Regulation of glycogen synthase kinase 3 in human platelets: a possible role in platelet function?[1]

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Abstract In this study we show that both glycogen synthase kinase 3 (GSK3) isoforms, GSK3α and GSK3β, are present in human platelets and are phosphorylated on Ser^{21} and Ser^{9}, respectively, in platelets stimulated with collagen, convulxin and thrombin. Phosphorylation of GSK3α/β was dependent on phosphoinositide 3-kinase (PI3K) activity and independent of platelet aggregation, and correlated with a decrease in GSK3 activity that was preserved by pre-incubating platelets with PI3K inhibitor LY294002. Three structurally distinct GSK3 inhibitors, lithium, SB415286 and TDZD-8, were found to inhibit platelet aggregation. This implicates GSK3 as a potential regulator of platelet function.

Key words: Platelet; Platelet activation; Platelet aggregation; Glycogen synthase kinase 3; Phosphoinositide 3-kinase; Protein kinase B

1. Introduction

Platelets play a pivotal role in haemostasis. Upon damage to the vascular wall platelets are exposed to extracellular matrix proteins, such as collagens, to which they adhere and aggregate together to form an haemostatic plug. Effective platelet responses are ensured through positive feedback mechanisms, due to the activity of molecules released by activated platelets. Precise regulation of platelet function is essential to prevent inappropriate activation of platelets and the occurrence of thrombosis.

Central to platelet activation are a number of cell surface receptors, including the collagen receptors, glycoprotein VI (GPVI) [1,2] and integrin $\alpha_2\beta_1$ [3], the thrombin receptors, PAR1 and PAR4 [4], and the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$ [5], amongst others. The integrin $\alpha_2\beta_1$ and GPVI act in concert to support the adhesion of platelets to collagen [6]. Collagen binding to GPVI results in receptor clustering and tyrosine phosphorylation of the Fc receptor γ-chain, an associated transmembrane protein. This results in the recruitment of signalling molecules leading to the activation of multiple signalling pathways that culminate in platelet shape change, secretion and aggregation [7,8]. Thrombin, generated at sites of tissue damage, is also a potent platelet agonist [4]. Although collagen and thrombin activate different receptor types, they share a number of common signalling components including the mobilisation of calcium from intracellular stores and the activation of phosphoinositide 3-kinase (PI3K) [9]. Despite the importance of PI3K in platelet regulation the functionally important effectors that lie downstream of PI3K in platelets are not understood. Protein kinase B (PKB/Akt), a known effector of PI3K, is regulated by a number of platelet agonists, including collagen, thrombin and thrombopoietin [10,11]. The potential importance of PKB in platelet function led us to examine a known substrate in other cell systems, glycogen synthase kinase 3 (GSK3).

GSK3 is a widely expressed cytoplasmic serine/threonine protein kinase. It exists as two highly homologous isoforms, GSK3α (51 kDa) and GSK3β (46 kDa), that are encoded by two different genes but are regulated in a similar manner [12]. Originally identified as one of several protein kinases that can phosphorylate glycogen synthase, GSK3 has become recognised to play a role in the regulation of many cell functions [12].

In this study we demonstrate the presence of both isoforms of GSK3 in human platelets and show regulation of GSK3 in response to a number of platelet agonists. We show that this leads to the phosphorylation of both GSK3α and GSK3β, the kinetics of which mirror that of the phosphorylation of PKB. Phosphorylation of GSK3 correlates with inhibition of its enzymic activity, which is also PI3K dependent. Furthermore, investigation of the effects of selective GSK3 inhibitors implicates GSK3 in the regulation of platelet function.

2. Materials and methods

Anti-phospho-GSK3α/β, anti-PKB and anti-phospho-PKB (Ser^{473}) antibodies were purchased from New England Biolabs (Hertfordshire, UK). Anti-GSK3α and anti-GSK3β antibodies and GSK3 substrate peptide 2 were purchased from Upstate Biotechnology (TCS Biologicals, Buckingham, UK). Horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Buckinghamshire, UK). LY294002, wortmannin and TDZD-8 were from Calbiochem (CN Biosciences, Nottingham, UK), Bradford protein assay reagent was from Bio-Rad (Hertfordshire, UK), and SB415286 was from Tocris (Bristol, UK). All other reagents were from previously described sources [13].

