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ARTICLE

Non-muscle myosin IIA is involved in focal adhesion and actin remodelling controlling glucose-stimulated insulin secretion

C. Arous · D. Rondas · P. A. Halban

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Abstract
Aims/hypothesis Actin and focal adhesion (FA) remodelling are essential for glucose-stimulated insulin secretion (GSIS). Non-muscle myosin II (NM II) isoforms have been implicated in such remodelling in other cell types, and myosin light chain kinase (MLCK) and Rho-associated coiled-coil-containing kinase (ROCK) are upstream regulators of NM II, which is known to be involved in GSIS. The aim of this work was to elucidate the implication and regulation of NM IIA and IIB in beta cell actin and FA remodelling, granule trafficking and GSIS.
Methods Inhibitors of MLCK, ROCK and NM II were used to study NM II activity, and knockdown of NM IIA and IIB to determine isoform specificity, using sorted primary rat beta cells. Insulin was measured by radioimmunoassay. Protein phosphorylation and subcellular distribution were determined by western blot and confocal immunofluorescence. Dynamic changes were monitored by live cell imaging and total internal reflection fluorescence microscopy using MIN6B1 cells.
Results NM II and MLCK inhibition decreased GSIS, associated with shortening of peripheral actin stress fibres, and reduced numbers of FAs and insulin granules in close proximity to the basal membrane. By contrast, ROCK inhibition increased GSIS and caused disassembly of glucose-induced central actin stress fibres, resulting in large FAs without any effect on FA number. Only glucose-induced NM IIA reorganisation was blunted by MLCK inhibition. NM IIA knockdown decreased GSIS, levels of FA proteins and glucose-induced extracellular signal-regulated kinase 1/2 phosphorylation.
Conclusions/interpretation Our data indicate that MLCK–NM IIA may modulate translocation of secretory granules, resulting in enhanced insulin secretion through actin and FA remodelling, and regulation of FA protein levels.

Keywords Actin remodelling · Beta cell · Focal adhesion · Insulin secretion · Non-muscle myosin II

Abbreviations

ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FA	Focal adhesion
F-actin	Filamentous actin
FAK	Focal adhesion protein kinase
GFP	Green fluorescent protein
GSIS	Glucose-stimulated insulin secretion
MHC	Myosin heavy chain
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NM II	Non-muscle myosin II
NPY	Neuropeptide Y
PAX	Paxillin
ROCK	Rho-associated coiled-coil-containing kinase
siRNA	Short interfering RNA
TIRF	Total internal reflection fluorescence

Introduction

The pathogenesis of beta cell dysfunction in type 2 diabetes is characterised by progressive loss of beta cell function and

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mass [1]. A better understanding of the beta cell secretory pathway might reveal new targets for the treatment of type 2 diabetes. Filamentous actin (F-actin) and focal adhesion (FA) remodelling in beta cells play a crucial role in insulin secretion [2, 3]. In beta cells, glucose-stimulated insulin secretion (GSIS) is triggered by phosphorylation and activation of focal adhesion protein kinase (FAK) and paxillin (PAX), and their recruitment into nascent lamellipodia linked to the activation of extracellular signal-regulated kinase (ERK) 1/2 through β1-integrin engaged with the extracellular matrix (ECM) [2–4]. F-actin reorganisation following glucose stimulation is a prerequisite for FA formation, allowing insulin granules to approach the plasma membrane and engage with components of the exocytotic machinery for docking and fusion [2, 4, 5]. A recent in vivo study using a beta cell-specific FAK knockout mouse confirmed these in vitro findings and further demonstrated a key role for FAK in beta cell viability through insulin signalling [6]. Nevertheless, the detailed regulation of FA and actin remodelling in beta cells, and the key molecules that couple this to GSIS, remain to be elucidated.

Non-muscle myosin II (NM II) is a major cytoskeletal protein that interacts with F-actin to contribute to cellular processes, such as migration and adhesion [7]. NM II has a functional role in FA and actin remodelling [7–9], and has been implicated in late events leading to exocytosis in other secretory cells [10, 11]. Previous studies have suggested that NM II could be an actin-motor protein implicated in insulin secretion [12, 13]. NM II consists of two myosin heavy chains (MHC), two essential light chains and two regulatory light chains (MLC). In mammals, three different genes encode NM II proteins, including MHC IIA (*Myh9*) and IIB (*Myh10*). These isoforms exhibit different enzymatic properties, subcellular localisation and tissue patterns [14–16], leading us to postulate isoform-specific roles in beta cell secretion. MHC IIA contributes to FA stability and maturation [17–19] and to Rho-associated coiled-coil-containing kinase (ROCK)-dependent functions including stress fibre organisation [20]. Furthermore, this isoform associates with actin fibres following KCl stimulation of RINm5F cells [13], suggesting a possible role in exocytosis. MHC IIB contributes to cell migration by controlling protrusion stability and also mediates stress fibre formation [21, 22].

Unlike other myosins, NM II is regulated by direct phosphorylation of both its heavy and light chains. Myosin light chain kinase (MLCK) and ROCK activate NM II through MLC phosphorylation, which induces FA maturation and contributes to F-actin polymerisation in stress fibres [7, 23]. In beta cells, Rho–ROCK signalling contributes to the stabilisation of the actin cytoskeleton and inhibits GSIS [24], while MLCK co-localises with insulin granules [25]. Other studies correlated insulin secretion with MHC phosphorylation and revealed co-localisation with F-actin [13, 26]; heavy chain phosphorylation seemed to be more important for insulin secretion than regulatory light chain phosphorylation. MLC phosphorylation by ROCK occurs centrally, and by MLCK towards the periphery, with distinct effects on plasma membrane ruffling and FA dynamics in fibroblasts [27].

We have studied the role of NM II isoforms and their upstream regulators MLCK and ROCK in beta cell function. We highlight the MLCK–MHC IIA–FAK–PAX–ERK pathway as a positive regulator of insulin secretion. MHC IIA emerges as a central regulatory molecule that serves to integrate and coordinate actin and FA remodelling, secretory signalling pathways and granule shuttling to the basal membrane, all key components of GSIS.

