Glucose Uptake via Glucose Transporter 3 by Human Platelets Is Regulated by Protein Kinase B*

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In insulin-responsive tissues, insulin is a potent activator of protein kinase B (PKB)-mediated glucose uptake through the facilitative glucose transporter GLUT4. In platelets, glucose uptake is mediated through GLUT3, which is present in plasma (15%) and intracellular α-granule (85%) membranes. Here we report the PKB-mediated glucose uptake by platelets by agents that do (thrombin) or do not (insulin) induce α-granule translocation to the plasma membrane. Both thrombin and insulin activate PKB and induce glucose uptake albeit with different kinetics. Inhibition of PKB by the pharmacological inhibitor ML-9 decreases thrombin-induced α-granule release and thrombin- and insulin-induced glucose uptake. At low glucose (0.1 mM), both agents stimulate glucose uptake by lowering the $K_m$ for glucose (thrombin and insulin) and increasing $V_{max}$ (thrombin). At high glucose (5 mM), stimulation of glucose uptake by insulin disappears, and insulin becomes an inhibitor of thrombin-induced glucose uptake via mechanisms independent of PKB. We conclude that in platelets glucose transport through GLUT3 is regulated by changes in surface expression and affinity modulation, which are both under control of PKB.

Platelet aggregation and secretion depend on plasma glucose as a source for anaerobic resynthesis of metabolic ATP (1). The facilitative glucose transporter 3 (GLUT3)* is the major GLUT subtype in human platelets. At resting conditions, ~85% of GLUT3 is located intracellularly in α-granule membranes, whereas the remainder is present in the plasma membrane (PM) (2). Upon platelet activation, GLUT3 containing α-granules fuse with the PM, and glucose uptake increases 2-fold (2, 3). GLUT3 is predominantly expressed in neuronal cells and platelets, but has also been found in granulocytes, monocytes, fibroblasts, pancreatic, endothelial, myocardial, and skeletal muscle cells (4–8). GLUT3 is predominantly expressed in neuronal cells and platelets, but has also been found in granulocytes, monocytes, fibroblasts, pancreatic, endothelial, myocardial, and skeletal muscle cells (4–8). GLUT3 has a $K_m$ for glucose of ~1.5 mM, and this parameter appears constant in different cell types (4–9). This implicates that under normal (~5 mM glucose) and pathological conditions (up to 20 mM glucose) this transporter functions near saturation. GLUT1 is a ubiquitously expressed glucose transporter functions near saturation. GLUT4 is determined by copy number at the PM and phosphorylation of PKB isoforms that contribute to platelet aggregation (19). Animal models show that both PKB-subtypes are crucial for the formation and stabilization of a thrombus in an arterial injury model (20, 21), suggesting that PKB is a critical component of PI3K-supported platelet activation. The activity of PKB depends on its phosphorylation at Ser473* and Thr308* for PKBα and PKBβ, respectively (22), and is finely regulated by PI3K-independent mechanisms that include Ca$^{2+}$-sensitive protein kinase C (19), integrin-linked kinase (23), phosphatidylyserine translocation (24), and the Src-family kinase Lyn (25).

By analogy with insulin-responsive tissues one would expect that platelets respond to insulin with an increase in glucose uptake via a mechanism controlled by PKB. Up-regulation of glucose uptake requires an increase in surface expression of GLUT3 (2, 3). Because this process depends on the fusion of α-granule membrane with the PM, and because secretion depends on an increase in cytosolic Ca$^{2+}$, one would expect that insulin supports secretion by enhancing Ca$^{2+}$ mobilization. This appears not to be the case (16).

The present study was undertaken to understand the apparent contradiction in insulin responses inflicted in platelets. The results show that insulin indeed triggers PKB activation as well as glucose uptake despite its capacity to inhibit thrombin-induced Ca$^{2+}$ mobilization. Whether insulin acts as an activator or inhibitor of PKB-mediated glucose uptake depends on the extracellular glucose concentration, which shifts the balance between activation and inhibition of platelets by insulin.

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2 The abbreviations used are: GLUT3, glucose transporter 3; PM, plasma membrane; PKB, protein kinase B; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; DOG, 2-deoxy-o-glucose; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine; RT, reverse transcription; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine; AU, arbitrary units; ns, not significant; BAPTA-AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester).

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EXPERIMENTAL PROCEDURES

Materials—We obtained prostacyclin (PGI₂) from Cayman Chemical (Ann Arbor, MI); α-thrombin (thrombin in short), human recombinant insulin, Fura-2-AM, 2-deoxy-d-glucose (DOG), 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), LY-294002, SQ22536, cytochalasin B, phloretin, BAPTA-AM, and primers from Sigma; 2-deoxy-[1-³H]-d-glucose ([³H]DOG) with a specific radioactivity of 37 Mø/ml from Amersham Biosciences; d-glucose (glucose in short) from BDH laboratory supplies (Poole, England); the microbicinchoninic acid protein assay kit from Pierce. The ADP receptor P2Y₁₂ antagonist AR-C69931MX was a kind gift from Astra Zeneca (Loughborough, UK). All other chemicals were of analytical grade.

