IRS-1 Mediates Inhibition of Ca\(^{2+}\) Mobilization by Insulin via the Inhibitory G-protein G\(_i\). *

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Platelet agonists initiate aggregation and secretion by activating receptors coupled to the G-protein Go\(_i\), thereby raising cytosolic Ca\(^{2+}\), [Ca\(^{2+}\)]. The rise in [Ca\(^{2+}\)] is facilitated via inhibition of cAMP formation by the inhibitory G-protein of adenylyl cyclase, G\(_i\). Since insulin attenuates platelet activation, we investigated whether insulin interferes with cAMP regulation. Here we report that insulin (0.5–200 nmol/liter) interferes with agonist-induced increases in [Ca\(^{2+}\)], (ADP, thrombin), cAMP suppression (thrombin), and aggregation (ADP). The effects of insulin are as follows: (i) independent of the P2Y\(_{12}\) receptor, which mediates ADP-induced cAMP lowering; (ii) not observed during G\(_i\)-mediated cAMP formation; (iii) unaffected by treatments that affect phosphodiesterases (3-isobutyl-1-methylxanthine); and (iv) not changed by interfering with NO-mediated regulation of cAMP degradation (N\(^\circ\)-monomethyl-L-arginine). Hence, insulin might interfere with G\(_i\). Indeed, insulin induces the following: (i) tyrosine phosphorylation of the insulin receptor, the insulin receptor sub- stance-1 (IRS-1) and G\(_{i0}\); (ii) co-precipitation of IRS-1 with G\(_{i0}\) but not with other G\(_\alpha\) subunits. Despite persistent receptor activation, the association of IRS-1 with G\(_{i0}\) is transient, being optimal at 5 min and 1 nmol/liter insulin, which is sufficient to suppress Ca\(^{2+}\) signaling by ADP, and at 10 min and 100 nmol/liter insulin, which is required to suppress Ca\(^{2+}\) signaling by thrombin. Epinephrine, a known platelet sensitizer and antagonist of insulin, abolishes the effect of insulin on [Ca\(^{2+}\)], tyrosine phosphorylation of G\(_{i0}\), and aggregation by interfering with the phosphorylation of the insulin receptor β subunit. We conclude that insulin attenuates platelet functions by interfering with cAMP suppression through IRS-1 and G\(_i\).

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Patients with diabetes mellitus have a 2–4-fold increased risk for coronary artery disease. They suffer from both microvascular (nephropathy and retinopathy) and macrovascular (peripheral artery disease) complications (1). Apart from increased concentrations of certain coagulation factors (2), patients with diabetes mellitus type I and II have platelets that show increased adhesion, aggregation, thromboxone production, and P-selectin expression (3). The hyperactivity might be caused by the absence of insulin inhibition, since intensive insulin treatment in diabetic patients reduced platelet aggregation (4).

The insulin receptor is a heterotetrameric transmembrane glycoprotein composed of two extracellular α subunits (135 kDa each) and two transmembrane β subunits (95 kDa each) that function as allosteric enzymes in which the α subunit inhibits the tyrosine kinase activity of the β subunit. Insulin binding to the α subunit relieves the inhibition of the kinase activity in the β subunit leading to autophosphorylation of the β subunits and a conformational change that further increases the kinase activity. The insulin receptor tyrosine kinase phosphorylates proteins such as Shc and the insulin receptor substrates IRS-1 (165–185 kDa) and IRS-2 (180–190 kDa). IRS-1 and IRS-2 have a highly conserved amino terminus, which contains a pleckstrin homology domain, a phosphotyrosine binding domain, and a carboxyl terminus with several tyrosine phosphorylation sites. IRS-1 and IRS-2 are complementary and act as “docking sites” to several Src homology 2 domains containing proteins, such as the regulatory subunits of phosphatidylinsosi- tol 3-kinase (PI3K) (5). GTP-binding proteins (G-proteins) can also act as signal transducers for the insulin receptor. G-proteins are guanine nucleotide-binding regulatory proteins that function as molecular switches between a GTP-bound “on state” and a GDP-bound “off state.” These proteins amplify, transmit, and integrate signals. The major G-proteins involved in platelet aggregation and secretion are G\(_{i}\), which mediates increases in cytosolic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)], and G\(_{i}\), which inhibits adenylyl cyclase thereby suppressing cAMP that is an inhibitor of platelets (6). Receptors that couple to G-proteins are generally seven-transmembrane proteins, but there are important exceptions. The insulin-like growth factor II receptor has a single transmembrane domain and couples directly to G\(_{i}\) in a manner similar to that of conventional G-protein-coupled receptors (7). Studies have been reported suggesting that the insulin receptor binds G\(_{i0}\) (8, 9).