Methods

Antibodies and reagents anti-FAK: Santa Cruz Biotechnology (Santa Cruz, CA, USA); -phospho(Y397)-FAK and -phospho (Y118)-paxillin: Invitrogen (Carlsbad, CA, USA); -paxillin: BD Transduction Laboratories (San Jose, CA, USA); -ERK1/2, -phospho(T202/Y204)-ERK1/2 and -actin: Cell Signaling Technology (Beverly, MA, USA); -γ-actin: a gift from C. Chaponnier (University of Geneva, Geneva, Switzerland); -MHC IIA and -MHC IIB: Covance Research Products (Emeryville, CA, USA); donkey anti-rabbit horseradish peroxidase (HRP) and sheep anti-mouse HRP: Amersham Biosciences (Uppsala, Sweden); donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555, Alexa Fluor 647-phalloidin: Invitrogen; Y-27362 and blebbistatin: Calbiochem (La Jolla, CA, USA); ML7: Sigma Chemical Company (St Louis, MO, USA).

Cells and culture conditions Rat islet isolation, beta cell sorting by FACS and monolayer culture on ECM from 804G cells (804G-ECM) were performed as previously described [28]. MIN6B1 cells were cultured as previously described [29] and plated on 35 mm glass-bottomed microwell dishes (MatTek, Ashland, MA, USA) coated with 804G-ECM for total internal reflection fluorescence (TIRF) microscopy and confocal live imaging [30].

Expression vectors The pEGFPC1-FAK plasmid was a gift from D. D. Schlaepfer (University of California, San Diego, CA, USA); paxillin-pEGFP was from AddGene (Cambridge, MA, USA); and neuropeptide Y (NPY)-Cherry was a gift from G. Rutter (Imperial College London, London, UK).

Ribonucleic acid interference-mediated silencing of MHC IIA and IIB MHC IIA and IIB levels were knocked down by transfecting primary rat beta cells twice with two specific short interfering RNA molecules (siRNAs) directed towards

rat MHC IIA or IIB messenger ribonucleic acid (electronic supplementary material [ESM] Table 1) (Microsynth AG, Balgach, Switzerland), using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated for 72 h to allow siRNA expression before cell treatment and analysis.

SDS-PAGE and western blotting Protein samples were prepared and immunoblots analysed as described [2]. Western blots were quantified by densitometry and band density of phosphoproteins normalised to that of the corresponding total protein and/or to total actin as indicated in the figure legends.

TIRF microscopy Cells were transfected using Lipofectamine 2000 reagent according to the manufacturer's instructions and incubated for 48 h to allow for DNA expression. TIRF images were obtained as earlier described [3].

Immunofluorescence and confocal microscopy Immunofluorescence was performed as previously described [4]. Cell basal membranes were observed by confocal microscopy using a Zeiss LSM510 Meta microscope with a ×63 oil immersion lens, and the images were acquired and processed using LSM510 software (Carl Zeiss, Iena, Germany). Confocal live imaging was performed with a Nikon A1r microscope (Nikon, Tokyo, Japan) equipped with a ×60 CFI plan Apo objective and a filter optimised for mCherry fluorescence. Cells on 804G-coated glass-bottomed culture dishes were maintained on the microscope stage at 37°C and 5% CO_2. Transfected cells were chosen at random for analysis, and images were captured every 60 s.

Insulin secretion assays Rat beta cells were washed, pre-incubated and incubated for insulin secretion assays, and insulin was measured by radioimmunoassay as previously described [4].

Statistical analysis Statistical significance for differences between experimental conditions was determined using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, USA) by one-way ANOVA when more than two conditions were compared and by Student's *t* test for unpaired groups for comparison of two conditions; *p* values less than 0.05 were considered significant.

Results

Impact of NM II function on GSIS in rat primary beta cells Various inhibitors were used to study the impact of NM II and its upstream regulators MLCK and ROCK on GSIS: blebbistatin is a myosin II-specific ATPase inhibitor that completely blocks enzyme activity [31]; ML7 is a selective inhibitor of MLCK; Y27632 is an inhibitor of ROCK. As shown in Fig. 1a, blebbistatin decreased GSIS in a concentration-dependent manner without affecting basal secretion. MLCK inhibition similarly decreased GSIS by 25.5±3.5% ($p<0.05$) without affecting basal secretion (Fig. 1b). By contrast, ROCK inhibition (Y27632) increased GSIS by 1.91±0.63-fold (data not shown), as previously demonstrated by our group [24]. There was no effect of the inhibitors on cellular insulin content (data not shown). These results suggested that MLCK–NM II signalling favours GSIS, whereas ROCK inhibits it, confirming the opposing role of these two kinases on cell function [27, 32].

Impact of inhibition of MLCK, NM II and ROCK on actin and FA remodelling in rat primary beta cells In view of the important role of FA and F-actin reorganisation in the regulation of GSIS and the known role of NM II in such reorganisation in other cell types, we next studied the effects of MLCK, NM II and ROCK inhibition using confocal immunofluorescence. ROCK inhibition by Y27632 revealed F-actin filaments in the central portion of the cell, whereas MLCK inhibition by ML7 led to the formation of actin bundles at the cell periphery; both inhibitors induced membrane protrusions compared with control conditions. Blebbistatin completely modified cell morphology, including formation of large blebs in the absence or presence of high glucose, blunted glucose-induced actin reorganisation and caused actin accumulation at the cell periphery (Fig. 2a). Glucose stimulation led to bundling of F-actin filaments and protrusion formation as previously demonstrated [2]. Upon glucose stimulation, ROCK inhibition caused disassembly of actin stress fibres in the central portion of the cell without affecting those located in the