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Abbreviations: GSK3, glycogen synthase kinase 3; GPVI, glycoprotein VI; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; DMSO, dimethyl sulphoxide

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2.1. Preparation and stimulation of platelets

Fresh human platelets were obtained from volunteers, prepared as described previously [14] and resuspended in Tyrodes–HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) containing 10 μM indomethacin to a density of 8×10^8 cells/ml. Platelets (450 μl) were stimulated with collagen, convulxin, or thrombin at 37°C in an aggregometer with continuous stirring (1200 rpm). For inhibitor studies platelets were incubated with the appropriate inhibitor [dissolved in dimethyl sulphoxide (DMSO), final concentration $<0.2\%$ (v/v)] for 15 min prior to stimulation. Where stimulations were performed under non-aggregating conditions, platelets were pre-incubated for 30 min with 1 mM EGTA.

2.2. Immunoblotting

Platelet stimulation was stopped by the addition of Laemmli sample buffer, proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes by Western blotting. After blocking by incubation in Tris-buffered saline-Tween [TBS-T; 20mM Tris, 137 mM NaCl, 0.1% (v/v) Tween 20, pH7.6] containing 5% (w/v) milk protein, membranes were incubated overnight at 4°C with primary antibody (1 μg/ml) in TBS-T containing 5% (w/v) milk protein. Subsequent to washing in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (0.1 μg/ml) in 5% (w/v) milk protein in TBS-T, and then washed with TBS-T. Membranes were incubated with ECL substrate reagent and exposed to X-ray film.

2.3. Immunoprecipitation

Cells were lysed using an equal volume of RIPA buffer [200 mM NaCl, 62.5 μM Na deoxycholate, 50 mM Tris, 0.5% (w/v) SDS, 0.5% (v/v) Nonidet P40, pH 7.4 containing 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μM okadaic acid and 2 mM Na_3VO_4] and incubated on ice for 15 min. Clarified supernatants were pre-cleared by incubation with protein A Sepharose [PAS; 20 μl of a 50% (v/v) suspension in TBS-T] for 30 min. Following removal of the PAS, 1 μg of the appropriate antibody was added to each sample and incubated for 1 h before the addition of 40 μl PAS. After a 1 h incubation PAS beads were washed three times in TBS-T, and Laemmli sample buffer added.

2.4. GSK3 kinase assay

GSK3 activity was measured in platelet lysates using the method described by Ryves et al. [15]. Platelets were stimulated for 5 min in the presence or absence of LY294002. The reaction was stopped by the addition of ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% (v/v) Triton X-100, containing 10 μM dithiothreitol (DTT), 100 nM okadaic acid, 0.5 mM Na_3VO_4, 1 mM benzamidine, 1 mM PMSF, 1μg/ml pepstatin and 1 μg/ml leupeptin]. Protein concentrations were determined using the Bradford microassay, and 10 μg of protein used for each kinase assay. To a duplicate set of samples 50 mM LiCl was added. Subsequently, 40 μM substrate peptide (derived from glycogen synthase; YRRAAVPPSPSLSRHSSPHQpSEDEEE) was added to each tube and the reaction started by addition of 3 μCi [^{32}P]ATP in kinase buffer [50 mM Tris–HCl (pH 7.5), 100 μM ATP, 12.5 mM $MgCl_2$, 0.5 mM EGTA and 2 mM DTT]. After 10 min incubation at 30°C the reaction was stopped by spotting 75% of the reaction mix onto prelabelled P81 phosphocellulose paper. The paper was washed repeatedly with 75 mM phosphoric acid and then allowed to dry for 1 h. ^{32}P incorporation into the GSK3 substrate was quantified by scintillation spectrometry of the immobilised peptide.