The insulin receptor is present on muscle, liver, and adipose tissue but also on endothelial cells, lymphocytes, erythrocytes, and platelets. A human platelet contains ~570 insulin receptors (10). Insulin binding induces phosphorylation of the β subunits (11, 12), demonstrating that the receptor is func-

The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; PDEs, phosphodiesterases; IBMX, 3-isobutyl-1-methylxanthine; PRP, platelet-rich plasma; PG, prostaglandin; NO, nitric oxide.

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In insulin-resistant patients, platelet inhibition by insulin is attenuated or absent (13, 14), suggesting that in healthy individuals insulin signals to mechanisms that suppress platelet functions.

Many platelet agonists initiate aggregation and secretion of granule contents via $G_q$ which induces $Ca^{2+}$ release from intracellular storage sites and an increase in $[Ca^{2+}]_i$. A rise in $[Ca^{2+}]_i$ is a key step in platelet activation. It triggers granule

**Fig. 1. Insulin inhibits ADP-induced calcium mobilization.** Fura 2-AM-loaded platelets were incubated with and without insulin and stimulated with 10 μmol/liter ADP at 20 °C. **A**, curve a represents ADP-induced $Ca^{2+}$ mobilization in the absence of insulin. Curve b represents the $Ca^{2+}$ mobilization after preincubation with 1 nmol/liter insulin for 5 min. Insulin alone did not change the basal $[Ca^{2+}]_i$ (19.4 ± 2.7 nmol/liter). **B**, platelets were incubated with 1 nmol/liter insulin for 0–15 min prior to stimulation with ADP. The ADP-induced $Ca^{2+}$ mobilization (218.0 ± 72.8 nmol/liter) was expressed as 100% and taken as control. Insulin inhibited the ADP-induced $Ca^{2+}$ mobilization optimally when incubated for 5 min (25 ± 6%, $p < 0.001$). **C**, platelets were incubated for 5 min with different concentrations of insulin prior to stimulation with ADP. Insulin inhibited dose-dependently the ADP-induced $Ca^{2+}$ response at 0.5 nmol/liter and more ($p < 0.01$). Data (means ± S.D., $n = 5$) are expressed as percentages of control. The asterisk indicates a significant difference compared with controls ($p < 0.05$).
secretion thereby releasing ADP, activates the fibrinogen receptor (integrin αIIbβ3) forming aggregates, and induces a procoagulant surface that facilitates the formation of thrombin. One of the mechanisms that suppress platelet activation is an increase in cAMP. The inhibition is mediated by the cAMP-dependent protein kinase A. This kinase interferes with multiple steps in platelet activation cascades, such as receptor-ligand interaction, the activity of G-proteins, and the activation of phospholipase Cβ, protein kinase C, and mitogen-activated protein kinases. Protein kinase A also interferes with the elevation of [Ca2+]i, and inhibits actin-binding protein and caldesmon that are involved in cytoskeletal reorganization (15). Because a small rise in cAMP already leads to a strong activation of protein kinase A, platelet-activating sequences are extremely sensitive to increases in cAMP (16, 17).

Optimal platelet functions require maximal stimulation of the Gq pathway and the concomitant inhibition of cAMP production. cAMP is formed from ATP through the action of adenylyl cyclase and is subsequently metabolized by phosphodiesterases (PDEs). Adenylyl cyclase is inhibited by Gβγ which makes this G-protein a key factor in the control of cAMP formation. Gi is activated either by direct interaction with the agonist receptor or via secreted granule ADP, which activates the P2Y12 receptor via an extracellular feedback loop (18-21). Since Gi is involved in insulin signaling, we addressed the question whether insulin inhibits platelet functions by interfering with the activity of Gi.

EXPERIMENTAL PROCEDURES

Materials—Prosta-5,13-dien-1-oic acid, 6,9-epoxy-11,15-dihydroxy-, monosodium salt, [5Z,9Z,11α,13E,15Z] COOH (prostacyclin, PGI2 sodium salt) was obtained from Cayman Chemical (Ann Arbor, MI). Human recombinant insulin, Fura 2-AM, α-thrombin (thrombin in short), 3-isobutyl-1-methylxanthine (IBMX), epinephrine, and protease inhibitor mixture were obtained from Sigma. ADP was purchased from Roche Applied Science. N\(^{-}\)N\(^{-}\)-Monomethyl-l-arginine (l-NMMA) was supplied by Calbiochem. The ADP receptor P2Y\(_{12}\) antagonist, the ATP analogue N\(^{-}\)N\(^{-}\)-[2-(methylisobutyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylen]e ATP (AR-C69931MX), was a kind gift from Astra Zeneca (Loughborough, UK). All other chemical reagents were of analytical grade.