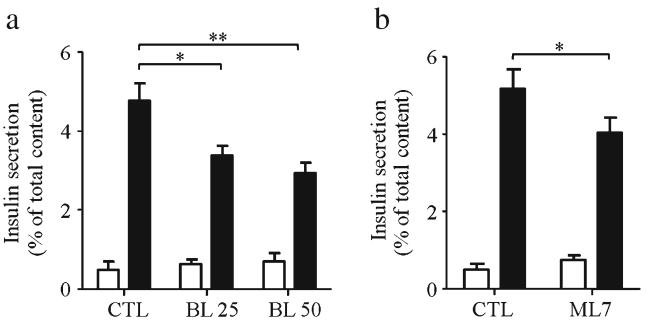

Fig. 1 Inhibition of myosin II and MLCK decreases GSIS. Cells were pre-incubated for 2 h at 2.8 mmol/l glucose, and basal secretion was measured during a further 1 h at the same glucose concentration in the continued presence, or not (CTL), of an inhibitor ([**a**] 25 μmol/l or 50 μmol/l blebbistatin (BL), [**b**] 20 μmol/l ML7), and then stimulated with high glucose (16.7 mmol/l) in the presence of the different inhibitors for 1 h (white bars, basal; black bars, stimulated). Data are mean±SEM from four independent experiments (*t* test, *$p<0.05$, **$p<0.01$)

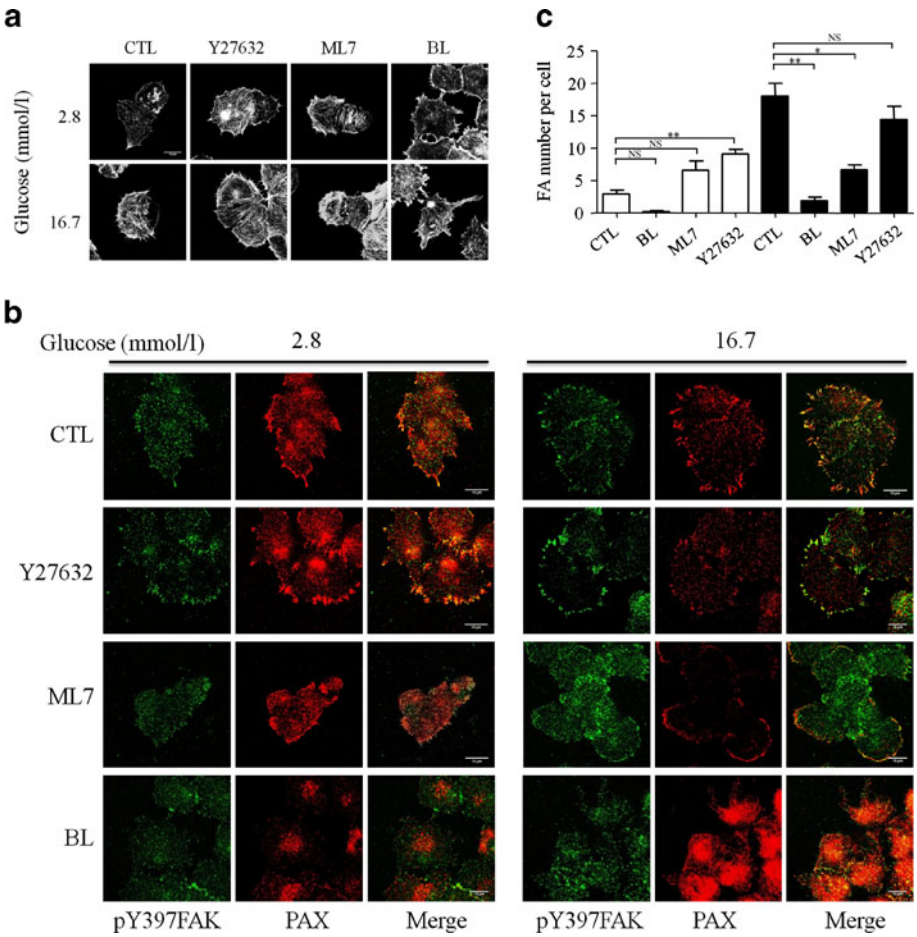

Fig. 2 Effect of the different inhibitors on actin remodelling and FA morphology and number. Primary beta cells were cultured for 3 h in low glucose (2.8 mmol/l) in the presence, or not (CTL), of an inhibitor (25 μmol/l blebbistatin [BL], 20 μmol/l ML7 or 50 μmol/l Y27632) and stimulated with high glucose (16.7 mmol/l) for 10 min in the presence, or not, of the respective inhibitor. (**a**) Cells were subsequently fixed and stained for actin (with phalloidin) or (**b**) using anti-pY397FAK (green) and PAX (red). All images are fully representative of four independent experiments. (**c**) Cells were subsequently fixed and stained for pY397FAK. FAs containing phosphorylated FAK were counted in each cell (white bars, 2.8 mmol/l; black bars, 16.7 mmol/l). Data are mean± SEM from three independent experiments (one-way ANOVA, *$p<0.05$, **$p<0.01$). Scale bar, 10 μm

periphery. However, MLCK inhibition seemed to have no effect on central actin remodelling but caused shortening of peripheral stress fibres (Fig. 2a).

Inhibition of MLCK and NM II caused profound changes in the intracellular distribution of pY397FAK and PAX in both basal and stimulated conditions (Fig. 2b), accompanied by a 54.1±5.1% ($p<0.05$) and 88.7±6.0% ($p<0.01$) decrease in the number of glucose-induced FAs, respectively (Fig. 2c). By contrast, ROCK inhibition induced large FAs containing pY397FAK and PAX, and an increase in FA number in the basal condition (2.8 mmol/l glucose), but was without significant effect after glucose stimulation (Fig. 2b, c).