2.5. Aggregation studies

Platelet aggregation was determined by optical aggregometry (Chronolog Corp., Havertown, PA, USA) at 37°C and with constant stirring.

3. Results

3.1. GSK3α and GSK3β are present in human platelets

Immunoblot analysis (Fig. 1) shows that both GSK3α and GSK3β are present in human platelet cell lysates, migrating on SDS–PAGE with apparent molecular masses of 51 and 46 kDa, respectively.

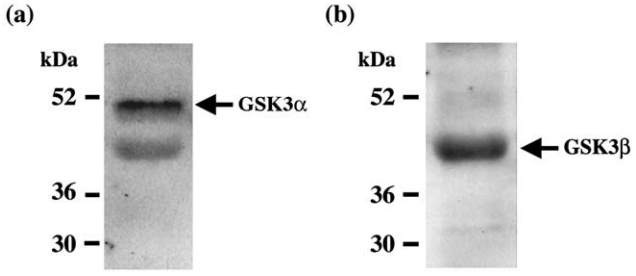

Fig. 1. GSK3α and GSK3β are present in platelets. Platelets proteins were separated by SDS–PAGE, transferred to PVDF membranes and immunoblotted using GSK3α-specific (a) and GSKβ-specific (b) antibodies.

3.2. Collagen, convulxin and thrombin induce phosphorylation of GSK3α/β

GSK3 activity is inhibited by phosphorylation of specific amino terminal serine residues, Ser^{21} on GSK3α and Ser^{9} on GSK3β [16,17]. The phosphorylation states of GSK3α and GSK3β in resting and activated platelets were examined by immunoblot analysis of platelet lysates employing a phos-

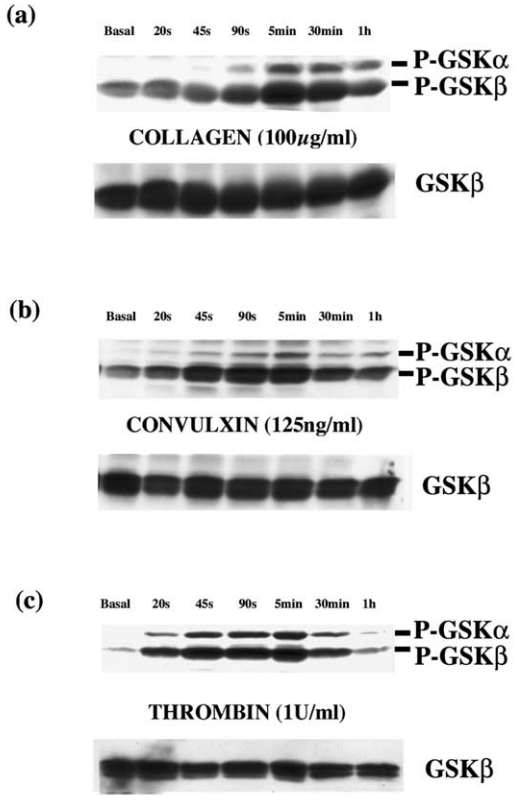

Fig. 2. Agonist-stimulated phosphorylation of GSK3α and GSK3β. Washed, indomethacin- and EGTA-treated platelets were stimulated for the times indicated with either (a) collagen (100 μg/ml), (b) convulxin (125 ng/ml) or (c) thrombin (1 U/ml). Samples were separated by SDS–PAGE, transferred to PVDF membranes and probed with phospho-specific GSK3 antibody (Ser^{21}, GSK3α; Ser^{9}, GSK3β). Data shown are representative of three separate experiments.

pho-specific antibody that recognises the phosphorylated forms of both Ser^{21} of GSKα and Ser^{9} on GSKβ. GSK3β was phosphorylated in resting platelets (Fig. 2). Stimulation of platelets with collagen, convulxin or thrombin resulted in a time- and concentration-dependent (data not shown) increase in the levels of phosphorylation of both GSKα and GSKβ (Fig. 2). Increases in phosphorylation levels were rapid and detectable within 20 s of stimulation. They continued to rise up to 5 min of stimulation and thereafter declined over a period of 1 h. Similar levels of GSK3 phosphorylation were observed in samples where platelets were allowed to aggregate through the omission of EGTA (data not shown).

