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Research Article

Ectoprotein kinase-mediated phosphorylation of FAT/CD36 regulates palmitate uptake by human platelets

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Abstract. Glycoprotein IV (FAT/CD36) has been shown to be phosphorylated by a cAMP-dependent, platelet membrane-bound ectokinase. In this study, we demonstrate that ectophosphorylation of FAT/CD36 regulates initial palmitate uptake. This is the first time that short-term regulation of the activity of a long-chain fatty acid carrier could be shown. Phosphorylation of FAT/CD36 was paralleled by a significant decrease in initial palmitate uptake by morphologically and functionally intact platelets. Maximum inhibition of palmitate uptake was achieved at 0.5 nM extracellular ATP, being significantly decreased to 72% compared to the control. Inhibition of palmitate uptake was abolished by co-incubation with the specific protein kinase A inhibitor peptide PKI or with β,γ-methylene-ATP, and was reversible upon addition of alkaline phosphatase. An extracellular ATP concentration above 5 µM completely prevented the ectophosphorylation-mediated inhibition of palmitate uptake. We conclude that FAT/CD36-mediated palmitate uptake by human platelets is short-term regulated via cAMP-dependent ectophosphorylation of FAT/CD36.

Key words. Ectoprotein kinase; purinergic receptor; adenylyl-(β,γ-methylene)triphosphate; long-chain fatty acid; transmembrane transport.

Long-chain fatty acids (LCFAs) diffuse passively across the membrane bilayer by a flip-flop mechanism [for a review see ref. 1]. In addition, there is extensive biochemical evidence for carrier-mediated transmembrane LCFA transport [for a review see ref. 2], and several LCFA-binding membrane proteins have been identified [3–7]. Harmon and co-workers labelled a membrane protein of rat adipocytes by derivatized LCFA which led to an inhibition of fatty acid uptake by 70%, and named it fatty acid translocase (FAT) [5]. Cloning of FAT revealed 85% identity to human platelet CD36 [8]. CD36 is a transmembrane glycoprotein of 88 kDa, first described and structurally characterised in platelets [9, 10]. It was previously named glycoprotein IIIb [11] and glycoprotein IV [for a review see ref. 12]. Based on the findings of Harmon and co-workers, FAT/CD36-mediated LCFA transport was identified in several cell types [13–15]. Whether there is a significant contribution of FAT/CD36 to transmembrane LCFA transport in vivo has been discussed controversially in the past. Several studies addressed this question, and different approaches revealed that FAT/CD36 plays a major role in cellular uptake of LCFA [16–18]. However, the importance of distinct routes of LCFA across the membrane may vary between different cell types and may depend on the metabolic state of the cell.

Regulation of FAT/CD36-mediated LCFA transport occurs via protein translocation between cytosol and membrane [19–21] and possibly via altered expression of FAT/CD36. We considered phosphorylation of FAT/CD36 to play a pivotal role in regulation of fatty acid uptake.

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Ectoprotein kinase A activity on human platelets was first described by Hatmi and co-workers, and CD36 was identified as the major substrate of ectophosphorylation [22]. The extracellular segment of FAT/CD36 exhibits at least two consensus protein kinase phosphorylation sites, Thr92 and Ser237. The first is recognised by protein kinase C and, to a lesser extent, also by protein kinase A (PKA) [22, 23]. In resting platelets, the Thr92 residue is constitutively phosphorylated. Dephosphorylation of FAT/CD36 by liberated acid phosphatase or in-site mutagenesis, when the threonine phosphorylation site is changed to alanine, promotes thrombospondin binding and suppresses collagen binding by human platelets [23]. Phosphorylation of the Ser237 residue, which is recognised by PKA, has not been demonstrated. Whether the phosphorylation of FAT/CD36 has any effect on its metabolic function has not been shown so far.