MLCK inhibition blunts glucose-induced FAK, PAX and ERK phosphorylation in rat primary beta cells To gain insight into a possible effect of MLCK, NM II and ROCK inhibition on the FAK signalling pathway, we studied the impact of the inhibitors on the glucose-induced phosphorylation of FAK and two of its known downstream targets, PAX and ERK1/2. FAK, PAX and ERK1/2 phosphorylation induced by high glucose (16.7 mmol/l; 10 min) decreased in the presence of ML7 by 43.5±3.8% ($p<0.05$), 40.9±22.7% ($p<0.05$) and 64.3±14.6% ($p<0.01$), respectively (Fig. 3a–c). There was no significant effect of blebbistatin because of greater variability in the data. To obtain sufficient numbers, MIN6B1 cells were used to monitor the time course of the effects of blebbistatin. This highly differentiated mouse beta cell line displays glucose-induced actin and FA remodelling similar to primary rat beta cells [24]. This time course showed a decrease in FAK, PAX and ERK1/2 phosphorylation in the presence of blebbistatin after 5 min of glucose stimulation, which was completely restored after 10 min (data not shown). The acute effects of blebbistatin on these molecules appear short-lived, possibly explaining inter-experimental variability at the 10 min time point studied using primary cells. ROCK inhibition had no significant effect on glucose-induced FAK, PAX and ERK phosphorylation (Fig. 3a–c). These results suggest other final effectors in charge of the GSIS signalling pathway regulated by both NM II and ROCK in an opposing way.

MLCK and myosin II are involved in granule movement and localisation in MIN6B1 cells Following previous reports indicating the involvement of actin remodelling and FA maturation in dense-core vesicle transport and localisation close to the basal membrane [3, 5], we investigated whether MLCK, myosin II or ROCK inhibition affected the shuttling of insulin granules and movement to the plasma membrane. Due to the

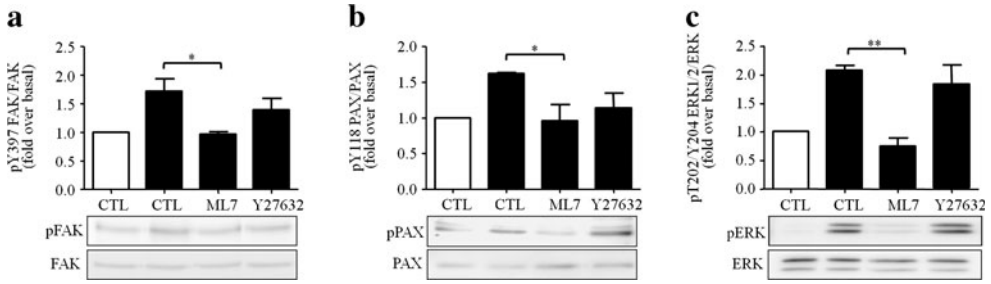

Fig. 3 ML7 inhibits glucose-induced FAK, PAX and ERK phosphorylation in rat primary beta cells. Primary beta cells were cultured for 3 h in low glucose (2.8 mmol/l) in the presence, or not (CTL), of an inhibitor (20 μmol/l ML7 or 50 μmol/l Y27632) and stimulated with high glucose (16.7 mmol/l) for 10 min in the presence, or not, of the respective inhibitor. Cell lysates were analysed by western blot, and the relative intensities of phosphorylated (p) and total protein bands were quantified by densitometry and expressed as a ratio in comparison with non-stimulated control cells, and normalised to total actin (white bars, 2.8 mmol/l; black bars, 16.7 mmol/l). (**a**) pY397FAK/FAK total; (**b**) pY118PAX/PAX total; (**c**) pT202/Y204 ERK1/2/ERK total. Data are mean±SEM from three independent experiments (one-way ANOVA, $*p<0.05$, $**p<0.01$)

low plasmid transfection rate in rat primary beta cells (less than 5%), we performed TIRF and live microscopy in MIN6B1 cells. Cells were transfected with a construct expressing the fluorescent fusion protein NPY-Cherry to label dense-core vesicles [33], FAK-green fluorescent protein (GFP) and PAX-GFP. TIRF microscopy showed a reduction in the number of plasma membrane-associated vesicles and FA formation in glucose-stimulated conditions with ML7 or blebbistatin (Fig. 4a). High glucose increased the number of granules in close proximity to the plasma membrane, but this was prevented by MLCK and NM II inhibition (Fig. 4b). These data suggest that active MLCK–NM II is implicated in glucose-induced delivery of insulin-containing granules to the plasma membrane and FA formation. As anticipated, ROCK inhibition did not affect the presence of insulin granules and FA formation at the basal membrane (Fig. 4a, b), confirming that MLCK–NM II and ROCK regulate beta cell secretory function in an opposing way.