Phosphorylation of GSK3α on Ser^{21} and GSK3β on Ser^{9} have been shown to be catalysed by a number of kinases, including PKB, that are dependent on PI3K activation [18]. Indeed, the kinetic profile of phosphorylation shown in Fig. 2 mirrors that observed for PKB phosphorylation in platelets stimulated with collagen, convulxin and thrombin at the concentrations shown [10]. In order to verify that a PI3K-dependent pathway was involved in the phosphorylation of GSK3 in activated platelets, structurally unrelated PI3K inhibitors, LY294002 and wortmannin, were used [19,20]. Platelets were pre-incubated with various concentrations of either inhibitor and then stimulated with collagen, convulxin or thrombin. Both PI3K inhibitors decreased substantially the phosphorylation of Ser^{9} on GSK3β, while inhibiting completely the detectable phosphorylation of Ser^{21} on GSK3α, in response to collagen, convulxin, and thrombin (Fig. 3). The levels of inhibition of GSK3 phosphorylation are similar to our reported levels of inhibition of PKB Ser^{473} and Thr^{308} phosphorylation at these concentrations of agonists and inhibitors [10]. This supports the notion that GSK3 may be a target of PKB in stimulated platelets.

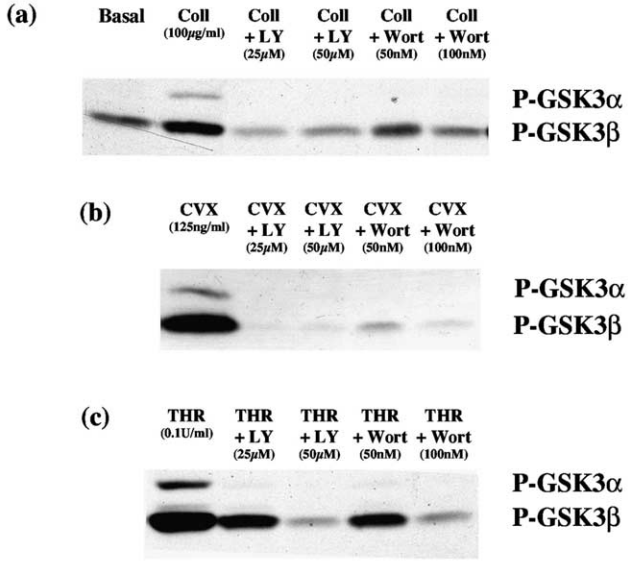

Fig. 3. Phosphorylation of platelet GSK3 is dependent on PI3K. Washed, indomethacin- and EGTA-treated platelets were incubated with varying concentrations of LY294002 or wortmannin, for 15 min prior to stimulation with collagen (Coll, 100 μg/ml), convulxin (Cvx, 125 ng/ml) or thrombin (Thr, 0.1 U/ml). Samples were separated by SDS–PAGE and immunoblotted to detect phospho-GSK3α/β. Results are representative of three separate experiments.