Antibodies—4G10 anti-phosphotyrosine was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), IRS-1 (C-20), G\(_{\alpha}\), I-20), and G\(_{\alpha}\) (T-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse immunoglobulin (GAMPO) were obtained from Dako A/S (Glostrup, Denmark). Anti-α-rabbit horseradish peroxidase was supplied by Cell Signaling Technology (Beverly, MA). Insulin receptor phospho-Tyr\(^{1158}\) antibodies were delivered by BIOSOURCE (Camarillo, CA). G\(_{\alpha}\) and G\(_{\alpha}\) antibodies were obtained from Calbiochem.

Platelet Isolation—All healthy, non-diabetic volunteers claimed not to have taken any medication 10 days prior to blood collection. After obtaining informed consent, freshly drawn venous blood was collected into 0.1 volume of 130 mmol/liter trisodium citrate. Citrated blood was centrifuged (150 \(\times\) g, 15 min, 20 °C), and the platelet-rich plasma (PRP) was collected and used for Ca2+ measurements in intact platelets with Fura 2-AM. For the preparation of washed platelets, PRP was supplemented with 0.1 volume of ACD (2.5% trisodium citrate, 1.5% citric acid, 2% d-glucose) for acidification to pH 6.5 and 0.001 volume of PGI\(_2\) (10 ng/ml final concentration), centrifuged again (330 \(\times\) g, 15 min, 20 °C), and resuspended in HEPES/Tyrode buffer (145 mmol/liter NaCl, 5 mmol/liter KCl, 0.5 mmol/liter Na\(_2\)HPO\(_4\), 1 mmol/liter MgSO\(_4\), 10 mmol/liter HEPES, pH 7.25) containing 5 mmol/liter d-glucose. The final platelet concentration was adjusted to 2.0 \(\times\) 10\(^{11}\) cells/liter. Prior to the experiments, platelets were kept at 20 °C for 45 min to ensure a resting state. In some experiments platelets were incubated with AR-C69931MX (50 mmol/liter, 30 s), IBMX (1 mmol/liter, 5 min), and l-NMMA (100 mmol/liter, 20 min) at 20 °C.

Measurement of Ca2+ Mobilization—PRP was incubated with 3 μM Fura 2-AM (45 min, 37 °C, light-protected). After incubation, PRP was acidified with ACD to pH 6.5, centrifuged again (330 \(\times\) g, 15 min, 20 °C), and resuspended in HEPES/Tyrode buffer (145 mmol/liter NaCl, 5 mmol/liter KCl, 0.5 mmol/liter Na\(_2\)HPO\(_4\), 1 mmol/liter MgSO\(_4\), 10 mmol/liter HEPES, pH 7.25) containing 5 mmol/liter d-glucose. The final platelet concentration was adjusted to 2.0 \(\times\) 10\(^{11}\) cells/liter. Fura-2 fluorescence was recorded in 1.0 ml aliquots of platelet suspension without additional Ca\(^{2+}\) 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 [Ca\(^{2+}\)] were monitored using the Fura-2 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (22).

Measurement of cAMP—Washed platelets were incubated at 20 °C. Samples were lysed in 0.33 volumes of 7% perchloric acid followed by centrifugation (11,000 \(\times\) g, 10 min, 4 °C). cAMP levels, [cAMP], were determined in a cAMP \(^{3}H\) assay system (Amersham Biosciences).

Immunoprecipitation—Washed platelets were incubated at 20 °C, and samples were collected in 10\(\times\) lysis buffer containing 1% w/v SDS, 5% w/v N-acyl glucoside, 0.5% SDS, and 10% v/v Nonidet P-40, with 10% v/v protease inhibitor mixture and 1 mmol/liter Na\(_2\)VO\(_3\). Detergent-insoluble material was sedimented by centrifugation for 1 min at

**Fig. 2. Effect of ADP receptor blockage on inhibition by insulin.** Fura 2-AM-loaded platelets were incubated for 5 min with and without 100 mmol/liter insulin prior to stimulation with 10 μmol/liter ADP in the absence and presence of 50 mmol/liter AR-C69931MX, an inhibitor of the P2Y\(_{12}\) receptor, at 20 °C. The ADP-induced Ca2+ mobilization was expressed as 100%. AR-C69931MX decreased the Ca2+ mobilization to 68 ± 13% (n = 3, p < 0.001) which is in the range of inhibition by insulin. A combination of insulin and AR-C69931MX did not induce further inhibition. Further details are in Fig. 1.
Immunoprecipitations were performed with 4G10 anti-phosphotyrosine, G_{i2} (T-19) or IRS-1 (C-20) antibodies. Immune complexes were collected with protein A-Sepharose, washed, solubilized in 3× sample buffer, and separated by SDS-PAGE on 12% gels.