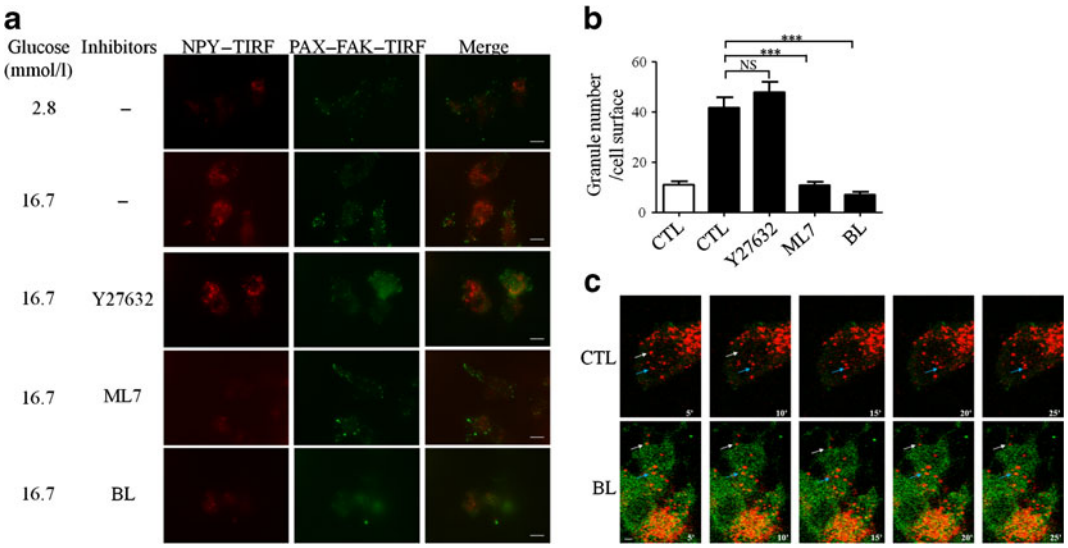

Fig. 4 MLCK and myosin II are implicated in beta cell exocytosis. MIN6B1 cells were co-transfected with NPY-Cherry, FAK-GFP and PAX-GFP plasmids and cultured for 48 h in normal medium. Cells were incubated with low glucose (2.8 mmol/l) in the presence, or not (CTL), of an inhibitor (25 μmol/l blebbistatin [BL], 20 μmol/l ML7 or 50 μmol/l Y27632) for 3 h and stimulated with high glucose (16.7 mmol/l) in the presence of the respective inhibitors for 10 min. (**a**, **b**) The effect of the different inhibitors on insulin granule number at the basal membrane was evaluated by TIRF microscopy on fixed cells. (**a**) Representative pictures of each condition. Red corresponds to dense-core insulin granules (NPY-Cherry) and green to FAK and PAX protein tagged with GFP. (**b**) The number of secretory vesicles detected by TIRF in the basal membrane and plasma membrane area were quantified with ImageJ software 1.33u (NIH, Bethesda, MD, USA) and expressed as a ratio of arbitrary units (white bar, 2.8 mmol/l; black bars, 16.7 mmol/l). Data are means±SEM from three independent experiments (one-way ANOVA, $***p<0.001$). (**c**) Insulin granule movement was observed in the presence of the different inhibitors using confocal microscope live cell imaging. After transfection, cells were treated without glucose in the presence, or not, of an inhibitor for 3 h and stimulated with high glucose (20 mmol/l) in the presence, or not (CTL), of inhibitors for 10 min. Pictures were taken every 5 s during 5 min (ESM Video 1 and ESM Video 2). All images are fully representative of three independent experiments. Scale bar, 10 μm

The effect of NM II on granule movement was evaluated by live confocal microscopy imaging of MIN6B1 cells transfected with NPY-Cherry, FAK-GFP and PAX-GFP. In Fig. 4c (see also ESM Videos 1, 2), control-stimulated cells presented an important rate of insulin granule movement. Blebbistatin completely blunted glucose-induced insulin granule movement while dramatically altering the distribution of FAK and PAX (Fig. 4c). Y27632 failed to influence granule movement to any apparent extent, contrary to ML7, which seemed to induce a decrease in insulin granule velocity (ESM Videos 1, 2).

MLCK inhibition blunts glucose-induced MHC IIA remodelling, and γ-actin protrusion formation and co-localisation with MHC IIA at the cell periphery of rat primary beta cells In view of the potential differential role of NM II isoforms in beta cell function, we next examined the effect of glucose on the distribution of the MHC of each isoform at the basal surface of rat primary beta cells by immunofluorescence using isoform-specific antibodies. As shown in Fig. 5a, b, at low glucose, MHC IIA and IIB were concentrated at the cell periphery and co-localised with F-actin with a more diffuse signal towards the centre of the cells. Furthermore, we observed co-localisation of MHC IIA and IIB with PAX in the cytoplasm. Upon short-term glucose stimulation, there was rapid reorganisation of both MHC IIA and IIB, coinciding with actin co-localisation in filaments (Fig. 5a, b). We did not observe co-localisation of PAX and MHC IIA or MHC IIB at filopodial extensions, suggesting an indirect role of MHC II on FA maturation and/or stabilisation.

We next examined the effect of MLCK, NM II and ROCK inhibition on MHC IIA and IIB distribution. As shown in Fig. 2a, ROCK inhibition caused disassembly of central glucose-induced actin stress fibres and induced actin concentration in FAs at the cell surface which co-localised with both MHC IIA and PAX (Fig. 5a). MLCK inhibition blunted the glucose effect on actomyosin IIA bundle formation, with an MHC IIA localisation similar to that of the low glucose condition, suggesting involvement of this kinase in glucose-induced MHC IIA remodelling. Analysis of MHC IIB distribution after glucose stimulation revealed no effect of MLCK and ROCK inhibition (Fig. 5b). Blebbistatin, however, blunted glucose-induced remodelling of both MHC IIA and MHC IIB in bundles with cytoplasmic localisation of both isoforms (Fig. 5a, b, lower pictures). Most interestingly, this set of images indicates that only glucose-induced MHC IIA reorganisation was blunted by MLCK inhibition and confirmed that MLCK–myosin IIA seems to

