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"Análise das vias de sinalização intracelulares em linhagens de células produtoras de insulina expostas ao INGAP-PP"

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RESUMO

Resumo

INGAP (*Islet Neogenesis Associated Protein*), um peptídeo primeiramente identificado em *hamsters,* cujo pâncreas foi previamente embrulhado em papel celofane, tem como função principal induzir neogênese e diferenciação de células beta pancreáticas, além de melhorar a secreção de insulina induzida por glicose e aminoácidos. Existem poucas informações a respeito dos mecanismos intracelulares desencadeados pelo INGAP em células beta. Assim, este trabalho teve como objetivo estudar tais mecanismos em 2 linhagens de células beta pancreáticas produtoras de insulina, denominadas RINm5F e MIN6. Para isso foi utilizado o INGAP-PP ($INGAP^{104-118}$) constituído por uma seqüência de 15 aminoácidos e que mantém as mesmas propriedades da molécula do INGAP. Durante o procedimento experimental foi realizado um "*screening*" dos elementos responsivos a alguns fatores de transcrição. A expressão de algumas proteínas como receptor muscarínico M3, p85, AKT, $p70^{S6k}$, PCNA e NFκB foi analisada, assim como a secreção de insulina estimulada por glicose, a mobilização de cálcio intracelular e a medida de viabilidade celular. A exposição das células MIN6 ao INGAP-PP aumentou a secreção de insulina induzida por glicose assim como a mobilização intracelular de cálcio. INGAP-PP ativou os fatores de transcrição c-Myc, SRE e em especial o NFκB nas duas linhagens celulares. A viabilidade celular também foi aumentada nas células expostas ao INGAP-PP a qual foi acompanhada de aumento na expressão de PCNA, proteína diretamente relacionada com a progressão do ciclo celular. Ainda, a expressão protéica do receptor muscarínico M3 foi aumentada na presença de INGAP-PP. Esse efeito foi bloqueado pela pré-exposição das células a um inibidor farmacológico do NFκB. Pode-se concluir que a ativação moderada do c-Myc e o aumento na expressão de PCNA estão relacionados com o aumento na viabilidade celular. Adicionalmente, conclui-se que a ativação moderada do NFκB induzida pelo INGAP-PP controla direta ou indiretamente a expressão do receptor M3 e tal processo pode estar relacionado sinergicamente com o aumento na proliferação celular.

ABSTRACT

Abstract

The pentadecapeptide comprising the 104-118 aminoacid sequence of the ilotropin-derived Reg3-related islet neogenesis associated protein (INGA-PP) has been implicated in pancreatic beta-cell neogenesis and enhancement of the insulin secretion in pancreatic islets. There is little information regarding the mechanism of action of the polypeptide. The aim of this study was to investigate intracellular pathways by which INGAP-PP signs insulin-producing cells. The results show that INGAP-PP increased the insulin secretion and induced mobilization of intracellular calcium in MIN6 cells. INGAP-PP exposure activated c-Myc, serum response element (SRE) and particularly nuclear factor kappa B (NFκB) in both MIN6 and RINm5F insulin-producing cells. There was an increase in the proliferation rate of viable cells that was accompanied by an increase in the proliferating cell nuclear antigen (PCNA) protein expression following INGAP-PP treatment. In addition, INGAP-PP increased the expression of the muscarinic M3 receptor subtype. This effect was impaired by blocking NFκB signaling pathway. Cells incubated in the presence of foetal calf serum (FCS) also showed increased M3 receptor expression. In conclusion, these data show that activation of c-Myc signaling pathway and increased PCNA expression might be involved in the increased proliferation rate of insulin-producing cells following incubation with INGAP-PP. NFκB signaling plays an essential role in controlling the expression of the acetylcholine M3 receptor.