Immunoblotting—Washed platelets were incubated at 20 °C, and samples were collected in 3× sample buffer. Aliquots were subjected to SDS-PAGE on 5% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in either 5% Protifar, 0.1% TBST or 4%

**Fig. 3. Insulin inhibits thrombin-induced calcium mobilization.** Fura 2-AM-loaded platelets were incubated with and without insulin prior to stimulation with 0.25 units/ml thrombin at 20 °C. A, curve a represents thrombin-induced Ca^{2+} mobilization. Curve b represents Ca^{2+} mobilization after preincubation with 100 nmol/liter insulin for 10 min. B, platelets were treated with 100 nmol/liter insulin for 0–20 min. The Ca^{2+} response by thrombin (592.6 ± 69.4 nmol/liter) was expressed as 100%. Insulin optimally inhibited the thrombin-induced Ca^{2+} response after 10 min of preincubation (25 ± 8%, p < 0.001). C, platelets were incubated for 10 min with different concentrations of insulin prior to stimulation with thrombin. Insulin dose-dependently decreased the thrombin-induced Ca^{2+} mobilization at 100 nmol/liter and more (p < 0.001). Further details are in Fig. 1.

4 °C. Immuno precipitations were performed with 4G10 anti-phosphotyrosine, G_{i2} (T-19) or IRS-1 (C-20) antibodies. Immune complexes were collected with protein A-Sepharose, washed, solubilized in 3× sample buffer, and separated by SDS-PAGE on 12% gels. **Immunoblotting—**Washed platelets were incubated at 20 °C, and samples were collected in 3× sample buffer. Aliquots were subjected to SDS-PAGE on 5% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in either 5% Protifar, 0.1% TBST or 4%
10 min with insulin prevented the fall in [cAMP] completely. Data are expressed as nanomoles of cAMP/10^11 platelets. Treatment with L-NMMA had no effect.

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the blots were subjected to the same procedure as described above.

Enhanced chemiluminescence with horseradish peroxidase-labeled

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the ADP-induced Ca^{2+} mobilization as insulin (100 nmol/liter). A combination of AR-C69931MX and insulin did not inhibit Ca^{2+} mobilization induced by thrombin. Stimulation with 0.25 units/ml thrombin induced a larger Ca^{2+} mobilization induced by thrombin. Simultaneous addition of insulin and ADP or incubation times of 15 min or longer failed to reveal the inhibition by insulin (Fig. 1B). To investigate the threshold above which insulin inhibited Ca^{2+} mobilization, platelets were incubated for 5 min with different concentrations of insulin followed by stimulation with ADP. There was a dose-dependent increase in the inhibition by insulin, which became significant at 0.5 nmol/liter insulin and more (Fig. 1C).

ADP activates platelets via the P2Y_{12} receptor, which is coupled to G_{i} and signals to [Ca^{2+}], while concurrently suppressing cAMP formation via the P2Y_{12} receptor and G_{i} (18). As shown in Fig. 2, the P2Y_{12} antagonist AR-C69931MX induced the same degree of inhibition of ADP-induced Ca^{2+} mobilization as insulin (100 nmol/liter). A combination of AR-C69931MX and insulin did not inhibit Ca^{2+} signaling stronger than each of these factors alone. Thus, insulin appears to inhibit ADP-induced Ca^{2+} mobilization by interfering with the regulation of G_{i}.