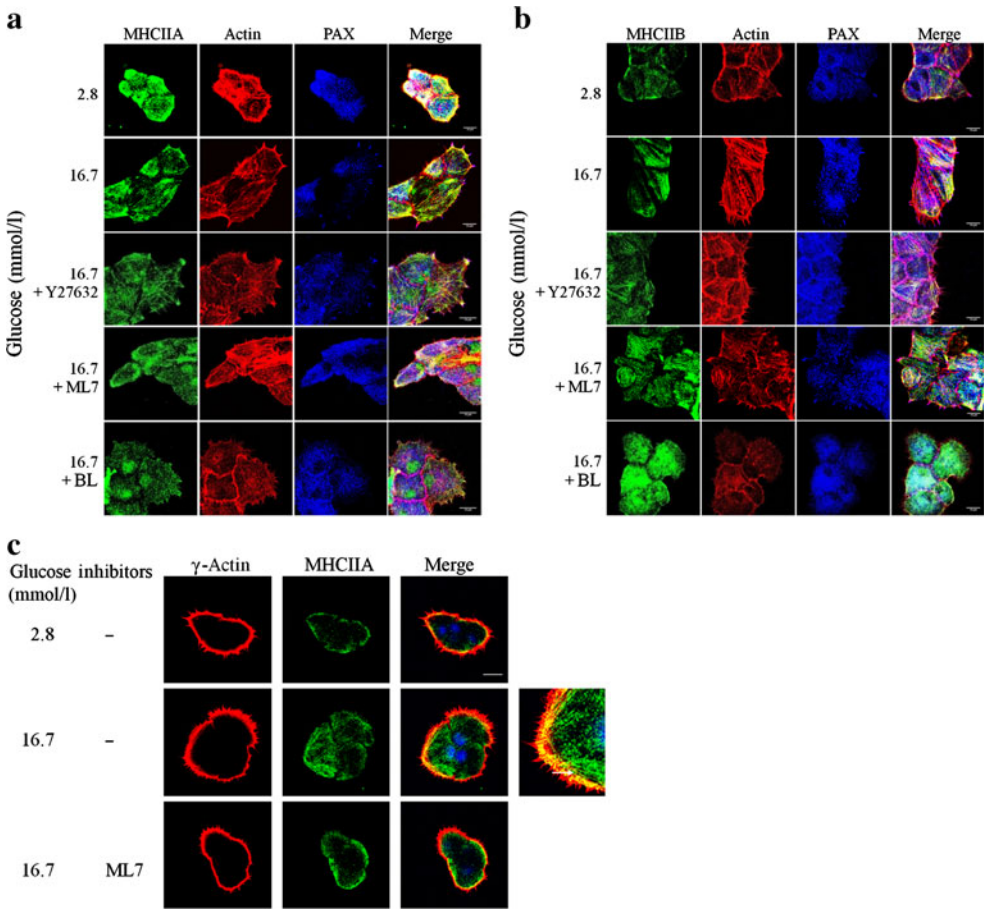

Fig. 5 MLCK inhibition blunts glucose-induced MHC IIA remodelling, glucose-induced γ-actin protrusion formation and co-localisation with MHC IIA at the cell periphery. Primary beta cell cells were cultured for 3 h in low glucose (2.8 mmol/l) in the presence, or not, of inhibitors (25 μmol/l blebbistatin [BL], 20 μmol/l ML7 or 50 μmol/l Y27632) and fixed or further stimulated with 16.7 mmol/l glucose for 10 min in the presence, or not, of inhibitors before fixation. Cells were subsequently fixed and stained for (**a**) MHC IIA or (**b**) MHC IIB (green), actin (phalloidin in red) and PAX (blue). (**c**) Cells were stained using specific γ-actin (red) and MHC IIA (green) antibodies. All images are fully representative of three independent experiments. Scale bar, 10 μm

be an important regulator of beta cell molecular modification induced by glucose. Preliminary studies (n=2; data not shown) suggest that glucose-induced MHC IIA reorganisation is Ca^{2+}-dependent, since this was prevented by the voltage-gated calcium channel blocker nifedipine (10 μmol/l).

Phalloidin does not stain all F-actin pools [34] and also fails to discriminate between actin isoforms. Since γ-actin has been localised in protrusions in other cell types [35], we investigated the effect of glucose and MLCK inhibition on its distribution. As predicted, there was indeed an increase in γ-actin-containing protrusions in response to glucose, and co-localisation with MHC IIA filaments at the cell periphery. MLCK inhibition induced a decrease in protrusions containing γ-actin and its co-localisation with MHC IIA filaments (Fig. 5c).

MHC IIA and IIB are necessary for glucose-induced actin remodelling and PAX localisation in rat primary beta cell protrusions In another strategy to discriminate between the roles of each NM II isoform in beta cell function, isoform-specific siRNAs were developed to characterise any loss-of-function associated phenotype. Ribonucleic acid interference-mediated silencing of NM II isoforms resulted in a 30.9±4.2% (p<0.05) and 36.6±2.2% (p<0.01) reduction in protein levels of MHC IIA (Fig. 6a) and MHC IIB (Fig. 6b), respectively. Co-transfection with siRNA against the two isoforms resulted in a 31.3±6.3% (p<0.05) and 44.0±8.9% (p<0.05) decrease in MHC IIA and IIB protein levels, respectively. We therefore decided not to include the double knockdown in the following experiments because it clearly induced cell death (data not shown). Immunofluorescence of actin and PAX demonstrated that MHC IIA knockdown strongly disrupted glucose-induced actin filaments and PAX recruitment to FAs without affecting MHC IIB distribution (Fig. 6c). Knockdown of MHC IIB had no influence on PAX-containing FA formation induced by glucose but blunted actin reorganisation, albeit with less impact than MHC IIA knockdown (Fig. 6d).

MHC IIA but not MHC IIB is necessary for insulin secretion through the FAK–PAX–ERK pathway in rat primary beta cells Our previous results suggest an involvement of the MLCK–MHC IIA axis in beta cell exocytosis. In view of this, we assessed insulin secretion (Fig. 7a) following knockdown of each myosin II isoform. First, we verified that scrambled siRNAs had no effect on GSIS, allowing them to be used as the control condition. Interestingly,