LISTA DE ABREVIATURAS

Lista de Abreviaturas

AKT - thymona viral oncogene homolog 1

AP-1 - activator protein 1

ATF-2 - activating transcription factor-2

ATP - adenosine triphosphate

c-Myc - myeloid leukemia in chicken

CRE - cAMP (cyclic adenosine monophosphate) response element

DAG – diacylglycerol

DNA - Deoxyribonucleic acid

EDTA - ethylenediamine tetraacetic acid

eNOS - endothelial nitric oxide synthase

ERK - extracellular signal-activated kinase

FCS - foetal calf serum

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMG-1 - high mobility group protein-1

HRP - horseradish peroxidise

IκB - inhibitor of NF-kappaB

IFN gamma - interferon gamma

IKK - inhibitor of NF-kappaB kinase

IL-1 beta - interleukin 1 beta

INGAP-PP - islet neogenesis associated protein polypeptide

iNOS - nitric oxide synthase

INS-1 - insulin-secreting cells

IP3 - inositol 1,4,5-triphosphate

IRF-6 - interferon regulatory transcription factor 6

IRS-1 - insulin receptor substrate 1

IRS-2 - insulin receptor substrate 2

JAK - Janus-activated kinase

JNK - jun N-terminal kinase

M3 - cholinergic muscarinic 3

MAPK - mitogen-activated protein kinase

MEKK-1 - mitogen-activated protein kinase kinase 1

MIN6 - mouse insulinoma 6

MTS-3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl) -2H-tetrazolium

NFAT - nuclear factor of activated T-cells

NFκB - nuclear factor kappa B

$p70^{S6K}$ - protein 70 S6 kinase

PBS - phosphate buffered saline

PCNA - proliferating cell nuclear antigen

PDTC - pyrrolidine dithiocarbamate

Pdx-1 - pancreatic duodenal homeobox

PI3K - phosphatidylinositol 3'kinase

PKC - protein kinase C

PLC - phospholipase C

PMA - phorbol 12-myristate 13-acetate

PMSF - phenylmethylsulphonyl fluoride

Reg proteins - regenerating proteins

RIA - radioimmunoassay

RINm5F - rat insulinoma m5F

RNA - ribonucleic acid

RPMI 1640 - Roswell Park Memorial Institute

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEAP - secreted alkaline phosphatase

SRE - serum response element

T1DM - type 1 diabetes mellitus

T2DM - type 2 diabetes mellitus

TNF alpha - tumor necrosis factor alpha

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INTRODUÇÃO

Introdução

1. *Pâncreas Endócrino e INGAP (Islet Neogenesis Associated Protein)*

O Diabetes mellitus, doença caracterizada por aumento nas concentrações de glicose plasmática, resulta de defeitos na secreção e/ou na ação da insulina, hormônio produzido e secretado pelas células beta pancreáticas e de fundamental importância na manutenção de concentrações adequadas de nutrientes circulantes, especialmente da glicose (Deeney *et al.,* 2000).

A conservação da massa adequada e funcional de células secretoras de insulina é essencial para o indivíduo responder à demanda desse hormônio nos vários períodos de vida (embrionário, fetal, neonatal e adulto) e em situações de alterações fisiológicas (jejum, pós-alimentar, estresse) em quantidade e tempo adequados, mantendo assim a normoglicemia. A formação dos diferentes tipos celulares durante a gênese das ilhotas pancreáticas depende de atuação dinâmica de vários fatores de transcrição, hormônios e sinais neuronais. Durante a vida adulta, a manutenção de massa insular adequada depende da reposição das células beta perdidas. Acredita-se que esse processo se deve à replicação de células beta pré-existentes e diferenciação de novas células a partir de precursores presentes nas células extra-ilhotas (Bouwens *et al.,* 1994; Butler *et al.,* 2003; Dor *et al.,* 2004, Rosenberg *et al.,* 2004). No entanto, neogênese considerável pode ser observada em pâncreas de animais adultos em situações de regeneração induzida por processos lesivos tais como: pancreatectomia parcial (Lee *et al.,* 1989), ligação do ducto pancreático (Wang *et al.,* 1995, Xu *et al.,* 2008) administração de estreptozotocina (Fernandes *et al.,* 1997) ou empacotamento da cabeça do pâncreas em papel celofane (Rosenberg *et al*., 1983).