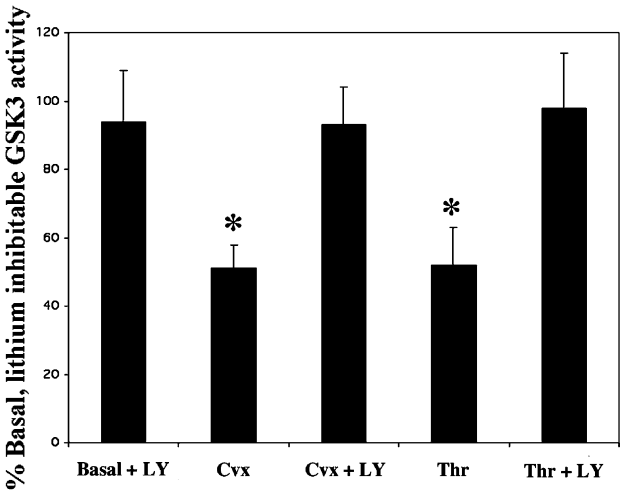

Fig. 4. GSK3 is inhibited on platelet activation. Platelets were stimulated with convulxin (Cvx, 125 ng/ml) or thrombin (Thr, 0.1 U/ml) for 5 min, either in the presence or absence of LY294002 (LY, 50 μM), and then lysed. GSK3 kinase assays were performed as described. Lithium-inhibitable GSK3 activity is expressed as a percentage of activity in unstimulated platelets. Results represent mean ± S.D. ($n = 3$). Student's t test was used in comparison of convulxin- and thrombin-stimulated samples with GSK3 activity in basal cells to determine statistical significance ($^{*}P < 0.01$).

3.3. GSK3 activity is regulated by stimulation of platelets

In vitro kinase assays were performed to determine whether the activity of GSK3 might be regulated during platelet activation. Resting and stimulated platelets were assayed for GSK3 activity either in the presence or absence of lithium chloride (50 mM). Lithium inhibits GSK3 specifically [20] and, combined with the use of a specific primed phosphopeptide substrate, was used to distinguish and correct for non-specific kinase activity present in the assays. Fig. 4 shows that the activity of GSK3 is inhibited significantly following stimulation of platelets with convulxin or thrombin. GSK3 activity was reduced to approximately 50% of basal levels following platelet stimulation, which is similar to reports of GSK3 inhibition by other agonists [21]. This reduction in activity was rescued by pre-incubating platelets with LY294002 (50 μM), confirming that GSK3 activity is regulated in a PI3K-dependent manner.

3.4. Selective inhibitors of GSK3 inhibit platelet aggregation

Platelets were incubated with lithium chloride before stimulation with collagen (0.5μg/ml). Lithium was found to cause a concentration-dependent inhibition of platelet aggregation (Fig. 5a), with complete inhibition at a concentration of 50 mM, where platelet GSK3 activity was found to be inhibited completely (data not shown). Although a recognised inhibitor of GSK3, lithium has been reported to affect the activity of other signalling enzymes. Platelets were therefore incubated with two structurally distinct selective GSK3 inhibitors, SB415286 and TDZD-8 [22,23], and the effect on platelet aggregation in response to collagen (0.5 μg/ml) and thrombin (0.05 U/ml) determined. Using concentrations of these inhibitors that have previously been shown to reduce GSK3 activity in whole cells [22,23], concentration-dependent inhibition of platelet aggregation in response to collagen and thrombin (Fig. 5b and data not shown) was observed. Platelet GSK3

