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Title page

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

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Abstract

Aims/hypothesis Actin and focal adhesion (FAs) remodelling are essential for glucose-stimulated insulin secretion (GSIS). Non-muscle myosin II (NMII) isoforms have been implicated in such remodelling in other cell types, and myosin light chain (MLCK) and Rho kinase (ROCK) are upstream regulators of NMII known to be involved in GSIS. The aim of this work was to elucidate the implication and the regulation of NM IIA and IIB in beta cell actin and FA remodelling, granule trafficking and GSIS.
Methods Inhibitors of MLCK, ROCK and NMII were used to study NMII 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 TIRF microscopy using MIN6B1 cells.
Results NMII and MLCK inhibition inhibited GSIS, associated with shortening of peripheral actin stress fibres, and decreased 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 reorganization was blunted by MLCK inhibition and NM IIA knockdown decreased GSIS, expression of FA proteins and glucose-induced ERK1/2 phosphorylation.
Conclusions 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 expression.

Keywords non-muscle myosin II, actin remodelling, focal adhesion, beta cell, insulin secretion

Abbreviations

FA	Focal adhesion
FAK	Focal adhesion kinase
GSIS	Glucose-stimulated insulin secretion
MHC	Myosin heavy chain
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NMII	Non-muscle myosin II
PAX	Paxillin
ROCK	Rho-associated coiled-coil containing kinase
TIRF	Total internal reflection fluorescence

Introduction

Pathogenesis of beta cell dysfunction in T2D is characterized by progressive loss of beta cell function and mass [1]. A better understanding of the beta cell secretory pathway might reveal new targets for the treatment of T2D. Filamentous actin (F-actin) and focal adhesion (FA) remodelling in beta cells play a crucial role in insulin secretion [2, 3]. In beta cells, 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 ERK1/2 through β1-integrin engaged with the extracellular matrix (ECM) [2-4]. F-actin reorganization following glucose stimulation is a pre-requisite 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 localization 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) dependant functions including stress fibre organization [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 Rho kinase (ROCK) activate NM II through MLC phosphorylation, which induces FA maturation and contributes to F-actin polymerization in stress fibres [7, 23]. In beta cells, Rho-ROCK signalling contributes to the stabilization of the actin cytoskeleton and inhibits GSIS [24] while MLCK co-localizes with insulin granules [25]. Other studies correlated insulin secretion with MHC phosphorylation and revealed co-localization 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 now studied the role of NM II isoforms and their upstream regulators MLCK and ROCK in beta cell function. We highlight the MLCK- NM IIA - FAK/PAX- ERK pathway as a positive regulator of insulin secretion. NM 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); -

phospho(Y397)FAK and -phospho(Y118)paxillin: Invitrogen (Carlsbad, CA); -paxillin: BD Transduction Laboratories (San Jose, CA); -ERK1/2, -phospho(T202/Y204)ERK1/2 and -actin: Cell Signalling Technology (Berverly, MA); -gamma-actin a gift from Dr. Christine Chaponnier (University of Geneva, Switzerland); -MHC IIA and -MHC IIB: Covance Research Products (Emeryville, CA); 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); ML7: Sigma Chemical Company (St. Louis, MO).

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 bottom microwell dishes (MatTek, Ashland, MA) coated with 804G-ECM for TIRF microscopy and confocal live imaging [30].

Expression vectors The pEGFPC1-FAK plasmid was a gift from Dr. David D. Schlaepfer (University of California, San Diego), paxillin-pEGFP was from AddGene (Cambridge, MA) and NPY-cherry was a gift from Dr. Guy Rutter (Imperial College, London, UK).

RNAi-mediated silencing of MHC IIA and IIB MHC IIA and IIB expression was knocked-down by transfecting primary rat beta cells twice with two specific siRNAs directed towards rat MHC IIA or IIB mRNA (ESM Table 1) (Microsynth AG, Balgach, Switzerland), using Lipofectamine^{TM} 2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were incubated 72 h to allow siRNA expression before cell treatment and analysis.

SDS-PAGE and Western blotting Protein samples were prepared and immunoblots analyzed as described [2]. Western blots were quantified by densitometry and band density of phosphoproteins normalized 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^{TM} 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 63x oil immersion lens and images acquired and processed using LSM510 software (Carl Zeiss AG, Germany). Confocal live imaging was performed with a Nikon A1r microscope (Nikon, Tokyo, Japan) equipped with a 60x CFI plan Apo objective and a filter optimized for mCherry fluorescence. Cells on 804G-coated glass-bottom 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, preincubated 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 Prim 5 software by one way ANOVA when more than two

conditions were compared and Student's *t* test for unpaired groups for comparison of two conditions. *P* values less than 0.05 were considered significant.