Antibodies—we obtained antibodies for P-selectin (C-20), PKBα/b (Akt1/2), and the phospho-specific p-PKBα/β/γ (Akt1/2/3) Ser⁴⁷³/⁴ from Santa Cruz Biotechnology (Santa Cruz, CA); P-selectin/RPE (CD-62P) from Immuno cytometry Systems, San Jose, CA). The samples were gated for

Measurement of Platelet Aggregation—Aliquots of 0.5 ml of washed platelets were warmed to 37 °C for 5 min, followed by stimulation with 0.25 unit/ml thrombin. Platelet aggregation was monitored continuously for 7 min at 900 rpm in an optical aggregometer (model 570 VS, Chrono-Log Corp., Havertown, PA).

RT-PCR—The megakaryocytic cell lines MEG-01, DAMI, and CHRF-288-11 resembling immature (26), intermediate (27), and mature megakaryocytes (28), respectively, were cultured as described previously (29). mRNA was isolated with the RNeasy mini kit from Qiagen and converted to cDNA using the SuperScript II Reverse Transcriptase kit from Invitrogen, according to the manufacturer’s instructions. cDNA from human umbilical vein endothelial cells, Wistar rat brain, and 3T3-L1 adipocytes was kindly provided by R. T. Urbanus (Department of Hematology, University Medical Center Utrecht, The Netherlands), I. W. M. Bos (Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, the Netherlands), and M. Bazuine (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands) and used as positive control for GLUT1, GLUT3 and GLUT4. To identify the distribution of GLUT subtypes the following degenerated oligonucleotide primers from conserved regions of human GLUT1, human and rat GLUT3, and human and mouse GLUT4 were designed: GLUT1, 5’-TCACTGT-GCTCTGTTGTCCTG-3’ (sense, positions human: 1489–1506) and 5’-CCTTGTCCTCGAGAGATTC-3’ (antisense, positions human: 1702–1721); GLUT3, 5’-AGTCGGTTGAAATGCTGAT-3’ (sense, positions human: 481–499 and rat: 428–446) and 5’-TAAGAACACAGCATGTACCC-3’ (antisense, positions human: 986–1006 and rat: 934–953); GLUT4, 5’-ATAGGACGTGGTGTTGTC-3’ (sense, positions human: 1124–1141 and mouse: 986–1003) and 5’-GCAAAAT-GAAAGGAAGACGA-3’ (antisense, positions human: 1487–1506 and mouse: 1349–1368). The resulting PCR products were 233 bp for GLUT1, 526 bp for GLUT3, and 383 bp for GLUT4.

The reaction was performed in 20 μl containing 2 μl of 10× PCR buffer II, 4 μl of MgCl₂ (25 mM), and 0.2 μl of AmpliTaq DNA polymerase (5 units/μl) from the GeneAMP kit obtained from Applied Biosystems (Foster City, CA), 1 μl of dNTP (dATP, dCTP, dGTP, and dTTP) of each 25 μM obtained from Amersham Biosciences, 6.8 μl of distilled water (9.8 μl for GLUT1), 2 μl of both sense and antisense primers (10 μM; 1 μl of each primer for GLUT1), and 2 μl cDNA for GLUT3 and GLUT4 (1 μl for GLUT1). After heating at 94 °C for 2 min, amplification proceeded for 43 (GLUT1) or 40 (GLUT3/4) cycles, with denaturation for 30 s at 94 °C, annealing of primers for 1 min at 62 °C (GLUT1) or 56 °C (GLUT3/4), and extension for 1 min at 72 °C. This was followed by a final extension step at 72 °C for 5 min. After the RT-PCR, 10 μl of the products was separated on a 2% agarose gel for GLUT1 and a 1% agarose gel for GLUT3 and GLUT4.

TaqMan-PCR—Real-time quantitative RT-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). Primers and TaqMan probes were designed with Primer Express software (Applied Biosystems, Foster City, CA). Primers designed for GLUT1 and GLUT3 amplified a 62-bp amplicon and a 63-bp amplicon, respectively, spanning the exon 4-exon 5 junction of each sequence. Both TaqMan probes were labeled with 6-carboxyfluorescein (FAM) as a reporter dye and tetramethylrhodamine (TAMRA) as a quenching dye. The following primers were used: GLUT1 forward: 5’-CAT CGT CGT CAT CCT-3’; GLUT1 reverse: 5’-CCT TGT TGC CCA TGA TGG A-3’; GLUT1 TaqMan probe: 5’-(FAM)-ATC GCC CAG GTT TTC-(TAMRA)-3’; GLUT3 forward: 5’-GTT GCC CCA CAT CTT TGG T-3’; GLUT3 reverse: 5’-CAG TAG CAG CGG CCA TAG C-3’; GLUT3 TaqMan probe: 5’-(FAM)-CTT CAG ACC

Chrono-Log Corp., Havertown, PA).
CAA GGA TGA-(TAMRA)-3'. The relative expression of GLUT1 and GLUT3 in cDNA of the aforementioned megakaryocytic cell lines was determined by comparing the cycles at which the reporter dye fluorescence surpassed an arbitrary threshold. The same amount of cDNA was used in each PCR reaction.