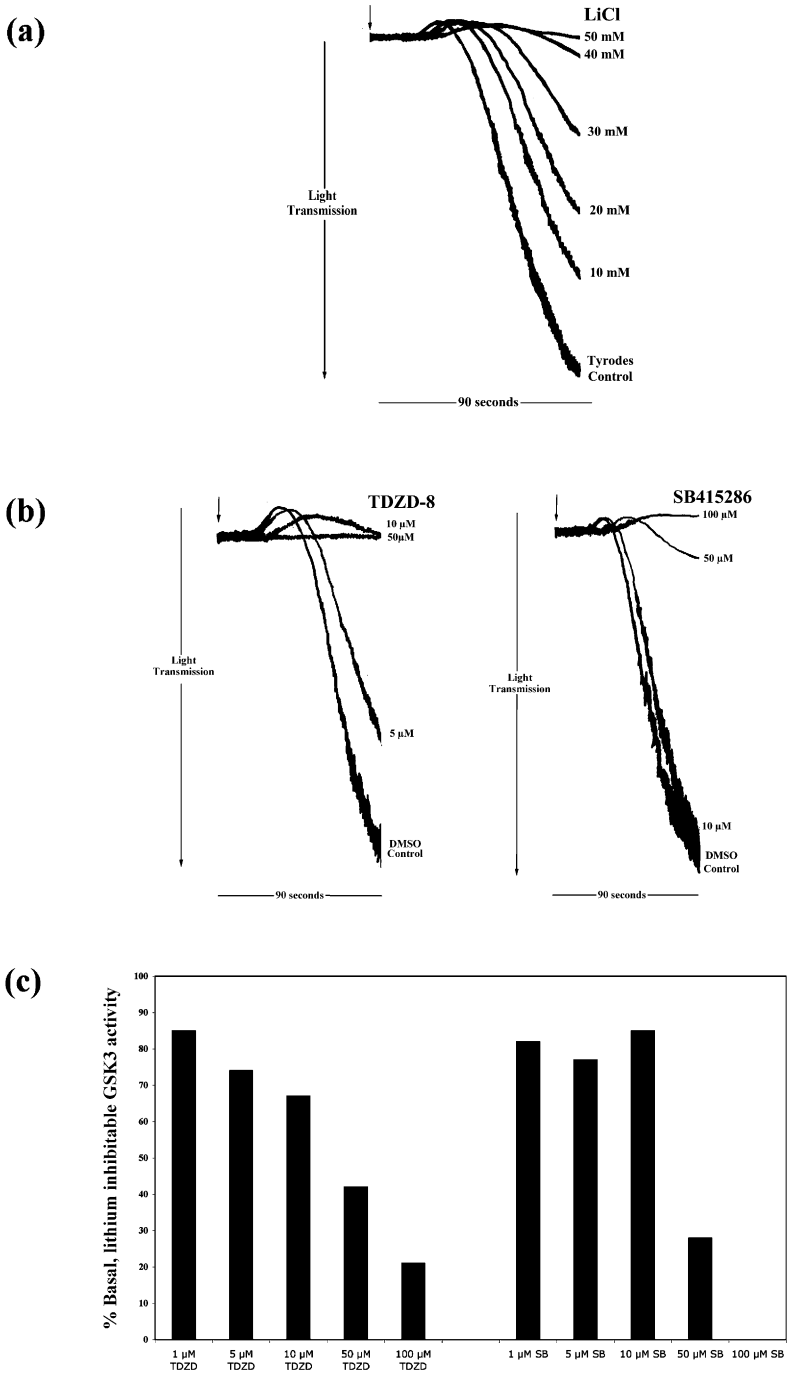

Fig. 5. Selective inhibitors of GSK3 inhibit platelet aggregation. a: Washed platelets were incubated with the indicated concentrations of lithium chloride for 2 min before stimulation of platelet aggregation with collagen (0.5 μg/ml). b: Washed platelets were incubated with DMSO alone or GSK3 inhibitors TDZD-8 and SB415286 at the concentrations shown for 15 min prior to stimulation with collagen (0.5 μg/ml). Aggregation was monitored by turbidometric aggregometry at 37°C under stirring conditions for 90 s. Traces are representative of three separate experiments. c: GSK3 activity in platelet lysates was determined following incubation of platelets with TDZD-8 and SB415286 at the concentrations shown. Results are expressed as % basal, lithium-inhibitable GSK3 activity.

activity was measured following incubation with TDZD-8 and SB415286 and representative concentration–inhibition relationships are shown in Fig. 5c. The profile of GSK3 inhibition with TDZD-8 and SB415286 correlated with the profile of inhibition of platelet aggregation, accounting for a degree of donor variability that is typical in aggregation responses to collagen.

4. Discussion

PI3Ks play a critical role in signalling mechanisms that underlie the regulation of platelet function [9], although little is known of the downstream signalling in these cells. We have recently shown that PKB, a key downstream effector of PI3K, is regulated in collagen-stimulated platelets and may play a

role in the regulation of platelet function [10]. Stimulation of platelets with thrombin and TPO also cause PKB phosphorylation and activation, although ADP and platelet activating factor do not [10,11]. Here we report that GSK3α and GSK3β, known PKB targets in other cell systems, are present in human platelets, are regulated following platelet activation, and through inhibitor studies implicate GSK3 in the regulation of platelet function. GSK3 is widely expressed, although it has not been previously shown to be present in platelets. It is linked with a variety of cellular functions including regulation of metabolism, the regulation of the transcription factors c-jun, c-myc and c-myb, and eukaryotic initiation factor 2B [24,25], the negative regulation of Wnt signalling [21] and modulation of the microtubule-associated protein tau [26].