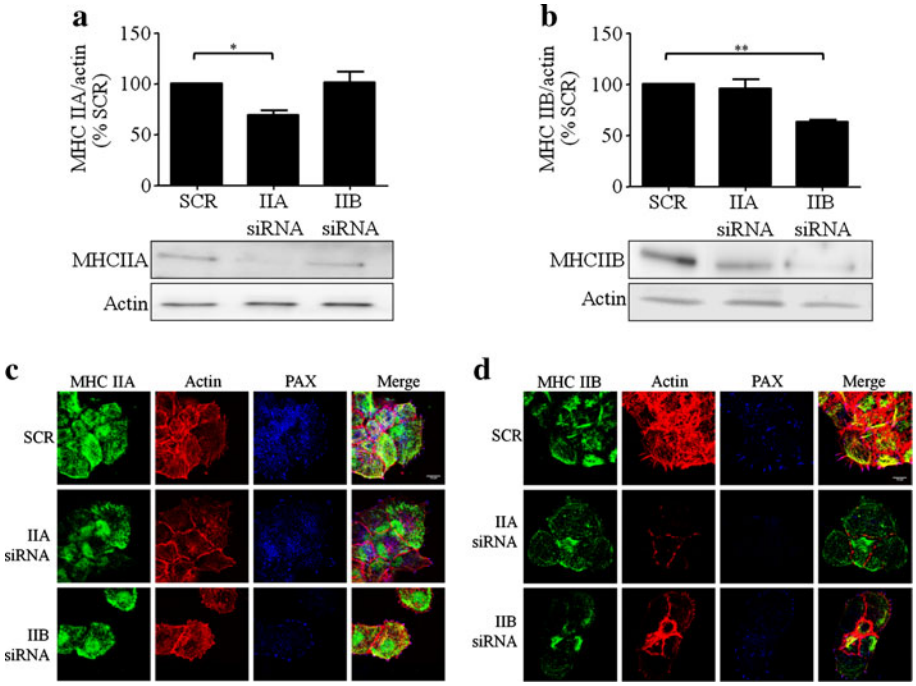

Fig. 6 MHC IIA and IIB are necessary for glucose-induced actin remodelling and PAX localisation in protrusions. Primary beta cells were transfected with siRNA against MHC IIA (IIA siRNA) or MHC IIB (IIB siRNA), or with a mixture of scrambled siRNAs designed for each specific isoform siRNA (SCR) and maintained in culture for 4 days. Cells were then cultured for 3 h in low glucose (2.8 mmol/l) and stimulated with high glucose (16.7 mmol/l) for 10 min. (**a**, **b**) Cell lysates were analysed by western blot, and the relative intensities of total protein MHC IIA and MHC IIB bands were quantified by densitometry and expressed as a ratio in comparison with the scrambled siRNA condition and normalised to total actin. Data are means±SEM from five independent experiments (one-way ANOVA, *p<0.05, **p<0.01). (**c**, **d**) After glucose stimulation, cells were fixed and stained for MHC IIA (**c**) or MHC IIB (**d**), actin (phalloidin) and PAX. All images are fully representative of three independent experiments

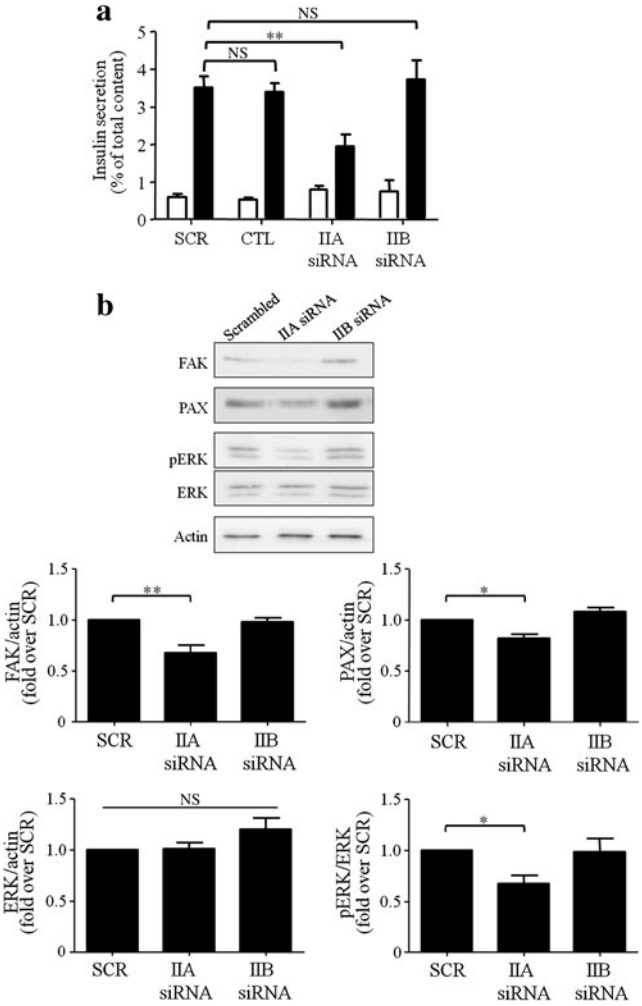

Fig. 7 MHC IIA but not MHC IIB is necessary for insulin secretion through FAK–PAX–ERK. Primary beta cells were transfected with siRNA against MHC IIA (IIA siRNA), MHC IIB (IIB siRNA) or scrambled siRNAs (SCR) and maintained in culture for 4 days. (**a**) Insulin secretion was measured in beta cells incubated with low glucose (2.8 mmol/l) and stimulated with high glucose (16.7 mmol/l) for 1 h (see legend to Fig. 1). Data are expressed as the percentage of total insulin content (white bars, basal; black bars, stimulated). (**b**) Cell lysates were analysed by western blot, and the relative intensities of FAK, PAX, ERK and phosphorylated (p) ERK were quantified by densitometry and expressed as a ratio in comparison with the control condition with scrambled siRNA and normalised to total actin. Data are mean±SEM from four independent experiments (one-way ANOVA, *$p<0.05$, **$p<0.01$)

GSIS was decreased by 42.3±8.03% ($p<0.01$) when MHC IIA had been knocked down, but there was no such inhibition after MHC IIB knockdown. Furthermore, there was no impact of either MHC isoform-specific siRNA on cellular insulin content (data not shown). To determine whether MHC IIA and/or IIB may regulate insulin secretion through FA protein regulation and downstream signalling, we compared the effect of MHC IIA and IIB knockdown on FAK, PAX and ERK1/2 protein levels (Fig. 7b). Only MHC IIA knockdown resulted in a decrease of FAK and PAX levels by 27.6±8.9% and 16.7±4.5% ($p<0.05$), respectively. Total ERK1/2 protein levels remained unchanged but there was a decrease in phosphorylated ERK1/2 (Fig. 7b). Taken together these results indicate that MHC IIA plays an important role in GSIS through the regulation of FAK and PAX protein levels and ERK phosphorylation necessary for FA maturation in response to glucose.