**Uptake of [3H]DOG by Platelets**—Washed platelets were resuspended in HEPES/Tyrode buffer, pH 7.25, with or without 5 mM glucose, which is referred to as low and high glucose, respectively. Glucose uptake by platelets was initiated by addition of [3H]DOG in the indicated concentrations prior to or after treatment with insulin or thrombin as outlined in the “Results.” Glucose transport was stopped by adding 5 ml of stop buffer (phosphate-buffered saline, with 10 μM cytochalasin B) as described previously (2, 3). Samples were centrifuged (3000 × g, 10 min, 20 °C), and the supernatant was replaced by 5 ml of stop buffer. After three washing steps, the supernatant was replaced with 500 μl of 0.1 M NaOH. The platelet pellet was dissolved overnight, and total protein content was determined to correct for variations in cell number. [3H]DOG uptake was determined in a liquid scintillation counter and expressed as arbitrary units (AU) defined as disintegrations per minute corrected for protein content (disintegrations per minute/μg of protein). Kinetic parameters for glucose transport were determined by a Hofstee plot with weighted linear regression analysis (30).

**Measurement of Ca2+ Mobilization**—Platelet-rich plasma was preincubated with 3 μM Fura-2 AM (45 min, 37 °C, light-protected). After incubation, platelet-rich plasma was supplemented with acid-citrate-dextran and prostaglandin I2, centrifuged again (330 × g, 15 min, 20 °C), and resuspended in HEPES/Tyrode buffer (pH 7.25) containing 5 mM glucose. The final platelet concentration was adjusted to 2.0 × 10^11 cells/liter. Fura-2 fluorescence was recorded in 1.0-ml aliquots of platelets without additional Ca2+ at 20 °C in a F-4500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and emission at 510 nm. Changes in [Ca2+]i were monitored using the Fura-2 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (31).

**Statistical Analysis**—Statistical analysis was performed using one-way analysis of variance with Tukey’s multiple comparisons test as post-test for repeated measurements unless stated otherwise. Results are expressed as means ± S.D. of five observations unless stated otherwise; ns indicates not significant. Differences were considered significant at p < 0.05. An asterisk indicates a significant difference with controls.

**RESULTS**

**ML-9 Inhibits Thrombin- and Insulin-induced PKB Phosphorylation**—As illustrated in Fig. 1 (A and B), thrombin induced a rapid phosphorylation of Ser473/4 confirming earlier observations (19). Interestingly, stimulation with insulin also initiated the phosphorylation of Ser473/4, illustrating that, in addition to inhibition of Ca2+ mobilization by interfering with P2Y12 signaling (16), ML-9 (100 μM) reduced P-selectin expression by 37.3% (Fig. 2A). Thus ML-9 was a better inhibitor of aggregation than secretion suggesting that apart from indirectly modulating aggregation via ADP release, PKB directly participates in the regulation of the aggregation response.

**Glucose Transport Supports Platelet Aggregation**—Apart from anaerobic and aerobic degradation of glucose as sources for resynthesis of metabolic ATP, platelets can maintain their energy content through oxidative phosphorylation of lipids (1). To assess the importance of glucose uptake in aggregation, platelets were preincubated with cytochalasin B and phloretin, which inhibit glucose transport by interfering with the intra- and extracellular glucose binding sites, respectively. These treatments induced a 30% fall in aggregation responses, illustrating that glucose transport is necessary for optimal platelet aggregation (Fig. 2, A and B).

**PKB Regulates Surface Expression of P-selectin and Platelet Aggregation**—Earlier studies showed that the uptake of glucose by platelets depends on an increase in GLUT3 surface expression that accompanies the secretion of α-granules (2, 3). To address the question whether PKB participates in the translocation of α-granules and thereby in the surface expression of GLUT3, the expression of P-selectin was measured, which is a second component of the α-granule membrane. There was a slight P-selectin expression on non-stimulated platelets, which was unaffected by ML-9. In thrombin-stimulated platelets, ML-9 (100 μM) reduced P-selectin expression by 37.3 ± 3.1% (Fig. 2A). These results suggest that PKB contributes to the secretion of α-granules thereby increasing surface expression of GLUT3.
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which is the major glucose transporter under control of insulin. To address this issue, GLUT subtype distribution was determined by RT-PCR in the megakaryocytic cell lines MEG-01, DAMI, and CHRF, which represent different stages in the maturation of normal megakaryocytes (26–28). Degenerated oligonucleotide primers against conserved sequence regions in GLUTs were used together with cDNA from the megakaryocytic cell lines, human umbilical vein endothelial cells, Wistar rat brain, and 3T3-L1 adipocytes. The primer combinations resulted in amplification of products with the expected sizes for GLUT1, GLUT3, and GLUT4. All megakaryocytic cell lines expressed GLUT1 and GLUT3 mRNA. No message was found for GLUT4 in the megakaryocytic cell lines in contrast to the positive controls (Fig. 4). The ratio of GLUT1 and GLUT3 mRNA was measured in the megakaryocytic cell lines MEG-01, DAMI, and CHRF by real-time quantitative RT-PCR using the TaqMan fluorogenic probe system. We observed 4.3 ± 0.2-, 2.6 ± 0.2-, and 13.2 ± 2.0-fold increases in GLUT3 mRNA in the MEG-01, DAMI, and CHRF cell lines, respectively, compared with GLUT1 mRNA (n = 3). These results suggest that platelets are deficient of GLUT4 and contain predominantly GLUT3.