Formado por 175 aminoácidos (mmlpmtlcrm swmllsclmf lswvegeesq kklpssritc pqgsvaygsy cyslilipqt wsnaelscqm hfsghlafll stgeitfvss lvknsltayq yiwiglhdps hgtlpngsgw kwsssnvltf ynwernpsia adrgycavls qksgfqkwrd fncenelpyi ckfkv), o INGAP é considerado membro da família

de proteínas relacionadas à regeneração (RegIII) (Taylor-Fishwick *et al.,* 2003) e componente da ilotropina, proteína associada ao crescimento e diferenciação do pâncreas endócrino.

O INGAP foi primeiramente identificado em *hamsters* cujo pâncreas foi previamente embrulhado em papel celofane (Pittenger *et al.,*1992), condição de hipóxia/estresse mecânico, que induz a formação de novas ilhotas a partir de células do ducto pancreático (Bonner-Weir *et al.,* 1993; Leahy, 1996). Sua principal função parece estar relacionada à neogênese e diferenciação de células beta pancreáticas, embora ligação fraca do INGAP a outros tecidos tais como fígado e duodeno, foi observada (Rafaeloff *et al.,* 1997; Flores *et al.,* 2003; Borelli *et al.,* 2007).

Evidências sugerem que células positivas para Pdx-1/INGAP presentes nas ilhotas pancreáticas representem uma população de precursores de células beta em estágios iniciais de desenvolvimento (Gagliardino *et al.,* 2003), indicando o envolvimento do INGAP no processo de neogênese.

Através de técnicas de clonagem e seqüenciamento, foi identificado um pentadecapeptídeo contendo o intervalo de seqüência de 104 a 118 resíduos de aminoácidos que constituem a molécula do INGAP. Denominado INGAP-PP ou $INGAP^{104-118}$, esse polipeptídeo, cuja seqüência apresenta os seguintes resíduos de aminoácidos (iglhdpshgt lpngs), possui as mesmas propriedades e reproduz o mesmo efeito da molécula intacta (Rafaeloff *et al.,* 1992).

Existem poucas informações sobre os mecanismos intracelulares envolvidos durante a atuação do INGAP sobre as células beta pancreáticas. Foi observado que ilhotas de ratos neonatos expostas ao INGAP-PP tiveram aumento na expressão de diversos genes, vários dos quais envolvidos na maturação, diferenciação e função das células beta pancreáticas, evidenciando papel importante desse polipeptídeo no processo de secreção de insulina (Barbosa *et al.,* 2006). Ilhotas humanas que adquiriram características de células ductais após 10 dias de cultivo em meio DMEM/F12 voltaram a apresentar características estruturais e secreção de insulina estimulada por glicose, semelhantes à de ilhotas normais recém isoladas após exposição ao INGAP-PP. Essa reconstituição da ilhota pancreática é dependente da ativação da via da PI3K (Jamal *et al.,* 2005).

Recentemente, foi demonstrado que a exposição aguda ao polipeptídeo INGAP de ilhotas de ratos neonatos ativa proteínas *downstream* da via de sinalização da insulina, assim como aumento na expressão de proteínas da via colinérgica, sugerindo que o efeito autócrino da via insulínica e o sistema nervoso parassimpático podem ser fatores responsáveis pela melhora na secreção de insulina após tratamento com esse polipeptídeo (Barbosa *et al.,* 2008).

2. *Secreção de Insulina e Via Colinérgica*

A síntese e secreção de insulina são processos regulados por inúmeros fatores. Os estimuladores mais importantes são nutrientes, como glicose e aminoácidos. Existe ainda a participação de hormônios, neurotransmissores e citocinas evidenciando um controle multifatorial sobre as células beta pancreática para proporcionar a homeostase glicêmica.