Insulin also inhibited Ca^{2+} mobilization induced by thrombin. Stimulation with 0.25 units/ml thrombin induced a larger increase in [Ca^{2+}], than 10 nmol/liter ADP, and more insulin was required to inhibit this response. When platelets were incubated with 100 nmol/liter insulin prior to stimulation with 0.25 units/ml thrombin, a 25 ± 8% fall in the Ca^{2+} response
IBMX, an inhibitor of PDEs. IBMX raised the basal [cAMP] to interfere with cAMP degradation, platelets were treated with addition of PGI2 (10 ng/ml) raised [cAMP] to 27.3. * appeared after 10 min of preincubation with insulin (Fig. 4). To investigate whether insulin interfered with the rise thrombin were added simultaneously and completely disappeared when insulin (100 nmol/liter) and thrombin (0.25 units/ml) induced a 60% decrease of basal cAMP, as in agreement with a previous publication (23). This reduction was smaller when insulin (100 nmol/liter) and thrombin were added simultaneously and completely disappeared after 10 min of preincubation with insulin (Fig. 4A). Addition of PGI2 (10 ng/ml) raised [cAMP] to 27.3 ± 2.0 nmol/10¹¹ platelets in 10 min. A similar rise was found when platelets were preincubated for 10 min with insulin (1, 10, and 100 nmol/liter). Thus, insulin failed to interfere with the activation of adenylyl cyclase by PGI2 (Fig. 4B). To assess whether insulin interfered with cAMP degradation, platelets were treated with IBMX, an inhibitor of PDEs. IBMX raised the basal [cAMP] to 12.5 ± 2.9 nmol/10¹¹ platelets in 5 min, and this effect was not disturbed by insulin. Again, thrombin interfered with the rise in cAMP, and insulin abolished the effect of thrombin. Thus, insulin only interfered with cAMP regulation in the presence of an agonist that activates Gt (Fig. 4C). Earlier studies with platelets suspended in plasma suggested that insulin inhibited platelets by raising [cAMP] (24). This effect was attributed to insulin-induced formation of nitric oxide (NO) via NO synthase and subsequent inhibition of PDE3b. To investigate whether a similar mechanism was present in isolated platelet suspensions, platelets were treated with L-NMMA, an inhibitor of NO synthase. L-NMMA did not change the basal [cAMP] or the rise induced by PGI2. Also the suppression of the [cAMP] increase induced by thrombin was left undisturbed. Again, insulin interfered with the fall in cAMP induced by thrombin, and this effect was the same in the absence and presence of L-NMMA (Fig. 4D). These data argue against a role for NO-mediated cAMP control in the present studies.

**Insulin Increases the Tyrosine Phosphorylation of Gtα2**—To address the question whether insulin interfered with the regulation of cAMP formation via Gt, immunoprecipitation studies were performed using an anti-Gtα2 antibody, and the tyrosine phosphorylation of Gtα2 on Western blot was measured using a 4G10 anti-phosphotyrosine antibody on blots with a Gtα2 antibody (Fig. 5B). Treatment of platelets with 1 nmol/liter insulin induced a transient increase in the tyrosine phosphorylation of Gtα2, with an optimal effect between 2 and 5 min (Fig. 5, A and C). These results were obtained in immunoprecipitates with 4G10 anti-phosphotyrosine antibody on Western blot with a Gtα2 antibody (Fig. 5B). A 5-min incubation period with increasing concentrations of insulin (0.5, 1, and 10 nmol/liter) showed a dose-dependent increase in the tyrosine phosphorylation of Gtα2 (see below). These results suggest that insulin interfered with the fall in cAMP via tyrosine phosphorylation of Gtα2.

**IRS-1 Co-precipitates with Gtα2**—To investigate whether IRS-1 played a role in insulin signaling to Gtα2, platelets were

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**Fig. 5. Insulin increases the tyrosine phosphorylation of Gtα2.** Washed platelets were treated with 1 nmol/liter insulin. At the indicated times, cells were lysed, and samples were collected for immunoprecipitation (IP) and Western blotting (WB). A, Gtα2 was immunoprecipitated with a 4G10 anti-phosphotyrosine or anti-Gtα2 antibody. The figures are representative for three observations with similar results. B, tyrosine-phosphorylated proteins were immunoprecipitated with 4G10 anti-phosphotyrosine followed by immunoblotting with an antibody against Gtα2. C, bands were scanned and quantified with ImageQuant software. Data are expressed as percentage of control. A dose of 1 nmol/liter insulin increased the tyrosine phosphorylation of Gtα2, being optimal at 2–5 min of incubation (281 ± 106 and 276 ± 58%, respectively, n = 3). Further details as in Fig. 1.
The co-association between IRS-1 and Gi protein increased at 100 nmol/liter insulin. Weak and short interaction at 1 nmol/liter insulin and a strong, persistent interaction at 100 nmol/liter insulin. The association depends on the insulin concentration showing a direct binding to the G-protein subunit and demonstrates that an antibody against phospho-Tyr 1158 of the insulin receptor.

IRS-1 Mediates Platelet Inhibition by Insulin

The transient nature of the tyrosine phosphorylation of the β subunit (Fig. 7A). Time courses over a 15-min incubation period showed that 1 nmol/liter insulin induced an increase in β subunit phosphorylation that reached a plateau after 5 min and that 100 nmol/liter insulin induced a 5-fold stronger phosphorylation that reached a plateau after 25 min (Fig. 7B and C and not shown). Importantly, there was no indication of receptor dephosphorylation during this period at either insulin concentration. Concurrent analysis of IRS-1 showed a dose-dependent phosphorylation induced by insulin. Both at 1 and 100 nmol/liter insulin, this phosphorylation was transient showing an optimum between 5 and 10 min and decreasing to pre-stimulating values after 15 min. Thus, the transient phosphorylation of IRS-1 and Gαs was not caused by receptor inactivation but was the result of interference with insulin signaling at a step downstream of receptor activation and upstream of the formation of an IRS-1-Gαs complex.