Glucose Uptake by Platelets in Medium with Low Glucose—To investigate the involvement of PKB in glucose transport, platelets were preincubated in medium containing either 0 mM glucose or 5 mM glucose (defined as low and high glucose, respectively). Subsequent addition of 0.1 mM [3H]DOG would reveal the regulation of both GLUT1 (Km = 29.1 mM) and GLUT3 (Km = 1.5 mM) under conditions of low glucose and predominantly of GLUT1 alone under conditions of high glucose concentration. Although the kinetic parameters of GLUTs in platelets have not been established, current literature data show these characteristics to be constant among the different cell types (4–11). To study glucose transport at low glucose, platelets were isolated in a glucose-free medium and preincubated with [3H]DOG for 3 min. Subsequently, thrombin, insulin, or vehicle was added, and glucose uptake was measured during a subsequent 30-min incubation. Basal glucose transport increased during the first 10 min following addition of vehicle and then reached a plateau (Fig. 5A). Thrombin increased the glucose uptake almost 2-fold and reached a plateau after 30 min. A similar increase was found following addition of insulin, but there was a clear delay of about 10 min before glucose uptake increased. A plot emphasizing the increase induced by thrombin and insulin revealed the rapid stimulation by thrombin in contrast to the slow enhancement by insulin (Fig. 5A, inset). These data show that both thrombin and insulin stimulate glucose transport in platelets albeit with different kinetics.

Because we reported earlier that insulin inhibits thrombin-induced increases in Ca2+, which would slow down secretion and thereby the surface expression of GLUT3 (16), a possible inhibition of thrombin-induced glucose uptake by insulin was investigated. Platelets were preincubated with insulin for 5 min, stimulated with thrombin, and glucose uptake was assessed 30 min later. Separate addition of thrombin and insulin increased basal glucose uptake to a similar degree. Insulin did

FIGURE 2. PKB regulates surface expression of P-selectin and platelet aggregation. A, platelets were preincubated with ML-9 and stimulated with thrombin and expression of P-selectin was determined by flow cytometry. Thrombin-increased P-selectin expression was defined as 100%. ML-9 inhibited thrombin-induced P-selectin expression to 62.7 ± 3.1% (p < 0.001). B, incubation with different concentrations ML-9 inhibited thrombin-induced aggregation to 87 ± 15% at 25 μM (ns), 66 ± 14% at 50 μM (p < 0.05), and 22 ± 13% at 100 μM (p < 0.001), n = 5.

FIGURE 3. Glucose transport supports platelet aggregation. Platelets were preincubated with cytochalasin B (CB) or phloretin (Phl) and aggregation was initiated with thrombin. A, shown is a typical aggregation curve. B, cytochalasin B and phloretin inhibited thrombin-induced aggregation to 72.7 ± 2.5% (p < 0.001) and 71.7 ± 7.6% (p < 0.001), respectively. Shown is a representative example for three experiments.

FIGURE 4. GLUT subtypes in megakaryocytes. mRNA isolated from the megakaryocytic cell lines MEG-01, DAMI, CHRF-288-11 was subjected to RT-PCR analysis using degenerate primers corresponding to common sequence motifs of GLUT1, GLUT3, and GLUT4. The PCR products were size-fractionated on an agarose gel. Bands for GLUT1 and GLUT3 were found in all megakaryocytic cell lines. No band for GLUT4 was found. Shown are representative examples for five experiments.
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not inhibit the thrombin-induced glucose uptake (Fig. 5B). These data suggest that the decrease in GLUT3 expression caused by the expected inhibition of thrombin-induced Ca\(^{2+}\) signaling by insulin was compensated by a second mechanism that enhanced the uptake of glucose.

To understand the role of PKB in thrombin- and insulin-induced glucose uptake, platelets in the presence of 100 μM ML-9 were preincubated with \(^{3}H\)DOG for 3 min and thereafter stimulated with thrombin or insulin for 30 min. Inhibition of PKB reduced basal glucose uptake by ~30%. In thrombin-stimulated platelets ML-9 induced a 30% decrease that is 60% of the extra glucose uptake induced by thrombin. In insulin-treated platelets the inhibition was stronger and fully abolished the extra glucose uptake induced by insulin (Fig. 5C). Together, these results indicate that PKB contributes to glucose uptake both in resting platelets and in platelets stimulated with thrombin or insulin.