The objective of this study was to test the hypothesis that the activity of the LCFA carrier FAT/CD36 is short term regulated, thereby altering cellular uptake of LCFA. Thus, we considered ectoprotein kinase-mediated phosphorylation of FAT/CD36 to be an important part of the regulation of LCFA uptake.

Materials and methods

Materials

Prostaglandin I_2 (PGI_2; Flolan) was obtained from Glaxo Wellcome (Oxford, UK). γ-[^{32}P]ATP (6000 Ci/mmol) was from Hartmann Analytic (Braunschweig, Germany). [^{3}H]palmitic acid (52 Ci/mmol) was from Amersham Biosciences Europe (Freiburg, Germany). Antibody H-300 was purchased from Santa Cruz (Heidelberg, Germany). N-glycosidase F and adenylyl-(β,γ-methylene) triphosphate (β,γ-ATP) were from Calbiochem (Bad Soden, Germany). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany), unless stated otherwise.

Isolation of human platelets

Blood from healthy, unmedicated donors was anticoagulated with 1/6 of its final volume of citric acid-citrate-dextrose (7 mM, 93 mM, 139 mM, respectively; pH 6.4) that contained heparin (20 IU/ml; Hoffmann-La Roche, Grenzach-Wyhlen, Germany). Washed platelets were prepared according to Mustard [24], but apyrase was replaced by PGI_2 (1 nM) during the first two washings. Finally, platelets were resuspended in Tyrode's buffer without PGI_2 and bovine serum albumin (BSA), and their concentration was adjusted to 2.5×10^8 cells/ml. As during the whole preparation process, the platelets were kept at 37 °C until use. A typical yield of platelet isolation was 10×10^8 platelets from 10 ml blood (60% of total platelets). Impurity was <0.2‰ leukocytes and <10‰ erythrocytes. Functional integrity of resting platelets was assured by aggregometry (Whole Blood Lumi-Aggregometer Type 560-CA; Chrono-Log, Havertown, PA.).

Measurement of palmitate uptake

Palmitic acid (460 µl, 1 mg/ml) with trace [^{3}H]palmitic acid (60 µl, 1 mCi/ml) was dissolved in ethanol and added to Celite (Supelco, Steinheim Germany). Ethanol was evaporated under nitrogen, and 8 ml of Tyrode's buffer and 880 µl of fatty-acid free BSA [500 µM in phosphate-buffered saline (PBS); Boehringer Mannheim, Germany] were added. After 1 h incubation at 37 °C, the Celite was removed by centrifugation, the supernatant was recentrifuged (1500 g, 10 min) and its volume adjusted to 100 ml by adding Tyrode's buffer. The final concentration of BSA was 4.4 µM and unbound palmitate was calculated to 5.1 nM [according to ref. 25].

Palmitate uptake

Freshly isolated platelets ($50 \times 10^{6)}$) were incubated for 20 s at 37 °C in the presence of 5.1 nM unbound palmitate. The uptake was stopped by addition of 2.5 ml ice-cold isotonic NaCl containing 1% (w/v) BSA (stop solution). Subsequently, the incubation medium was removed by filtration of the suspension through a Cyclopore filter (Whatman, Maidstone, UK). The platelets lying on the filter were washed twice with stop solution. Filters were transferred into scintillation vials, covered with 8 ml of scintillation cocktail (Wallac OptiPhase HiSafe 3; Fisher Chemicals, Loughborough, UK), and after extensive agitation of the vials, the radioactivity was counted.

Phosphorylation of membrane-bound proteins

Platelets (5×10^8/500 µl Tyrode's buffer) were kept at 37 °C. For a 5-min preincubation, cAMP was added to the medium (final concentration 5 µM); controls received no cAMP. Subsequently, γ-[^{32}P]ATP was added (35 µCi, final concentration 15 nM) and incubated for 10 min. The phosphorylation reaction was stopped with ice-cold Tyrode's buffer containing 10 mM paranitrophenyl phosphate. Platelets were quickly spun down, and the sediment was lysed in separation buffer for SDS-PAGE according to Laemmli [26].