Effect of Epinephrine on Insulin-induced Platelet Inhibition—Epinephrine is known to enhance the sensitivity of platelets to activating agents by reducing the level of cAMP (23, 25, 26) and to antagonize the effect of insulin in rat skeletal muscle by decreasing the IRS-1-associated activity of PI3K (27). To investigate whether epinephrine interfered with the effects of insulin on [Ca2+]i, platelets were incubated for 5 min with insulin (1 and 10 nmol/liter), and epinephrine (10 μmol/liter) was added 1 min prior to stimulation with ADP. Epinephrine decreased ADP-induced Ca2+ mobilization by about 20% (Fig. 8A). The lower Ca2+ increase in the presence of insulin (1 and 10 nmol/liter) completely normalized in the presence of epinephrine. Thus, epinephrine abolished the inhibition by insu-
The measured. Insulin increased the tyrosine phosphorylation of induced a dose-dependent increase in G_\text{i} \text{subunit}, which was abolished by the addition of epinephrine. Preincubation with sodium vanadate, an inhibitor of protein-tyrosine phosphatases, partially reversed the effects of insulin received tyrosine phosphorylation of the insulin receptor at Tyr^{1158} was dose-dependent. To investigate whether this effect of insulin on the decrease in cAMP was measured in platelets preincubated with increasing concentrations of insulin (100 nmol/liter) and cells were lysed at the indicated times for Western blotting (WB) for insulin receptor phospho-Tyr^{1158} and IRS-1 immunoprecipitation (IP). IRS-1 immunoprecipitates were subjected to Western blotting for 4G10 anti-phosphotyrosine. Bands were scanned and quantified, and curves were fitted with non-linear regression. The intensity of the bands at 5 min of incubation and 1 nmol/liter insulin was expressed as 100 arbitrary units (indicated with open square). Insulin receptor phospho-Tyr^{1158} was optimal after an incubation of 15 (1 nmol/liter insulin) and 25 min (100 nmol/liter insulin) and at 10 min and 100 nmol/liter insulin. Tyrosine phosphorylation of IRS-1 had disappeared completely after 15 min of incubation. Further details are in Fig. 5.

Effect of Insulin on Platelet Aggregation in PRP—To address the question whether this mechanism was also functional in PRP, an aggregation assay was performed at 37 °C. PRP was preincubated for 2 min with 1 nmol/liter insulin and 10 μmol/liter epinephrine was added 1 min prior to initiation of aggregation with 10 μmol/liter ADP. Epinephrine enhanced the aggregation response to ADP. Insulin inhibited ADP-induced platelet aggregation without affecting the shape change response. The addition of epinephrine neutralized the inhibition of insulin such that the difference in platelet aggregation proved not to be significant (Fig. 9). These results illustrate that platelet inhibition by insulin is also present in PRP.

Sensitivity of Ca^{2+} Increases to Inhibition by cAMP—As illustrated in Figs. 1 and 3, ADP-induced Ca^{2+} rises were inhibited following a short (about 5 min) incubation with 1 nmol/liter insulin. In contrast, inhibition of thrombin-induced Ca^{2+} rises required a longer (about 10 min) incubation with a higher dose of insulin (100 nmol/liter). To investigate whether this difference was caused by a factor downstream of the formation of cAMP, Ca^{2+} rises induced by ADP and thrombin were measured in platelets preincubated with increasing concentrations of PGI_2. As shown in Fig. 10, Ca^{2+} rises induced by 10 μmol/liter ADP were strongly inhibited by small increases in PGI_2 leading to complete inhibition at 1 ng/ml PGI_2. In contrast, Ca^{2+} rises induced by 0.25 units/ml thrombin were resistant to these PGI_2 concentrations, although at higher concentrations (10 ng/ml) complete inhibition was observed.
When the thrombin concentration was lowered to the range where a similar Ca$^{2+}$/H$^{+}$ increase was found as induced by ADP, both responses were equally sensitive to PGI$_2$. The presence of the P2Y$_{12}$ receptor blocker AR-C69931MX led to a 45% fall in Ca$^{2+}$/H$^{+}$ response illustrating a major contribution of secreted ADP in thrombin-induced Ca$^{2+}$/H$^{+}$ rises. Collectively, these data indicate that the differences in preincubation time and insulin concentration required for inhibition of Ca$^{2+}$/H$^{+}$ rises reflect the weaker activation by ADP compared with a more persistent activation by thrombin in combination with granule-released ADP.