We report that platelet GSK3α and GSK3β are phosphorylated on their N-terminal regulatory sites in a time- and concentration-dependent manner following stimulation with collagen, convulxin or thrombin. Of the two isoforms, the phosphorylation of GSK3β was more prominent, but this may be a reflection of differential expression levels between the isoforms in platelets, or differential antibody binding properties to the two serine phosphorylated isoforms.

Several kinases can phosphorylate GSK3α on Ser^{21} and GSK3β on Ser^{9}, including PKB [18]. Regulation of PKB in platelets is dependent on the 3′ phosphorylated phosphoinositide products of PI3K. Fig. 3 shows that incubation of platelets with either LY294002 or wortmannin prior to platelet stimulation with collagen, convulxin or thrombin reduced or abolished GSK3 phosphorylation. However, higher concentrations of thrombin were able to overcome the effects of both inhibitors indicating the presence of a PI3K-independent pathway. Indeed, thrombin-stimulated PKB regulation has been previously reported to involve both PI3K-dependent and PI3K-independent mechanisms [11].

Phosphorylation of GSK3α on Ser^{21} and GSK3β on Ser^{9} has been shown to result in a decrease in GSK3 activity [16,17]. In the present study the stimulation of GPVI and thrombin receptors on platelets resulted in attenuation of GSK3 activity, falling to 50% of basal levels (Fig. 4). This is similar in magnitude to the reduction in GSK3 activity reported for insulin [21]. Pre-incubation of the samples with LY294002 rescued the agonist-induced decrease in GSK3 activity, confirming a role for PI3K.

To begin to examine the role of GSK3 in platelet function, the effects of three structurally distinct GSK3 inhibitors, lithium, TDZD-8 and SB415286 [22,23], were explored. Each of these inhibitors caused a concentration-dependent attenuation of platelet aggregation in response to collagen (Fig. 5) and thrombin (data not shown). These results were surprising since GSK3 activity was reduced during platelet activation and PI3K-dependent signalling has been shown to be required for platelet aggregation. However, while each of these inhibitors is unlikely to be 100% selective for GSK3, the similar effects of three structurally distinct inhibitors are strongly supportive of the role for GSK3 in the regulation of platelet function. How might this apparent paradox be resolved? Given that GSK3 is active in unstimulated cells it is possible that active GSK3 is necessary for priming platelets for aggregation. The signal for aggregation comes in part via the activation of the PI3K–PKB pathway but does not require GSK3. However, activation of PI3K–PKB pathway results in inhibition of GSK3 activity, which may contribute to negative feedback regulation of platelet function. This latter point may be considered analogous to the stimulation of inhibitory signalling by platelet endothelial cell adhesion molecule-1 following stimulation of platelets with collagen or thrombin [13].

As noted in [12] the full complement of in vivo GSK3 substrates remains to be determined. Known substrates include proteins involved in microtubule binding, protein scaffolds, cellular adhesion, metabolic enzymes, transcription factors and components of cell cycle. While transcription factors and cell cycle machinery are unlikely to be involved in platelets, GSK3 substrates linked in other systems to regulation of adhesion, cytoskeleton and metabolism might play a role. It is possible that GSK3 is involved in the cytoskeletal reorganisation that platelets undergo when they are activated since both aggregation and shape change are inhibited by TDZD-8 (Fig. 5b). Known GSK3 targets here include APC, MAP1B, β-catenin, tau and presenilin-1. Of these proteins, all but tau have been shown to be expressed in platelets [27,28]. Investigations are currently underway to address these questions and to identify and determine the physiologic function of substrates of GSK3 in platelet function.

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