Digestion by N-glycosidase F

The total cellular protein of 7×10^8 platelets was deglycosylated as recommended by the manufacturer. Briefly, platelets were lysed in 0.1% SDS (w/v, containing 50 mM β-mercaptoethanol) and heated to 100 °C for 5 min. After addition of Triton-X100 (final concentration 5%) and 50 units N-glycosidase F, the sample was incubated for 3 h at 37 °C.

Autoradiography and immunoblotting
After separation of total platelet proteins by 4.5/8% (w/v) discontinous SDS-PAGE, protein was transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semidry transfer at 2.5 mA/cm^2 for 1 h. The blots were exposed to Kodak BioMax-MR-1 films for 4 days at –20 °C. For immunostaining, membranes were blocked overnight with 5% (w/v) powdered milk in tris-buffered saline [TBS; pH 7.4, containing 0.1% (v/v) Tween 20]. Blots were probed for 1 h with anti-CD36 (H-300), diluted 1:500 in TBS [pH 7.4, containing 5 g/l Chemoluminescence Blotting Substrate POD (Roche Diagnostics, Mannheim, Germany)] and 0.1% (v/v) Tween 20). After washing in TBS (containing 0.1% (v/v) Tween 20], immunoreactive bands were visualised with a peroxidase anti-rabbit IgG (diluted 1:5000 as described above) and Pierce SuperSignal Chemoluminescent Substrate (Perbio Science, Bonn, Germany).

Results

Palmitate uptake
Inhibition of palmitate uptake occurs only in the presence of both cAMP and ATP. Addition of extracellular protein kinase inhibitor peptide 5–24 (PKI) or alkaline phosphatase (AP) prevents an impact on palmitate uptake. The ATP analogue β,γ-ATP, resistant to cleavage between β- and γ-phosphate groups, was without effect on palmitate uptake in the presence of cAMP and, in addition, abolished the inhibitory effect of ATP and cAMP on palmitate uptake (fig. 1; see legend for concentrations).
Incubation of resting platelets under conditions described for ectophosphorylation, i.e. extracellular concentration of 5 µM cAMP and ATP each, did not change the initial uptake of palmitate in our experimental design. Only reduction of extracellular ATP to nanomolar concentrations revealed a significant decrease in initial palmitate uptake. The maximum effect on palmitate uptake was a decrease to 72.4 ± 8.9% compared to the control and was achieved at 0.5 nM ATP (fig. 2). The effect was significant from 15 nM to 10 pM of extracellular ATP concentration ($p < 0.025$, n = 3).
In a more complex approach, we successfully showed the reversibility of the ectophosphorylation-mediated inhibition of palmitate uptake. We added cAMP (5 µM), ATP (0.5 nM) and AP (3 U/ml) one after another to suspended platelets. After each incubation, the initial palmitate uptake by platelets was determined by taking an aliquot out of the suspension. Compared to the control, the uptake was 73 ± 12% after incubation with both cAMP and ATP, and reversed to 96 ± 4.6% after subsequent incubation with AP (mean ± SD, n = 4). This indicates that the inhibitory effect of ectophosphorylation on palmitate uptake is fully reversible by extracellular dephosphorylation of platelet proteins. Extracellular dephosphorylation of membrane-bound proteins by incubation of resting platelets with AP alone did not affect palmitate uptake compared to the control (fig. 1).