**DISCUSSION**

The present study reveals a novel mechanism by which insulin inhibits the responsiveness of platelets for activating agents. The decrease in responsiveness is illustrated by a 17% lower ADP-induced Ca$^{2+}$/H$^{+}$ mobilization (0.5 nmol/liter insulin) and a 25% lower thrombin-induced Ca$^{2+}$/H$^{+}$ mobilization (100 nmol/liter insulin). The Ca$^{2+}$/H$^{+}$ mobilization induced by thrombin is substantially larger than the ADP-induced Ca$^{2+}$/H$^{+}$ mobilization, and consequently more insulin is required to inhibit the rise in [Ca$^{2+}$/H$^{+}$]. In addition, there is the release of ADP from thrombin-stimulated platelets which contributes to the increase in [Ca$^{2+}$/H$^{+}$]. The effect of insulin is transient and depends on association and tyrosine phosphorylation of IRS-1 and G$_i$. Apparently, the result is loss of G$_i$ activity as expressed by an impaired reduction of cAMP and a weaker Ca$^{2+}$/H$^{+}$ response than observed in the absence of insulin. Interestingly, epinephrine, an activator of G$_i$ proteins and inhibitor of IRS-1/PI3K activity in rat skeletal muscle (27), abolishes the effect of insulin on cAMP regulation, Ca$^{2+}$/H$^{+}$ mobilization, and aggregation. It also abolishes the insulin-induced tyrosine phosphorylation of G$_i$, again suggesting that tyrosine phosphorylation of G$_i$ leads to inhibition of the G-protein. Epinephrine abolishes insulin signaling in platelets by interfering with the phosphorylation of the insulin receptor $\beta$ subunit. ADP is known to activate platelets via the P2Y$_1$ receptor,
which is coupled to Go and signals to Ca\(^{2+}\) mobilization, aggregation, and secretion. These responses are facilitated by concurrent binding of ADP to the P2Y\(_{12}\) receptor, which is coupled to Go and suppresses cAMP formation (18). Thrombin activates platelets by binding to members of the protease-activated receptors 1 and 4. It is a potent inducer of secretion of dense granule contents, leading to liberation of ADP and subsequent activation of the P2Y\(_{12}\) receptor (28). Our present findings are in accord with this concept and show qualitatively similar effects of insulin on Ca\(^{2+}\) mobilization by ADP and thrombin. The differences in dose inhibition studies of insulin for ADP- and thrombin-induced Ca\(^{2+}\) mobilization reflect the stronger and more persistent activation by 0.25 units/ml thrombin compared with 10 \(\mu\)mol/liter ADP. Consequently, the fall in cAMP is also stronger with thrombin than with ADP, thus making it possible to evaluate interference by insulin. It was impossible to analyze the effect of insulin on cAMP in platelets stimulated with ADP or with the low thrombin (0.08 units/ml) concentration, but in view of the similarities with the effects induced by the high thrombin concentration a similar mechanism is likely to be operational.

For inhibition of ADP-induced Ca\(^{2+}\) mobilization a 5-min preincubation with 1 nmol/liter insulin was sufficient. For inhibition of Ca\(^{2+}\) mobilization induced by a relatively high thrombin concentration (to reveal the effect of insulin on cAMP), a longer preincubation with a high insulin concentration was required. These differences are reflected in the coassociation of IRS-1 with Go\(_{\alpha}\). Under conditions that interfered with ADP signaling, IRS-1-Go\(_{\alpha}\) interaction was optimal after 5 min and rapidly declined thereafter. Under conditions that interfered with thrombin signaling, the association was more pronounced and lasted longer. Tyrosine phosphorylation of Go\(_{\alpha}\) correlated with binding of IRS-1 to Go\(_{\alpha}\). Thus, differences in Ca\(^{2+}\) inhibition were the result of differences in the binding of IRS-1 to Go\(_{\alpha}\) and the resulting tyrosine phosphorylation of the Go subunit caused by this association.