We reported earlier that in thrombin-stimulated platelets activation of PKB is mediated via Ca\(^{2+}\)-dependent protein kinase C subtypes (19). This property together with the Ca\(^{2+}\)-dependent secretion of α-granules would make the surface expression of GLUT3 strongly dependent on an increase in Ca\(^{2+}\). Fig. 6A shows that the Ca\(^{2+}\) chelator BAPTA-AM reduced the basal uptake by ~70%, illustrating that in resting platelets glucose uptake is Ca\(^{2+}\)-dependent. The additional uptake induced by thrombin was completely blocked by Ca\(^{2+}\) chelation. Concurrent analysis of P-selectin expression showed a 50% fall induced by BAPTA-AM, which is in agreement with a previous publication (34) (Fig. 6B). Thus, in addition to supporting secretion, Ca\(^{2+}\) contributes to glucose uptake via a second mechanism possibly reflecting the regulation of PKB.

Having established that Ca\(^{2+}\) was important for PKB signaling to secretion and glucose uptake, the possibility that PKB had an effect on the regulation of GLUT3 was investigated. Treatment with ML-9 did not change the Ca\(^{2+}\) level in resting platelets, and the thrombin-induced increase in Ca\(^{2+}\) was left undisturbed (Fig. 6C). These data suggest that Ca\(^{2+}\) homeostasis is insensitive to changes in PKB activity.

The observation that both thrombin, an inducer of GLUT3 surface expression through α-granule release, and insulin, known not to induce α-granule release, stimulate glucose uptake indicated that changes in surface expression and in affinity of the transporter for glucose contribute to uptake regulation. Kinetic analysis of the uptake of \(^{3}H\)DOG in concentrations ranging between 0.1 and 2 mM revealed a \(K_m\) for glucose of ~1.2 mM under conditions that both GLUT1 and GLUT3 were functional (Fig. 7 and TABLE ONE). Initial velocities were determined and plotted against substrate concentrations according to Woolls as advocated by Hofstee (30). Treatment with thrombin and insulin decreased the \(K_m\) for glucose transport to ~0.5 mM, indicating that both agonists induced a strong increase in the affinity of the transporter for glucose. In addition, thrombin induced a 1.5-fold increase in \(V_{max}\), probably reflecting the increase in surface expression of GLUT3. In contrast, insulin did not change the \(V_{max}\) in agreement with its inability to trigger secretion. Because GLUT1 is known to have a constant surface expression and is insensitive to regulation by intracellular signaling (35), the changes in kinetic parameters are likely to reflect the properties of GLUT3 (4–11).

Glucose Uptake by Platelets in Medium with High Glucose—To further separate the regulation of GLUT1 and GLUT3, platelets were preincubated in a medium with 5 mM glucose. This is ~4 times the \(K_m\) for glucose of GLUT3 making this transporter less susceptible to affinity regulation without disturbing the control of surface expression.

Platelets were preincubated with \(^{3}H\)DOG for 3 min, stimulated with thrombin and insulin, and glucose transport was monitored during a subsequent 30-min incubation (Fig. 8A). Basal glucose transport was constant throughout this period and remained the same upon addition of insulin. In contrast, thrombin induced about 3-fold increase in \(^{3}H\)DOG uptake. These results support the concept that an increase in glucose uptake at physiological glucose concentration is mainly mediated through an increase in surface expression of GLUT3. ML-9 and BAPTA-AM reduced glucose uptake by resting platelets as observed in a low glucose medium. In thrombin-stimulated cells both inhibitors completely blocked the extra glucose uptake induced by thrombin (Fig. 8B). These data illustrate the major role of PKB and Ca\(^{2+}\) in thrombin-induced surface expression of GLUT3.

To evaluate a possible inhibition by insulin under conditions that kept affinity changes in GLUT3 to a minimum, platelets were preincubated with insulin for 10 min to induce maximal inhibition of thrombin-induced Ca\(^{2+}\) increases (16). Again this treatment did not change the basal glucose uptake in a high glucose medium. Subsequent stimulation with thrombin increased \(^{3}H\)DOG uptake to 190.1 ± 7.0%. This increase was dose-dependently inhibited by insulin reducing thrombin-stimulated glucose transport to 18.8 ± 4.4% at 100 nM insulin (Fig. 8C).