Identification of the ectophosphorylated protein
Under conditions similar to those that preceded the palmitate uptake experiments, we obtained a pronounced band on autoradiography with a molecular mass of about 90 kDa. Occurrence of this band clearly depends on co-incubation with cAMP (fig. 3 A). Immunostaining of the same blots with antibodies directed against CD36 revealed bands of the same molecular mass in preparations

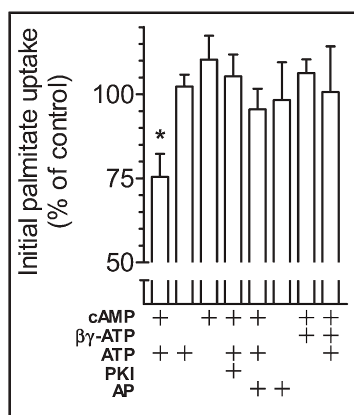

Figure 1. Effect of ectokinase A-mediated phosphorylation on initial palmitate uptake by platelets. 5 × 10^7 platelets were incubated as described in Materials and methods in the presence of 5 µM cAMP, 5 µM β,γ-ATP, 1 nM ATP, 5 µg/ml PKI and 3 U/ml AP in combinations as indicated. Columns represent the mean ± SD of three independent experiments (column 1, n = 4). The asterisk indicates a significant difference compared to the control ($p < 0.05$).

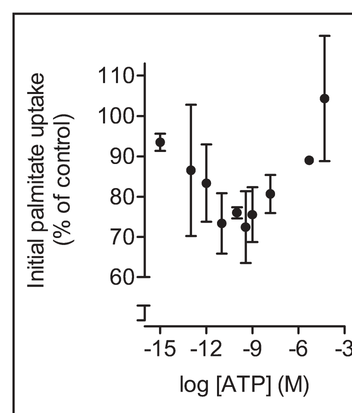

Figure 2. Ectophosphorylation-mediated inhibition of initial palmitate uptake depends on the extracellular ATP concentration. Uptake experiments as described in Materials and methods were performed in the presence of 5 µM cAMP and of the indicated ATP concentration. Values represent the mean ± SD of three independent experiments.

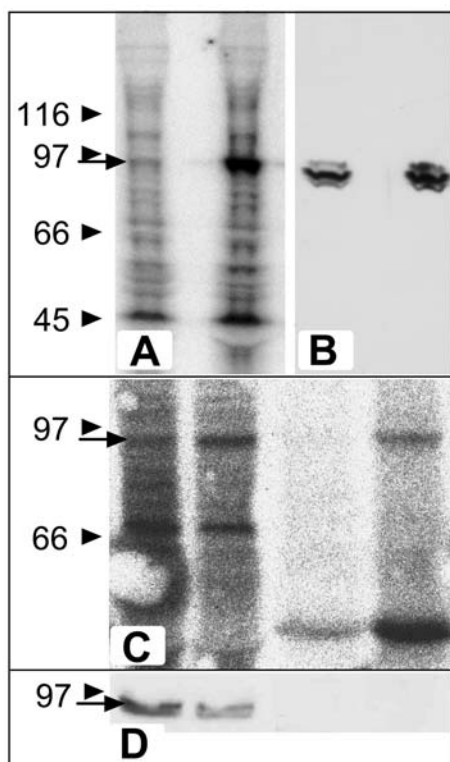

Figure 3. Identification of the ectophosphorylated protein. (*A*) Autoradiography: incubation of 5×10^8 platelets as described in Materials and methods with lane 1: 15 nM γ-[P^{32}]ATP only, lane 2: 15 nM γ-[P^{32}]ATP and 5 µM cAMP. (*B*) Immunostaining; blot as in *A* was stained with CD36 antibody H-300. (*C*) Autoradiography; incubation of 7×10^8 platelets with lane 1:5 nM γ-[P^{32}]ATP only, lane 2: 15 nM γ-[P^{32}]ATP and 5 µM cAMP, lanes 3 and 4: the same conditions as for lanes 1, 2, respectively, after subsequent deglycosylation with N-glycosidase F as indicated in Materials and methods. (*D*) Immunostaining, blot as in *C* was stained with the CD36 antibody H-300.

with and without cAMP (fig. 3B). For more evidence, we incubated the cell lysate with N-glycosidase F. The bands described above were no longer detectable by autoradiography (fig. 3C) or by immunoblotting against CD36 (fig. 3D). On autoradiography, another band with an apparent molecular mass of 50–60 kD was visible, which corresponds to the deduced mass of the deglycosylated amino acid backbone of FAT/CD36 (fig. 3C, lanes 3 and 4). As with the autoradiography of the non-deglycosylated proteins (fig. 3A), the occurrence of the band depends on the presence of cAMP (fig. 3C).