Results

Impact of NMII 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 dependant 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 shown previously by us [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, NMII and ROCK on actin and FA remodelling in rat primary beta cells In view of the important role of FA and F-actin reorganization in the regulation of GSIS and the known role of NMII in such reorganization in other cell types, we next studied the effects of MLCK, NMII 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 formation of actin bundles at the cell periphery; both inhibitors induced membrane protrusions compared to control conditions. Blebbistatin completely modified cell morphology including formation of large blebs in absence or in presence of high glucose, blunted glucose-induced actin reorganization 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 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 NMII 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 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, NMII 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 (16.7mmol/l; 10 min) decreased in 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 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 GSIS signalling pathway regulated by both NMII and ROCK in an opposite 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 localization 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 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-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 NMII inhibition (Fig. 4b). These data suggest that active MLCK-NMII 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-NMII and ROCK regulate beta cell secretory function in an opposing way.

The effect of NMII 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 seems to induce a decrease in insulin granule velocity (ESM Videos 1-2).

MLCK inhibition blunts glucose-induced MHC IIA remodelling, and gamma-actin protrusion formation and co-localization with MHC IIA at the cell periphery of rat primary beta cells In view of the potential differential role of NMII 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 (hereafter referred to as NMIIA and NMIIB) were concentrated at the cell periphery and co-localize with F-actin with a more diffuse signal towards the centre of the cells. Furthermore, we observed co-localization of MHC IIA and IIB with PAX in the cytoplasm. Upon short-term glucose stimulation, there was rapid reorganization of both MHC IIA and IIB, coinciding with actin co-localization in filaments (Fig. 5a-b). We did not observe co-localization of PAX and MHC IIA or MHC IIB at filopodial extensions suggesting an indirect role of MHC II on FA maturation and/or stabilization. We next examined the effect of MLCK, NMII 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-localized with both MHC IIA and PAX (Fig. 5a). MLCK inhibition blunted the glucose effect on actomyosin IIA bundle formation with a MHC IIA localization similar to 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 localization of both isoforms (Fig. 5a and 5b lower picture). Most interestingly, this set of images indicates that only glucose-induced MHC IIA reorganization was blunted by MLCK inhibition and confirmed that MLCK-myosin IIA seems to 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 gamma actin has been localized 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 of gamma-actin containing protrusions in response to glucose, and co-localization with MHC IIA filaments at the cell periphery. MLCK inhibition induced a decrease in protrusions containing gamma-actin and its co-localization with MHC IIA filaments (Fig. 5c).

MHC IIA and IIB are necessary for glucose-induced actin remodelling and PAX localization in rat primary beta cell protrusions. In another strategy to discriminate between the roles of each NMII isoform in beta cell function, isoform specific siRNAs were developed to characterize any loss-of-function associated phenotype. RNAi-mediated silencing of NMII isoforms resulted in 30.9±4.2% ($p<0.05$) and 36.6±2.2% ($p<0.01$) reduction in protein expression level 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 expression level respectively but also clearly induced cell death (data not shown). We therefore decided to not include the double knockdown in the following experiments. 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-d). Knockdown of MHC IIB had no influence on PAX-containing FA formation induced by glucose but blunted actin reorganization, 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, 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 expression 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. 7c). Taken together these results indicate that MHC IIA plays an important role in GSIS through the regulation of FAK and PAX protein expression and ERK phosphorylation necessary for FA maturation in response to glucose.

Discussion

Taken together, the results indicate a central role for NMIIA in beta cell function, serving as a point of convergence between glucose-induced actin and FA remodelling, and GSIS. NMII regulates actin organization as well as the subcellular localization 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-localize with F-actin, only MHC IIA appears critical for the regulation of FAs and GSIS. MLCK has been shown to regulate NMII [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 involve 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 that regulates gamma-actin protrusion and its co-localization 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 beta cells. By contrast, ROCK inhibition has no effect on the number of glucose-induced peripheral FAs. MLCK is thus suggested to positively regulate gamma-actin protrusion and FA formation which are implicated in activation of GSIS through NMII 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 was previously described [25, 38] but our findings add a specific role for NMII to this. We also observed that upon glucose stimulation MLCK regulates specifically remodelling of MHC IIA, presumably secondary to MLC phosphorylation. NMII 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 NMII as a mediator of granule shuttling at the basal membrane. NMII 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 NMII 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 vesicles 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 NMII inhibition decreased insulin granule

movement and localization 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 the Y27632 suggest that ROCK probably controls central actin remodelling to restrain insulin granule access towards the membrane. NMII 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 expression. 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 expression in beta cells.

