Regulation of L-arginine uptake by Ca^{2+} in human platelets

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Received 19 August 1999; received in revised form 13 October 1999

Abstract L-Arginine uptake and Ca^{2+} changes in unstirred platelets activated by thrombin, collagen and Ca^{2+} ionophore A23187 were evaluated. Thrombin did not affect L-arginine uptake at short incubation times (2–15 min), but at prolonged times slowed down the amino acid transport. Collagen was ineffective. A23187 decreased the L-arginine uptake in a dose-dependent manner, producing the maximal inhibition at 5 μM. In FURA 2-loaded platelets collagen did not modify Ca^{2+} basal level, thrombin induced a late Ca^{2+} rise and A23187 dose-dependently increased cytosolic Ca^{2+}, eliciting the highest increase at 5 μM. It is likely that L-arginine uptake is inversely modulated by Ca^{2+} concentrations and is inhibited during platelet stimulation with agonists which induce cytosolic Ca^{2+} elevation.

Key words: L-Arginine uptake; Ca^{2+}; Platelet activation; A23187; Collagen; Thrombin

1. Introduction

Nitric oxide (NO) is one of the most complex and widely used messenger molecules in animals [1,2] and among its various activities it exerts an inhibitory effect on platelet activation [3]. The regulation of platelet function is crucial to prevent platelet hyperaggregation, thrombus formation and strokes [4] and seems to be partially due to both endothelium and endogenous derived NO [5,6]. In the last years an endogenous isoform of NO synthase (NOS) was purified and characterised in platelets. This Ca^{2+}/calmodulin dependent enzyme is active in the presence of the same cofactors as the other constitutive NOS but it has a different molecular weight [7]. Despite the clear anti-aggregating effect of NO, the effective role of the endogenous enzyme is still unclear. Moreover, a particularly complicated problem is the regulation of the NO pathway inside platelets. In fact, the presence of a Ca^{2+}-dependent isoform is in contrast with the virtual absence of endogenous NO production as a consequence of specific Ca^{2+}-mobilising stimuli. Lantoine et al. [8] observed the production of endogenous NO in platelets stimulated with collagen, while thrombin was unable to do it. The same authors and other investigators observed that collagen does not mobilise Ca^{2+}, while thrombin is known to increase intracellular Ca^{2+} concentration in platelets [8,9]. Similarly, Radomski et al. observed that L-arginine, the natural substrate of NOS, was able to inhibit, in a concentration-dependent manner, platelet aggregation after collagen stimulation but curiously the phenomenon did not occur after thrombin or A23187 stimulation [10].

Since the production of NO is strictly related to the availability of L-arginine, a possible mechanism of regulation of NO synthesis could be related to the L-arginine transport system.

In recent years, attention on L-arginine transport inside the mammalian cells has been increasing [11]. There are various known L-arginine plasma membrane transport systems and their expression varies among different tissues and cell types. System y^+ has been identified as a specific transport system for L-arginine in human platelets, it is sodium-independent and encoded by the CAT gene family [12]. Yet, little is known about regulation of platelet L-arginine transport [12,13].

The experiments described in this paper were undertaken to evaluate the role of Ca^{2+} in the control of L-arginine uptake in stimulated platelets. Agonists triggering different transduction pathways have been tested: thrombin, which activates phospholipase C pathway and elevates cytosolic Ca^{2+} by an inositol 1,4,5-triphosphate-dependent release from internal stores [14,15], collagen that can mobilise Ca^{2+} only at high concentrations [16] and A23187 which bypasses receptor-coupling mechanisms and directly carries Ca^{2+} across membranes.

2. Materials and methods

2.1. Chemicals

L-Arginine, A23187, ionomycin, thrombin, Dowex 1×8-200 basic anion exchanger resin, digitonin, EGTA and PGE_1 were from Sigma. 1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethylester (Fura 2/AM) was from Calbiochem-Novabiochem. L-[2,3,4-^{3}H]Arginine was from NEN Life Science Products. Collagen was from Mascia Brunelli S.p.A. Titertek filters were from Flow Laboratories.

2.2. Blood collection and preparative procedures

Human blood from normal healthy volunteers, who declared to have not taken drugs known to interfere with platelet function, was collected in 130 mM aqueous trisodium citrate anticoagulant solution (9:1). Washed platelets were prepared as previously described [17]. Briefly, platelet-rich plasma, obtained by centrifugation of the whole blood at 100×*g* for 25 min, was centrifuged at 1000×g for 20 min. The pellet was washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose). Platelets obtained by centrifugation at 1000×*g* for 20 min were resuspended in Ca^{2+} free pH 7.4 HEPES buffer, (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Glucose, 10 mM HEPES).