The catalytic loops within the tyrosine kinase domain of the insulin receptor contain the three tyrosine motifs Tyr\(^{1158}\), Tyr\(^{1162}\), and Tyr\(^{1163}\). The general concept is that autophosphorylation within the activation loop of the insulin receptor involves the initial phosphorylation of Tyr\(^{1162}\) followed by Tyr\(^{1158}\) and Tyr\(^{1163}\), upon which the insulin receptor becomes fully active. Insulin induced a dose-dependent phosphorylation of the receptor \(\beta\) subunit, which reached a plateau after 5 (1 nmol/liter insulin) to 25 min (100 nmol/liter insulin). These kinetics differ strongly with the transient nature of the phosphorylation of IRS-1, the formation of an IRS-1-Go\(_{\alpha}\) complex, and the phosphorylation of Go\(_{\alpha}\). Apparently, there is a crucial role for a tyrosine phosphatase that dephosphorylates IRS-1. It is known from animal studies that disruption of the gene encoding PTP1B leads to a state of increased insulin-dependent tyrosine phosphorylation of the insulin receptor and IRS proteins (5), suggesting that a single phosphatase controls the phosphorylation state of both proteins. In addition, both the insulin receptor and IRS proteins undergo serine phosphorylation, which may attenuate signaling by decreasing the tyrosine phosphorylation. Several kinases have been implicated in this process, including PI3K, protein kinase B, protein kinase
C, glycogen synthase kinase-3, and mammalian target of rapamycin (5). Similar mechanisms may operate in platelets with the important restriction that they leave receptor phosphorylation undisturbed.

Trovati et al. (24) reported earlier that platelets suspended in plasma are inhibited by insulin through a rise in cAMP. Also cAMP production induced by the stable PGI₂ analogue iloprost and the adenyl cyclase activator forskolin was enhanced by insulin. The effect was attributed to insulin-induced production of NO, which would activate guanylyl cyclase and raise cGMP. In turn, cGMP would inhibit cAMP degradation by inhibiting PDE3b. Our present studies based on platelets suspended in buffer do not support these observations. First, inhibition of [Ca²⁺], increases caused by stimulation of cAMP production (PGI₂) or inhibition of its degradation (IBMX). Instead, inhibition by insulin only becomes apparent during agonist-stimulated activation of Gᵢ, indicating that it is restricted to conditions where Gᵢ is activated by the PZ₂₁₂ Receptor. Other reports already indicated that Gᵢ might play an important role in the signaling effects by insulin. Studies in mice with genetically compromised Gᵢσ₂ expression showed hyperinsulinemia, impaired glucose tolerance, and resistance to insulin, which are characteristic for diabetes mellitus type II. In addition, there was abolished counterregulation of lipolysis by insulin, insulin-stimulated glucose transport and recruitment of GLUT-4, impaired insulin-stimulated tyrosine phosphorylation of IRS-1, and an elevated cellular phosphothreonine phosphatase activity (29). In human adipocytes the synergistic activation of NADPH-dependent H₂O₂ generation in vitro by Mn²⁺ and insulin was mediated by a co-association of the insulin receptor with Gᵢσ₂ (30).

Epinephrine enhances platelet activation by other agonists via binding to ω₁₆-adrenergic receptors and G-α-mediated inhibition of adenyl cyclase (31). The Gᵢ family includes the ubiquitously expressed Gᵢ₁₂,2,3 as well as several members with a more restricted expression, such as Gᵢ₆. There are ample evidence that Gᵢσ₂ is a major mediator in epinephrine-induced adenyl cyclase inhibition (31, 32), but recent evidence suggests that also Gᵢ₂ contributes to cAMP control. In Gᵢ₂ knockout mice other Gᵢ proteins can functionally replace Gᵢ₂-mediated signal transduction (21). Comparisons between wild type and Gᵢ₂ knockout mice reveal a role for both Gᵢ and other Gᵢ members in the regulation of cAMP (25). Thus, it is important to establish the relative contributions of the Gᵢ members in cAMP regulation with respect to the inhibitory role of insulin. In rat skeletal muscle epinephrine suppresses insulin-induced glucose uptake by decreasing the IRS-1 associated activity of PI3K (27). By analogy, IRS-1 might be a target for epinephrine in platelets especially since IRS-1 can bind to Gᵢ proteins via pleckstrin homology domains (33). The present data show that instead of interfering with IRS-1, epinephrine interferes with the phosphorylation of the insulin receptor, thereby preventing tyrosine phosphorylation of Gᵢα and attenuating the rise in [Ca²⁺].

Patients with a defect in the PZ₂₁₂ receptor have an increased tendency to bleed, indicating that suppression of cAMP is vital for normal hemostasis. The present study reveals a similar but transient modulation of cAMP regulation by insulin. The inhibition of Gᵢ activity by insulin is in the same range as found with a PZ₂₁₂ receptor antagonist and results in a decrease in Ca²⁺ mobilization of about 20% (34) and reduced adhesion and aggregation (18, 19). Conversely, one might speculate that the hyperresponsiveness of platelets in diabetes mellitus type I and II illustrates the absence of the platelet inhibition by insulin. These findings illustrate the importance of Gᵢ-mediated suppression of cAMP accumulation for optimal platelet function in vivo.

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IRS-1 Mediates Inhibition of Ca\(^{2+}\) Mobilization by Insulin via the Inhibitory G-protein G\(_i\)

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