To confirm that the reduced glucose uptake following preincubation with insulin was caused by inhibition of surface expression mediated via α-granule release, the expression of P-selectin was determined. As expected, insulin inhibited thrombin-induced P-selectin expression reaching 17.9 ± 4.2% inhibition at 100 nM insulin...
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![FIGURE 6. Thrombin-induced glucose transport in medium with low glucose. Platelets were isolated in a low glucose medium. A, platelets were preincubated with BAPTA-AM. Glucose transport was initiated by [3H]DOG addition followed by stimulation with thrombin. Thrombin-induced glucose uptake was expressed as 100%. BAPTA-AM inhibited basal glucose uptake (15.0 ± 7.5% and 47.9 ± 11.4%, respectively; n = 5, p < 0.05), and thrombin-induced glucose uptake (49.0 ± 10.8%, p < 0.001). B, platelets were preincubated with BAPTA-AM and stimulated with thrombin. Expression of P-selectin was determined by flow cytometry. BAPTA-AM did not modulate basal P-selectin expression compared with control (6.3 ± 3.2% and 6.1 ± 3.5%, respectively, n = 5, ns). C, platelets were preincubated with different concentrations ML-9. ML-9 (100 μM) did not change basal Ca²⁺ levels (35.5 ± 2.1 nM and 325 ± 3.5 nM, respectively; n = 5, ns) or thrombin-induced Ca²⁺ mobilization (101.2 ± 10.8% of control; n = 5, ns).](image)

![FIGURE 7. Effect of thrombin and insulin on glucose uptake. Platelets were isolated in a glucose-free medium. Shown is a representative Hofste plot of [3H]DOG transport of four experiments in platelets. A summary of all experiments of this type is presented in TABLE ONE.](image)

(Fig. 8D). Together, these data show that, at physiological glucose levels, glucose uptake is mainly regulated via changes in surface expression as a result of α-granule release. They also demonstrate that relative small changes in surface expression can be accomplished by large changes in glucose uptake.

To determine the contribution of GLUT1 to glucose uptake in platelets, platelets were incubated with different concentrations of glucose (0–30 mM) for 10 min, and glucose uptake was measured. At low glucose concentrations (0.5–2 mM), uptake was substantial. At high glucose concentrations (25–30 mM), uptake was close to the Kₘ of GLUT3. At 5–30 mM glucose, with 30 mM being close to the Kₘ of GLUT1, a slight increase in glucose uptake was observed. The results were analyzed using non-linear regression. They showed a slightly better fit with a two-site binding model (R² = 0.8079) than with a one-site binding model (R² = 0.8061) indicating the presence of two glucose transporters (Fig. 9). Thus, although a contribution of GLUT-1 cannot be excluded, these data show that glucose uptake by platelets is predominantly via GLUT3.

**PKB Does Not Regulate Platelet Inhibition by Insulin**—The inhibition of thrombin-stimulated glucose uptake by insulin at physiological glucose concentration (Fig. 8C) agrees with the inhibition of Ca²⁺ mobilization and aggregation by interference with signaling through P2Y₁₂ (16). Because insulin is also an activator of PKB, we investigated whether PKB plays a role in the effect of insulin on P2Y₁₂ signaling. Fig. 10 (A and B) shows that the adenyl cyclase inhibitor SQ22536 fully blocks the inhibition of insulin, such an effect also seen with the P2Y₁₂ antagonist ARC69931MX (16, 36) illustrating the importance of cAMP regulation through P2Y₁₂. Direct analysis of Ca²⁺ changes in thrombin-stimulated platelets confirmed the decrease induced by preincubation with insulin. This inhibition was unaffected by ML-9 (Fig. 10C). Thus, both thrombin-induced Ca²⁺ changes and the effect of insulin on that response are unaffected by PKB.

**DISCUSSION**

The present study shows that platelet stimulation with thrombin and insulin activate PKB, an enzyme intimately involved in the regulation of glucose uptake by insulin responsive tissues, such as adipocytes and muscle cells (15). Both agonists also stimulate the uptake of glucose by platelets. This has been demonstrated earlier for thrombin and shown to be caused by translocation of GLUT3 from α-granule membranes to the platelet surface upon induction of secretion (2, 3). The finding that also insulin increases glucose uptake is therefore surprising, because it is incapable of inducing α-granule release. On the other hand, these findings agree with observations in many insulin-responsive tissues where insulin triggers a strong, PKB-mediated, glucose uptake via signaling through PI3K (15). In adipocytes, glucose uptake by GLUT4 is up-regulated by an increase in surface expression, together with an increase in affinity for glucose (14).

Data in resting platelets reveal a role for PKB in basal glucose transport. The PKB inhibitor ML-9 induces a 30% reduction suggesting that the basal activity of PKB-Stat4 is phosphorylation is important for glucose uptake by resting platelets. In theory, this uptake is the result of glucose transport through both GLUT1, a ubiquitously expressed transporter in the PM, and through GLUT3 of which ~15% of the cellular pool is present in the PM. Because GLUT1 is insensitive to control by PKB (35) and the contribution to glucose uptake in platelets is minimal, the inhibition induced by ML-9 probably reflects the participation of GLUT3. Furthermore, the low Kₘ of basal glucose transport and 13-fold increase in GLUT3 mRNA compared with GLUT1 mRNA in the mature megakaryocytic cell line CHRF also support the predominant role of GLUT3 in glucose uptake by platelets.