Discussion

We clearly demonstrate an inhibitory effect of ectokinase-mediated phosphorylation of FAT/CD36 on palmitate uptake by human platelets. The maximum inhibition achievable by ectophosphorylation in vivo is difficult to assess and may depend on the initial phosphorylation status of FAT/CD36 in isolated platelets. Thus, ectophosphorylation of FAT/CD36 may result in an even higher inhibition of fatty acid uptake, if the protein were completely dephosphorylated initially, but our data did not support that idea. Although it fully reversed the inhibitory effect of ectophosphorylation, dephosphorylation of platelet proteins by AP did not result in increased palmitate uptake when done on resting platelets prior to uptake experiments.

To rule out artefactual detection of protein phosphorylation by intracellular protein kinases, we used freshly isolated, resting platelets which were incubated for short times (10 min) in serum-free medium at a low ATP concentration (15 nM). In addition, we probed three substances for their ability to prevent the phosphorylation-mediated decrease of fatty acid uptake: β,γ-ATP, a slowly degradable ATP analogue, inhibited the effect on palmitate uptake; incubation with the cell-impermeable specific PKA-inhibitor peptide (PKI) or with AP reversed the inhibition of palmitate uptake we obtained under phosphorylation conditions (fig. 2). We thus met criteria for detection of ectoprotein kinase activity proposed by Redegeld and co-authors [27].

Physiological conditions in which ATP and cAMP are present outside the platelet simultaneously have been discussed in detail by a number of authors [22, 27]. The extracellular nucleotide level is thought to be controlled by ATP diphosphohydrolases (apyrases) which are thereby involved in the regulation of many physiological processes, including platelet aggregation [for reviews see refs. 28–30]. Our data show that the cAMP-dependent inhibition of palmitate uptake is sensitive to the extracellular ATP concentration. An increasing concentration of ATP (>15 nM) diminished the ectophosphorylation-mediated effect, which was no longer detectable at 5 µM ATP. Abolition of the inhibitory effect by a higher concentration of ATP due to degradation products of ATP could be ruled out, because the slow degradable analogue β,γ-ATP also abolished the ectophosphorylation-mediated effect at a concentration of 5 µM. Additionally, in the presence of ADP (5 µM), the ectophosphorylation-mediated effect on palmitate uptake occurs unimpaired (data not shown). ATP and β,γ-ATP are agonists of the purinergic receptor $P2X_1$ which is expressed in platelets. The $P2X_1$ receptor was recently shown to be activated by ATP with an EC_{50} of 0.6 µM [31]. We hypothesise that there is an increasing $P2X_1$ receptor-mediated signal transduction at ATP concentrations above 15 nM which may counteract the ectophosphorylation-mediated inhibitory effect on palmitate uptake of platelets. So far, the verification of this hypothesis using $P2X_1$ receptor antagonists (suramin, NF279) failed due to their high affinity to albumin, which drastically increased the concentration of unbound palmitate in our uptake experiments by displacement of palmitate from albumin.

In summary, the present study links the previously shown ectokinase-mediated phosphorylation of FAT/CD36 [22] with the regulation of transmembrane palmitate transport, an important function of this protein. Inhibition of palmitate uptake by ectophosphorylation of FAT/CD36 occurs at a low concentration of ATP, whereas concentrations of ATP above 15 nM abolish this effect. These data point to a specific, probably $P2X_1$-mediated effect of ATP as the underlying mechanism that modulates the ectophosphorylation-mediated inhibition of palmitate uptake.

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