2.3. Purification of commercial L-[2,3,4-^{3}H]arginine

Commercial L-[2,3,4-^{3}H]arginine was purified to eliminate every contaminating product such as ornitine and citrulline by chromatog-

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; FURA 2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethylester

0014-5793/99/$20.00
PII: S0014-5793(99)01422-2

raphy on Dowex 1×8-200 basic anion exchanger resin. After the consequent lyophilisation, purified L-[2,3,4-^{3}H]arginine was resuspended to 1.0×10^6 cpm/μl in 2% ethanol aqueous solution.

2.4. Measurement of L-arginine uptake

Washed platelets, resuspended in pH 7.4 HEPES buffer at 2.0×10^8/ml, were incubated with PBS or agonists in the presence of 40 μM L-arginine and 1 μCi/ml L-[2,3,4-^{3}H]arginine in a Dubnoff water bath under gentle shaking, without stirring, at 37°C. In these conditions platelet activation, but not aggregation was induced by agonists. At the indicated times aliquots of 1.0 ml incubation mixture were withdrawn, immediately filtered through a Titertek filter and washed twice with PBS. The radioactivity corresponding to the incorporated L-[2,3,4-^{3}H]arginine was directly measured by liquid scintillation counting of the filter in a Packard Tricarb 1600 TR.

2.5. Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured according to Rotondo et al. [18]. Washed platelets (3.0×10^8/ml), resuspended in Ca^{2+} free pH 7.4 HEPES buffer, were incubated with 1 μM FURA 2/AM, for 45 min at 37°C. 2 μM PGE_1 and 1.0 mM EGTA were added before centrifuging for 15 min at $1100\times g$. The pellet, resuspended at 2.0×10^8/ml in pH 7.4 HEPES buffer, was preincubated at 37°C for 2 min before the addition of the agonists. FURA 2-loaded platelet fluorescence followed, 60 min at 37°C in unstirred conditions in a Perkin-Elmer fluorescence spectrometer model LS50B with excitations at 340 nm and 380 nm and emission at 510 nm. The fluorescence of fully saturated FURA 2 (F_{max}) was obtained by lysing the cells with 50 μM digitonin in the presence of 2.0 mM Ca^{2+}, while F_{min} was determined by exposing the lysed platelets to 20 mM EGTA. The fluorescence was fully quenched with 5 mM Mn^{2+} to give the autofluorescence value. A computer program combined with the fluorescence spectrometer converted data into cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The K_d value for FURA 2 and Ca^{2+} was 140 nM.

3. Results

3.1. L-Arginine uptake in resting and activated platelets

The time-course of L-arginine uptake in resting and stimulated platelets was studied. The data (Fig. 1A) show that L-arginine uptake was not significantly modified in platelets challenged by collagen or thrombin at short incubation times (2–15 min). At prolonged incubation times (30–60 min) L-arginine uptake dose-dependently slowed down in thrombin-treated platelets, while it behaved similarly to controls in collagen-stimulated samples. The Ca^{2+} ionophore A23187 blocked the transport activity almost completely, inducing a limited increase only in platelets incubated for 2 min at 37°C.

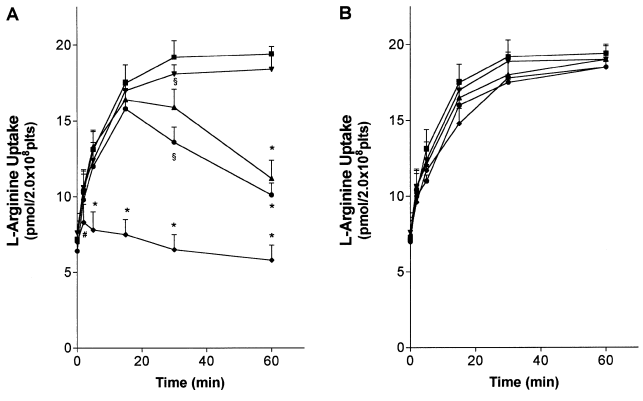

Fig. 1. Time-course of L-arginine uptake in resting and activated platelets. Washed platelets (2.0×10^8/ml) were incubated with PBS (■), 5 μg collagen (▼), 0.1 U/ml thrombin (▲), 5.0 U/ml thrombin (●) or 5 μM Ca^{2+} ionophore A23187 (♦) in the absence (A) or in the presence of 2.0 mM EGTA (B). Data are the mean ± S.D. of four experiments. * $P<0.0005$; § $P<0.0025$; # $P<0.05$ vs control.