The rapid increase in glucose uptake following stimulation by thrombin agrees with rapid activation of PKB and the immediate induction of GLUT3. The slower stimulation of glucose uptake by insulin agrees with the slower activation of PKB. Because insulin does not induce secretion, the increased uptake must reflect an affinity change in GLUT3. Indeed, insulin induced a 2-fold decrease in the Kₘ of glucose without changing the Vₘₚ₅. Apparently, insulin increases the GLUT3 affinity in platelets via a similar mechanism that induces the increase in GLUT4 affinity in adipocytes (14). GLUT3 is a 12-transmembrane (TM)-spanning protein and one has proposed that changes in key TM helices modulate glucose transport (37).

Studies on insulin-induced glucose transport in platelets are compli-
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The question whether in addition to an increase in GLUT3 expression, thrombin induces an affinity change was addressed by incubating platelets in a low glucose medium where glucose uptake depends both on GLUT1 and predominantly on GLUT3. Kinetic analysis in thrombin-stimulated platelets revealed both an increase in $V_{\text{max}}$ and an alteration in the $P_{\text{Km}}$ of glucose transport. To determine this change, a two-site binding model was used.

Table One

| Condition | $K_{\text{m}}$ (mM) | $p$ | $V_{\text{max}}$ (AU) | $p$ |
|-----------|---------------------|-----|-----------------------|-----|
| Control   | 1.22 ± 0.572        | <0.05 | 1                      | <0.05 |
| Insulin   | 0.51 ± 0.11         | <0.05 | 1.07 ± 0.24            | ns |
| Thrombin  | 0.48 ± 0.17         | <0.05 | 1.50 ± 0.42            | <0.05 |

a AU, arbitrary units.

b ns, not significant.

FIGURE 8. Glucose transport by platelets in medium with high glucose. Platelets were isolated in a medium containing 5 mM glucose. A, glucose transport was initiated with $[^{3}H]$DOG, and platelets were stimulated with thrombin and insulin. Glucose transport was monitored for 30 min. Basal glucose uptake was constant and not increased by insulin (21.8 ± 12.0 and 10.4 ± 4.4 AU, respectively; ns). Thrombin increased basal glucose uptake (57.8 ± 7.3 AU; p < 0.05). B, platelets were preincubated with ML-9 or BAPTA-AM and stimulated by thrombin for 10 min. Basal glucose uptake was expressed as 100%, ML-9 and BAPTA-AM inhibited basal glucose uptake to 58.2% (p < 0.001). ML-9 and BAPTA-AM inhibited thrombin-induced uptake to 95.8 ± 10.1% and 115.3 ± 12.2% (p < 0.001). C, platelets were preincubated with insulin and stimulated by thrombin. Basal glucose uptake was expressed as 100%. Insulin did not change basal glucose-uptake (100.7 ± 13.2%, ns). Thrombin increased basal glucose uptake to 190.1 ± 7.0% (p < 0.001). Insulin inhibited thrombin-induced glucose uptake to 169.7%, respectively. The increase in glucose uptake was pronounced in the low concentrations (0.5–30 mM) and continued for 10 min. Glucose transport at 0.5 mM was expressed as 100%. ML-9 and BAPTA-AM inhibited basal glucose uptake to 58.2 ± 8.0%, p < 0.05 and 62.7 ± 3.0%, p < 0.01, respectively. Thrombin increased basal glucose uptake to 133.8 ± 19.8% (p < 0.001). ML-9 and BAPTA-AM inhibited thrombin-induced uptake to 95.8 ± 10.1% and 115.3 ± 12.2% (p < 0.001).

FIGURE 9. Glucose uptake at different glucose concentrations. Platelets were isolated in a medium without glucose. Glucose transport was initiated with different $[^{3}H]$DOG concentrations (0.5–30 mM) and continued for 10 min. Glucose transport at 0.5 mM was expressed as 100%. Glucose uptake at 0, 0.5, 1, 2, 5, 10, 20, and 30 mM was 11.1 ± 0.8%, 100%, 132.3 ± 9.2%, 311.2 ± 83.8%, 371.2 ± 101.2%, 429.3 ± 104.8, 491.6 ± 150.5%, and 553.7 ± 169.7%, respectively. The increase in glucose uptake was pronounced in the low glucose concentration range (0.5–2 mM). Uptake at 1 mM glucose differed from uptake at 0.5 mM (p < 0.0001), and 2 mM from 1 mM (p < 0.0006). Glucose uptake leveled off at 5 mM glucose (p = 0.39). Uptake at 30 mM glucose did not differ from uptake at 5 mM (p = 0.16). The graph was fitted by non-linear regression using a two-site binding model ($R^2 = 0.8079$). Data are means ± S.D., n = 6.

cated by the fact that insulin is an inhibitor of Ca$^{2+}$ mobilization through interference with P2Y$_{12}$ signaling, which supports platelet activation upon release of ADP. Interference by insulin is absent in the absence of a secretion-inducing agents, and insulin alone neither changes the basal cAMP level nor Ca$^{2+}$ homeostasis (16). In experiments with co-stimulation by thrombin and insulin, one might therefore expect inhibition of thrombin-induced glucose uptake. In low glucose medium such an inhibition is absent suggesting that a possible decrease in GLUT3 surface expression is compensated by an increase in transport affinity. In high glucose medium, such an inhibition by insulin is apparent leading to a dose-dependent reduction in thrombin-induced glucose uptake. At this condition GLUT3 functions near saturation, indicating that the fall in glucose uptake reflects a fall in GLUT3 surface expression.