Discussion

Our results indicate a central role for MHC IIA in beta cell function, serving as a point of convergence between glucose-induced actin and FA remodelling, and GSIS. NM II regulates actin organisation as well as the subcellular localisation of two key FA proteins, FAK and PAX, and is involved in granule shuttling and movement to the basal membrane in response to glucose. While glucose induces both MHC IIA and IIB to bundle and co-localise with F-actin, only MHC IIA appears critical for the regulation of FAs and GSIS. MLCK has been shown to regulate NM II [36] and is also involved in the regulation of GSIS. We now show that MLCK is involved in FA formation and in glucose-induced remodelling of MHC IIA, without affecting the IIB isoform. These results allowed us to identify MLCK–myosin IIA–FAK–PAX–ERK as a new pathway involved in the regulation of GSIS.

In other cell types, MLC phosphorylation by ROCK occurs towards the centre of the cell, and by MLCK towards the periphery, with distinct effects on plasma membrane ruffling and FA dynamics [32]. In support of this, our findings in beta cells indicate that ROCK appears to regulate glucose-induced central actin filaments contrary to MLCK, which regulates γ-actin protrusion and its co-localisation with MHC IIA at the cell periphery. In fibroblasts, dynamic turnover of peripheral FAs is mediated by MLC phosphorylation through MLCK [32], and, in a similar fashion, ML7 inhibited glucose-induced FA formation and FA protein phosphorylation at the periphery of the beta cells. By contrast, ROCK inhibition has no effect on the number of glucose-induced peripheral FAs. MLCK is thus suggested positively to regulate γ-actin protrusion and FA formation, which are implicated in the activation of GSIS through NM II remodelling. Numerous studies confirm the heterogeneous character and dynamics of FAs, with divergent functions in response to an upstream stimulus depending on the orderly recruitment of myriad proteins and adaptors centred around FAK [36]. In rat primary beta cells, ROCK inhibition increases GSIS but not basal secretion [24]. However, in the present study there was no effect of ROCK inhibition on peripheral FA number or FA protein phosphorylation following glucose stimulation. This suggests that FA composition and disposition, rather than number per se, govern secretion, as described in previous reports [36, 37].

Myosin II activity is determined by phosphorylation of its regulatory MLC by MLCK or ROCK [7]. Involvement of MLCK in insulin secretion has been previously described [25, 38], but our findings add a specific role for NM II. We also observed that upon glucose stimulation MLCK specifically regulates remodelling of MHC IIA, presumably secondary to MLC phosphorylation. NM II regulation may also occur directly through phosphorylation of heavy chains but this remains controversial. A couple of interesting studies in beta cell lines have shown that MHC modifications do play a major role in insulin secretion [26, 39]. The possible interplay between MLC phosphorylation by MLCK and MHC phosphorylation merits further investigation but is beyond the scope of the present study.

Our results also point toward NM II as a mediator of granule shuttling at the basal membrane. NM II could act either directly on granule fusion or indirectly by regulation of molecular mechanisms involved in secretion such as actin and FA remodelling [37, 40]. Our results suggest that NM II regulates glucose-induced exocytosis through actin remodelling. As suggested by previous studies, secretory granules use actomyosin filaments as 'rails' to move to the basal membrane [41], but actomyosin could also act as a 'barrier' regulating secretory granule pool accessibility to the membrane [42]. These two hypotheses are not mutually exclusive, particularly since two insulin vesicle pools exist that are differentially regulated through actin remodelling [43]. We show that glucose induces MLCK-mediated formation of actomyosin IIA filaments and that MLCK and NM II inhibition decreased insulin granule movement and localisation at the basal membrane. Our data suggest that insulin granules use actomyosin II filamentous rails for movement towards the basal membrane after glucose stimulation, with MLCK as an upstream regulator. On the other hand, the inhibitory action of ROCK on GSIS [24] and the disassembly of central actin stress fibres induced by Y27632 suggest that ROCK probably controls central actin remodelling to restrain insulin granule access towards the membrane. NM II could also recruit FA proteins to the basal membrane, albeit without direct interaction between PAX and MHC II. Surprisingly, we show that MHC IIA is also involved in the regulation of FAK and PAX protein levels. As previously observed in other secretory cells [40], these data suggest a role for actomyosin II in the transport of insulin granules to the plasma membrane also through the regulation of FA formation and FA protein levels in beta cells.

Some cellular functions of NM II are isoform-specific, whereas others are redundant [36]. MHC IIA has been suggested to have a major role in GSIS [13]. We hereby confirm this and provide a novel insight into its mode of action. Although both isoforms regulate glucose-induced actin remodelling, only MHC IIA controls GSIS, FA formation, and FAK and PAX protein levels without compensatory or additive effects between the two isoforms. While each isoform may thus be subject to differential regulation and/or impact on different downstream pathways in beta cells, MHC IIA is involved in two essential secretory processes: it is an upstream regulator of the FA signalling pathway and modulates actin remodelling upon glucose stimulation.

We show that glucose-induced activation of the MLCK–MHC IIA–FAK–PAX–ERK signalling pathway promotes insulin secretion, leading to the hypothesis that this pathway may be perturbed in type 2 diabetes. Although there is decreased beta cell mass in type 2 diabetes [44], this probably does not account for the observed beta cell dysfunction, suggesting another mechanism [1]. Most recently, it has been proposed that the decrease in insulin-positive cells in type 2 diabetes is due to beta cell dedifferentiation and reversion to progenitor-like cells [45]. Although involvement of NM in dedifferentiation is poorly documented, non-muscle MHC is used as a marker of dedifferentiation in vascular smooth muscle cells [46], and NM II production is upregulated during differentiation of mesenchymal stem cells [19, 47]. Analysis of NM II isoform levels and activity in type 2 diabetic beta cells and their potential role in beta cell failure thus warrants further investigation.

In conclusion, NM II manages several major processes involved in the beta cell response to glucose, including γ-actin protrusion and remodelling, FA assembly and turnover, and the secretory granule approach to the basal membrane. Our findings thereby further identify MHC IIA as a master regulator of beta cell secretory function.

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Contribution statement CA designed the experiments, researched the data, contributed to the discussion and wrote the manuscript. DR researched the data, contributed to the discussion and reviewed the manuscript. PAH contributed to the study design and discussion, and reviewed the manuscript. All authors provided final approval of the manuscript.

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