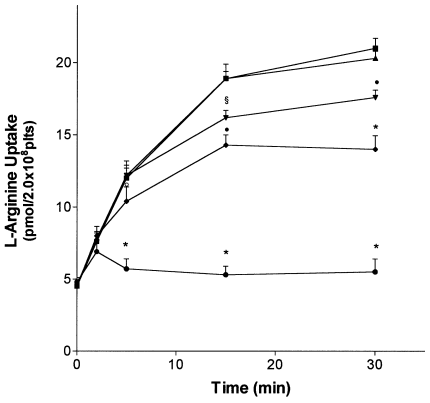

Fig. 2. Effect of Ca^{2+} ionophore A23187 on L-arginine uptake. Washed platelets (2.0×10^8/ml) were incubated with PBS (■), 0.05 μM A23187 (▲), 0.1 μM A23187 (▼), 1.0 μM A23187 (♦), 5.0 μM A23187 (●). Reported data are means ± S.D. of experiments performed three times with similar results. * $P<0.0005$; • $P<0.0025$; ° $P<0.005$; § $P<0.05$ vs control.

Addition of 2.0 mM EGTA to platelet suspensions completely reversed the inhibition produced by thrombin or A23187 (Fig. 1B). The data suggest that Ca^{2+} could be involved in the L-arginine uptake regulation. Therefore, the effects of A23187 were analysed. As shown in Fig. 2, L-arginine uptake was inhibited in a concentration dependent manner by A23187. The Ca^{2+} ionophore produced the maximal inhibition at 5 μM and higher concentrations up to 20 μM did not change the inhibitory profile. The same effects as A23187 were obtained in platelets stimulated by ionomycin (data not shown). All these experiments were performed incubating platelets in the presence of 40 μM L-arginine, but closely similar inhibition profiles were observed at physiological concentration (100 μM) [19] of L-arginine. (data not shown).

3.2. Cytosolic Ca^{2+} changes in agonist-stimulated platelets

To investigate the relationship between L-arginine uptake and cytosolic Ca^{2+} concentrations, Ca^{2+} changes were followed for 60 min in unstirred platelet suspensions challenged by the agonists, in the absence of external Ca^{2+}. Data shown in Fig. 3 have been obtained after 30 min of incubation. Stimulation of platelets by 5 μg collagen did not increase cytosolic Ca^{2+} basal level (46 ± 12 nM). Platelet treatment with A23187 produced a dose-dependent effect on cytosolic Ca^{2+} elevation, that, at 5 μM A23187, reached the maximal level corresponding to 151 ± 30 nM. Moreover, thrombin induced a $[Ca^{2+}]_i$ rise corresponding to 78 ± 8 nM (0.1 U/ml thrombin) or 110 ± 12 nM (5.0 U/ml thrombin). Nevertheless, a different time course of $[Ca^{2+}]_i$ during 60 min was found in platelets stimulated by A23187 or thrombin. In the first case (A23187) $[Ca^{2+}]_i$ rose very rapidly (1 min) and was unchanged during the whole incubation period. On the contrary, $[Ca^{2+}]_i$ slowly increased, varying from 46 ± 12 nM to 105 ± 9 nM or 130 ± 15 nM after 60 min in platelets treated with 0.1 U/ml or 5.0 U/ml thrombin, respectively. In addition, under stirring conditions, Ca^{2+} rise was very early and reached 250 ± 75 nM or 550 ± 93 nM in the presence of the above-mentioned thrombin concentrations.