The question whether in addition to an increase in GLUT3 expression, thrombin induces an affinity change was addressed by incubating platelets in a low glucose medium where glucose uptake depends both on GLUT1 and predominantly on GLUT3. Kinetic analysis in thrombin-stimulated platelets revealed both an increase in $V_{\text{max}}$ and a decrease in the $K_{\text{m}}$ for glucose. Again, these properties likely reflect changes in GLUT3, because GLUT1 is not known to change its subcellular localization or its affinity to glucose (10, 11). Assuming that the surface expression of P-selectin is a reliable marker for expression of GLUT3 located in the $\alpha$-granule membrane, one might expect that changes in P-selectin expression and glucose transport go hand in hand.
PKB Regulates Glucose Transport in Platelets

This appears not to be the case. Compared with resting platelets, thrombin induced a 12-fold increase in P-selectin expression and only a 2-fold increase in glucose transport. Insulin inhibited thrombin-glucose uptake to levels of resting platelets while decreasing P-selectin expression by not more than 20%. A similar discrepancy between glucose uptake and GLUT expression has been observed for GLUT4 in muscle cells, which has been explained by assuming that additional factors modulate the full functional expression of GLUTs at the cell surface (14).

The characteristics of GLUT3 regulation in platelets resemble those of GLUT4 in adipocytes and muscle cells. In addition to being stimulated by insulin, GLUT3 and GLUT4 share the soluble N-ethylmaleimide-sensitive attachment protein receptors (SNAREs) that mediate the fusion of GLUT3 and GLUT4 with the PM (34, 38). These SNAREs are located in lipid rafts, which are detergent-resistant and cholesterol- and sphingolipid-rich membrane domains involved in important cellular processes such as signal transduction and intracellular trafficking (39). Interestingly, a major part of the proteins described in this study such as GLUT3 (39), the IR-IRS-1 complex (40), Gβ (39), and PKB (41) are associated with lipid rafts revealing a highly organized signaling complex that regulates glucose uptake.

Even though PKB is crucial in insulin-induced glucose uptake, inhibition of the kinase does not abolish the inhibitory properties of insulin. The PKB inhibitor ML-9 left thrombin-induced Ca2+ mobilization and its interference by insulin undisturbed. The inhibition of P2Y12 signaling through inactivation of Gβλ by insulin accords with the anti-lipolytic effect of insulin in 3T3-L1 adipocytes. Both effects are independent of signaling through PI3K but result from Gα2 inactivation by the IR-IRS-1 complex (16, 42). Thus, the signaling events triggered by insulin can be separated in those directed at the maintenance of glucose homeostasis, in which activation of PKB is crucial, and those directed at the regulation of platelet activity, in which the activity of Gβ plays a vital role.

A comparison between the effects of ML-9 on the different platelets responses learns that in addition to glucose uptake, PKB plays a role in α-granule release and aggregation. However, a concentration that induces complete inhibition of PKB (100 μM), leads to complete inhibition of aggregation but incomplete inhibition of glucose uptake and P-selectin expression. It is clear that in addition to roles in secretion and glucose uptake, which are both important for aggregation, PKB has other effects that directly contribute to the aggregation response. Examples are a role in regulation of integrin β3 activity (43) and re-assembly of the actin cytoskeleton (44). The fact that insulin activates PKB without inducing aggregation illustrates that PKB activation alone is an insufficient stimulus to induce platelet-platelet interaction.

A response that is clearly independent of PKB is the mobilization of Ca2+ induced by thrombin. PKB is activated via PI3K and Ca2+−dependent protein kinase C subtypes (19). Recent studies show that inhibition of PI3K with LY294002 inhibited Ca2+ mobilization (36) and that PI3K regulates the entry of Ca2+ in the endoplasmic reticulum (45). Further studies are therefore required to clarify the roles of PKB and its upstream regulators in more detail.

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FIGURE 10. PKB does not regulate platelet inhibition by insulin. A and B, washed platelets were preincubated with the adenylyl cyclase inhibitor SQ22536, and insulin and aggregation was initiated with thrombin. The aggregation curves shown are representative for five similar observations. Inhibition of thrombin-induced aggregation by insulin was absent in the presence of SQ22536. C, platelets were preincubated with insulin and ML-9, and Ca2+ mobilization was initiated with thrombin. Insulin inhibited Ca2+ mobilization to 75.2 ± 9.33% (p < 0.05). ML-9 did not affect Ca2+ mobilization in the absence (106.8 ± 6.5%; ns) or presence of insulin (68.8 ± 19.4%; p < 0.01). n = 5.
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