0.64 \pm 0.25$
TAE (0.5 mM)	$0.29 \pm 0.10^{**}$
TAE (0.1 mM)	$0.41 \pm 0.13^{**}$
TAE (0.01 mM)	$0.51 \pm 0.08^*$
BAE (0.5 mM)	$0.67 \pm 0.13^*$
BAE (0.1 mM)	$0.71 \pm 0.14$
TAME (1 mM)	$0.29 \pm 0.10^{***}$
TAME (0.1 mM)	$0.33 \pm 0.12^*$
L-ArgOMe (10 mM)	$0.29 \pm 0.06^{**}$
D-ArgOMe (10 mM)	$0.34 \pm 0.15^*$
L-Arg (20 mM)	$1.04 \pm 0.31$
D-Arg (20 mM)	$0.66 \pm 0.26$

For abbreviations, see text.

Values are mean  $\pm$  s.e.mean of at least 3 different experiments.

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

$\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase in platelets in a dose-dependent way, suggesting that an increase in platelet cyclic GMP content (Figure 4a) reduces  $[Ca^{2+}]_i$  increase induced by  $\alpha$ -thrombin stimulation.

The suggestion that some L-arginine esters might influence cellular function by increasing nitric oxide synthesis (see Introduction) seems an unsatisfactory explanation of the inhibitory effects observed in our experimental conditions. In fact, a high concentration (100  $\mu$ M) of the NO-synthase inhibitor L-NMMA did not modify the inhibition induced by the compounds. Moreover, L-arginine esters did not increase platelet cyclic GMP content either in the presence of indomethacin and apyrase or in their absence (Figure 4a and b).

On the other hand, in the experimental conditions in which we measured  $[Ca^{2+}]_i$  (i.e. in the presence of indomethacin and apyrase), the NO-synthase pathway from L-arginine seems not to be activated. L-Arginine did not in fact inhibit the  $[Ca^{2+}]_i$  increase induced by  $\alpha$ -thrombin, either with or without a 30 min preincubation time at 37°C. Moreover, 100  $\mu$ M L-NMMA did not modify  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase. Furthermore, although 100  $\mu$ M L-arginine (preincubated 20 min) and  $\alpha$ -thrombin induced an increase in platelet cyclic GMP content in the absence of indomethacin and apyrase (Figure 4b), this increase seems to be too small to reduce  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase. In fact, a small dose of sodium nitroprusside (1  $\mu$ M), which induced a similar increase in platelet cyclic GMP content (52%, measured in the same experimental conditions as  $[Ca^{2+}]_i$  determination) hardly affected  $\alpha$ -thrombin-induced  $[Ca^{2+}]_i$  increase ( $\leq 10\%$  inhibition). This result is compatible with the weak inhibition by L-arginine of thrombin-induced aggregation of human platelets (Radomski *et al.*, 1990a,b).

We have shown that some of the L- and D-arginine esters inhibit Na<sup>+</sup>/H<sup>+</sup> antiporter activity, as demonstrated by analysing the early phase of Na<sup>+</sup>/H<sup>+</sup> activation. Furthermore, the potencies for the inhibition of the Na<sup>+</sup>/H<sup>+</sup> antiporter were correlated with potencies for inhibiting the  $[Ca^{2+}]_i$  increase induced by  $\alpha$ -thrombin, the most effective of the esters being TAE. More intriguing is the inhibitory effect of T(d)AME on the  $[Ca^{2+}]_i$  increase induced by  $\alpha$ -thrombin, as the Na<sup>+</sup>/H<sup>+</sup> antiporter activity was not affected by 1 mM or 0.1 mM T(d)AME (94% and 92% of the control, respectively). Further research will be necessary in order to clarify the effect of this compound.

Although the role of  $\text{Na}^+/\text{H}^+$  antiporter activation in the induction of aggregation and  $[\text{Ca}^{2+}]_i$  increase in platelets is a matter of debate (Siffert & Akkermann, 1987; Simpson & Rink, 1987; Hunyady *et al.*, 1987; Zavoico & Cragoe, 1988; Ghigo *et al.*, 1988; Sanchez *et al.*, 1988; Siffert *et al.*, 1989), we suggest that the effect of L-arginine esters on the  $\text{Na}^+/\text{H}^+$  antiporter activity may be the basis of their inhibitory action on  $\alpha$ -thrombin-induced  $[\text{Ca}^{2+}]_i$  increase and aggregation; indeed the prototype inhibitor of the  $\text{Na}^+/\text{H}^+$  antiporter, amiloride, also inhibits platelet aggregation (Siffert *et al.*, 1986).

The L-arginine esters were also inhibitors of  $\alpha$ -thrombin-induced aggregation,  $\text{IC}_{50}$  values being 15.2  $\mu\text{M}$ , 18.0  $\mu\text{M}$  and 26.3  $\mu\text{M}$  for TAAE, TAME and BAAE, respectively (results not shown). In contrast, concentrations up to 500  $\mu\text{M}$  of TAAE, TAME and BAAE were completely ineffective as inhibitors of collagen-induced aggregation, a mechanism of platelet activation which is independent of the activation of the  $\text{Na}^+/\text{H}^+$  antiporter (Joseph *et al.*, 1990). All these data support the hypothesis that the mechanism of action of L-arginine esters is the inhibition of  $\text{Na}^+/\text{H}^+$  antiporter activity.

Recently, the thrombin human platelet receptor has been cloned and functionally characterized (Vu *et al.*, 1991). It seems that thrombin binding with its receptor sites is characterized by a partial proteolytic digestion of an extraplatelet

domain. Although we cannot rule out the possibility that the antithrombin effect of L-arginine esters is an antiproteolytic process, preliminary results indicate that L-arginine esters inhibit the proteolytic effect of  $\alpha$ -thrombin only at concentrations which are 10 times greater than those required to inhibit the  $[\text{Ca}^{2+}]_i$  increase after  $\alpha$ -thrombin (unpublished observations). Therefore, this property is unlikely to explain the inhibitory effect of the compounds. In fact, subthreshold antiproteolytic concentrations are fully active in inhibiting the  $[\text{Ca}^{2+}]_i$  increase induced by  $\alpha$ -thrombin. Moreover, in PRP, BAAE inhibits thrombin-induced platelet aggregation without inhibiting fibrin clot formation (Spurej *et al.*, 1990).

In conclusion, our observations are consistent with the interpretation that pharmacological actions of some arginine esters may be linked to their inhibitory effect on platelet  $\text{Na}^+/\text{H}^+$  antiporter activity. For instance, this property might also explain some of the endothelium-independent (or only partially endothelium-dependent) effects of L-arginine esters described by other authors (White, 1988; Schmidt *et al.*, 1990; Farhat *et al.*, 1990; Fasehun *et al.*, 1990).

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