4. Discussion

NO plays a fundamental role in the regulation of platelet

aggregation [3]. A few years ago some authors evidenced that NO production in these cells is catalysed by a Ca^{2+}-dependent NOS activity [7,8,20,21]. Moreover, Radomsky et al. described L-arginine's ability to inhibit platelet aggregation induced by arachidonic acid or ADP [10]. The same authors also observed that L-arginine was unable to inhibit platelet aggregation after thrombin or A23187 stimulation [10]. Similarly Lantoine et al. measured the formation of NO in collagen-stimulated platelets, while no traces of NO were detected following thrombin activation [8]. These results suggest an important role of $[Ca^{2+}]_i$ in the control of NO formation. Therefore, we performed a series of experiments to demonstrate that $[Ca^{2+}]_i$ regulates the L-arginine transport. The data presented in this work, obtained in platelets incubated without stirring in order to generate cell activation but not aggregation, show a dose-dependent negative effect on L-arginine uptake by A23187 (Fig. 2), a complete failure of collagen to modify L-arginine transport and a late inhibition of this transport after thrombin stimulation (Fig. 1A). The abrogation by EGTA of the effects generated by these agonists suggests a basic role of $[Ca^{2+}]_i$ in the regulation of the L-arginine uptake (Fig. 1B). Parallel experiments, carried out in platelets activated with collagen, thrombin or various ionophore concentrations were performed to evaluate $[Ca^{2+}]_i$. Data showed the absence of $[Ca^{2+}]_i$ increase in the presence of collagen, while the dose-dependent A23187 effects were rapid and constant. The late inhibition of the L-arginine uptake after thrombin stimulation could be due to gradual and long-time prolonged Ca^{2+} rise. In fact, in platelets incubated without stirring, the Ca^{2+} rise during the first 30 min of incubation was too low to induce any inhibitory effect, while only at longer incubation times (30–60 min) the Ca^{2+} increase was enough to modify the L-arginine uptake. In particular, at 30 min of incubation the $[Ca^{2+}]_i$ in thrombin-activated platelets ranged with the values obtained in platelets stimulated by 0.1 μM and 0.5 μM A23187 (Fig. 3). Moreover, intracellular arginine concentrations measured in samples treated with 0.1 U/ml and 5.0 U/ml thrombin (Fig. 1A) were similar to those obtained in 0.1μM and 0.5 μM A23187-stimulated platelets (Fig. 2). The increase

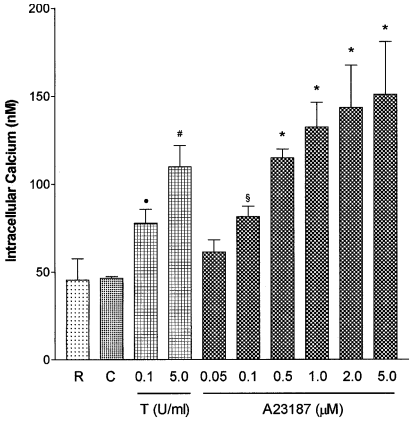

Fig. 3. Intracellular Ca^{2+} elevation induced by agonists. FURA-2 loaded platelets (2.0×10^8/ml) were preincubated for 2 min at 37°C prior to addition of saline (R), 5 μg collagen (C), 0.1 U/ml or 5.0 U/ml thrombin (T), or varying concentrations of Ca^{2+} ionophore A23187. Experiments were carried out in unstirred conditions. Data shown have been obtained after 30 min incubation and are the mean ± S.D. of three experiments. * $P<0.0005$; # $P<0.0025$; § $P<0.005$; • $P<0.01$ vs resting platelets.

of $[Ca^{2+}]_i$ elicited by 5 μM A23187 (151 ± 30 nM) was lower than that mobilised during platelet aggregation [22], thereby suggesting a complete stop of L-arginine uptake during this process. Finally, the observed decrease in arginine intracellular concentration in platelets treated with thrombin could be the consequence of both a reduced uptake and an increased efflux. This hypothesis is suggested by data concerning y^+ arginine transporter described in mouse hepatocytes [23,24] in which an arginine bidirectional flux was shown.

All these data strengthen the hypothesis of a very refined control system of the L-arginine/NO pathway in human platelets, in which $[Ca^{2+}]_i$ is at the same time the activator of NOS and the inhibitor of the L-arginine uptake. This peculiar behaviour may be justified by the analysis of the functions of free intracellular Ca^{2+}, which is a potent stimulator of many activation pathways [22] and may inversely control the NO production after activation by specific agonists.

The y^+ L-arginine transport system is present in other cell types, such as macrophages, in which it is subjected to a transcriptional control [24]. It is interesting to notice that the biosynthesis of the transporter is stimulated by lipopolysaccharides, which is also a potent stimulator of inducible NOS (iNOS) transcription [25]. Therefore, in macrophages a positive modulator of iNOS is also a positive modulator of the L-arginine transporter. On the contrary, in platelets the positive modulator of NOS is a negative modulator of the L-arginine transport. These findings stress the recent observations that strictly correlate the L-arginine metabolism with the NO production in many cells operating in the circulatory system [11]. In particular these considerations suggest the fundamental role of the L-arginine transport system in the various NO-producing cells and specifically in platelets in which the rapidity of events during the aggregation process requires simple and multifunctional messengers.

Finally, the recent considerable interest in the potential therapeutic properties of L-arginine in modulating NO bioavailability in the vascular system and its possible applications in patients with cardiovascular diseases [26] suggest further experiments to better understand the molecular basis of this peculiar control of L-arginine transport firstly described in platelets.

Acknowledgements: This work was supported by a grant from the Ministero della Ricerca Scientifica e Tecnologica to G.L. We are indebted to Prof A. De Flora and U. Benatti for stimulating discussions and critical reading of the manuscript.

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