Some cellular functions of NMII 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 while providing 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 expression 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 might be perturbed in T2D. Although there is decreased beta cell mass in T2D [44], this likely 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 T2D is due to beta cell dedifferentiation and reversion to progenitor-like cells [45]. Although involvement of NM in dedifferentiation is poorly documented, nonmuscle MHC is used as a marker of dedifferentiation in vascular smooth muscle cells [46], and NMII expression is upregulated during differentiation of mesenchymal stem cells [19, 47]. Analysis of NMII isoform expression and activity in T2D beta cells and their potential role in beta cell failure thus warrants further investigation.

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

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manuscript.

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

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Figure Legends

Fig. 1 Inhibition of Myosin II and MLCK decreases glucose-stimulated insulin secretion. Cells were preincubated 2 h at 2.8 mmol/l glucose and basal secretion measured during a further 1 h at this same glucose concentration in the continued presence or not (CTL) of inhibitor ((a) 25µmol/l or 50 µmol/l blebbistatin, (b) 20µmol/l ML7), and then stimulated with high glucose (16.7 mmol/l) in presence of the different inhibitors for 1 h (white bars: basal, black bars: stimulated). Data are mean ±SEM, n=4 independent experiments (t-test; *p<0.05; **p<0.01).

Fig. 2 Effect of the different inhibitors on actin remodelling and focal adhesion morphology and number. Primary beta cell cells were cultured for 3 h in low glucose (2.8mmol/l) in presence of inhibitor (25µmol/l blebbistatin, 20µmol/l ML7 or 50µmol/l Y27632) or not (CTL), and stimulated with high glucose (16.7mmol/l) for 10 min in the presence of the respective inhibitor or not. (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. Focal adhesions containing pFAK were counted in each cell (white bars: 2.8mmol/l, black bars: 16.7mol/l). Data are mean ± SEM, n=3 independent experiments (one way ANOVA *p<0.05; **p<0.01, ns: non significant). Scale bar: 10µm.

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 (2.8mmol/l) in presence of inhibitor (20µmol/l ML7 or 50µmol/l Y27632), or not (CTL), and stimulated with high glucose (16.7mmol/l) for 10 min in the presence of the respective inhibitor or not. Cell lysates were analyzed by western blot, and the relative intensities of phosphorylated and total protein bands were quantified by densitometry and expressed as a ratio in comparison to control non-stimulated cells, and normalized to total actin (white bars: 2.8mmol/l, black bars: 16.7mol/l). (a) pY397FAK/FAK total (b) pY118PAX/PAX total (c) pT202/Y204 ERK1/2/ERK total. Data are mean ± SEM, n=3 independent experiments (one way ANOVA *p<0.05; **p<0.01).

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 (2.8mmol/l) in presence of inhibitor (25µmol/l blebbistatin, 20µmol/l ML7 or 50µmol/l Y27632) or not (CTL) for 3 h, and stimulated with high glucose (16.7mmol/l) in 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 colour 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 at the basal membrane and the plasma membrane area were quantified by Image J and expressed as a ratio of arbitrary units (white bar: 2.8mmol/l, black bars: 16.7mol/l). Data are means ± SEM, n=3 independent experiments (one way ANOVA ***p<0.001, ns: non significant). (c) Insulin granule movement was observed in presence of the different inhibitors using confocal microscope live cell imaging. After transfection, cells were treated without glucose in presence of inhibitor or not for 3 h, and stimulated with high glucose (20mmol/l) in presence of inhibitors or not (CTL) for 10 min. Pictures were taken every 5 seconds during 5 min. All images are fully representative of 3 independent experiments. Scale bar: 10µm.

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

Fig. 6 MHC IIA and IIB are necessary for glucose-induced actin remodelling and PAX localization 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. Then, cells were cultured for 3 h in low glucose (2.8mmol/l) and stimulated with high glucose (16.7mmol/l) for 10 min. (a) Cell lysates were analyzed 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 to scrambled siRNA condition and normalized to total actin. Data are means ± SEM, n=5 independent experiments (one way ANOVA *$p<0.05$; **$p<0.01$). (b) After glucose stimulation, cells were fixed and stained for MHC IIA or MHC IIB, actin (phalloidin) and Paxillin. All images are fully representative of 3 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 MHCIIA (IIA siRNA), MHCIIB (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 (2.8mmol/l) and stimulated with high glucose (16.7mmol/l) for 1h (see Legend to Fig. 1). Data are expressed as percentage of total insulin content (white bars: basal, black bars: stimulated). (b) Cell lysates were analyzed by western blot, and the relative intensities of FAK, PAX, ERK and phospho-ERK were quantified by densitometry and expressed as a ratio in comparison to the control condition with scrambled siRNA and normalized to total actin. Data are mean ±SEM from four independent experiments (one way ANOVA *$p<0.05$, **$p<0.01$).