O sistema nervoso autonômico desempenha função importante na secreção de insulina. O pâncreas endócrino recebe nervos provenientes do sistema nervoso simpático (adrenérgico) e parassimpático (colinérgico). Enquanto o primeiro possui atividade redutora na secreção, agindo por meio do receptor adrenérgico alfa2, o segundo possui atividade potencializadora, agindo por meio do receptor colinérgico M3 (Boschero *et al.,* 1995; Ahren, 2000; Gautam *et al.,* 2006). O principal neurotransmissor secretado pelas fibras vagais é a acetilcolina (Bloom *et al.,* 1985; Verchere *et al.,* 1991).

A acetilcolina e seu análogo carbacol agem por meio de receptores muscarínicos acoplados à proteína G (Karlsson *et al.,* 1998; Renuka *et al.,* 2006). Foram descritos 5 subtipos de receptores muscarínicos, denominados de M1 a M5. Os receptores muscarínicos M1 e M3 são os subtipos predominantes nas ilhotas pancreáticas (Boschero *et al.,* 1995; Zawalich *et al.,* 1989) sendo que o M3 desempenha função importante no processo de secreção de insulina, através da ativação da fosfolipase C (PLC), que hidrolisa fosfoinositídeos de membrana liberando diacilglicerol (DAG) e inositol 3-fosfato (IP3). O IP3 liberado é responsável pela mobilização dos íons cálcio do retículo

endoplasmático para o citoplasma. Esses íons ligam-se ao DAG formando o complexo DAG-cálcio-fosfatidilserina, essencial para a ativação da proteína cinase C (PKC), que promove fosforilação de elementos fundamentais na exocitose dos grânulos de insulina aumentando a liberação do conteúdo granular para o meio extracelular (Huang, 1989; Rhee *et al.,* 1992).

Estimuladores da via colinérgica que agem sobre receptores muscarínicos têm participação sobre o crescimento e viabilidade celulares em diferentes tecidos, modulando diversas vias intracelulares. Foi demonstrado que exposição ao carbacol de linhagem humana de câncer de mama aumentou a mobilização de cálcio intracelular e a fosforilação de MAPK/ERK com conseqüente aumento da síntese protéica e da proliferação celular (Jimenez *et al.,* 2005). Astrocitomas apresentaram aumento da atividade da $p70^{S6k1}$ via PI3K através da ativação do receptor muscarínico M3 pelo carbacol (Tang *et al.,* 2003). Recentemente, foi descoberta uma nova via de sinalização estimulada por acetilcolina que envolve a participação das proteínas JAK-2/IRS-1/PI3K/AKT1/eNOS/ERK1-2 na aorta de ratos obesos que são simultaneamente resistentes a insulina e a acetilcolina (Zecchin *et al.,* 2007). O aumento na expressão de receptores muscarínicos M1 e M3 está relacionado tanto ao aumento na proliferação celular da ilhota durante a regeneração pancreática quanto ao aumento na secreção de insulina (Renuka *et al.,* 2005), que por sua vez pode modular o crescimento e diferenciação de célula beta via ativação de IRS-2 (Velloso *et al.,* 1995). Após exposição à oxotremorina M (agonista do receptor M3), neuroblastomas apresentaram aumento na atividade do fator de transcrição NFκB (Choi *et al.,* 2006).

O controle da expressão do receptor muscarínico M3 é um processo complexo que compreende a ação de estímulos externos e internos resultando na modulação da sua transcrição gênica. Muitas seqüências de reconhecimento para diversos moduladores (co-fatores, estimuladores, repressores) localizadas *upstream* ao gene do receptor M3, incluindo elementos proximais e distais, podem estar envolvidos na regulação desse processo (Forsythe *et al.,* 2002).

3. Fator de Transcrição NFκB (Nuclear Factor kappa B)

Os fatores de transcrição fazem parte de uma classe de proteínas que tem como função principal auxiliar no posicionamento correto da RNA polimerase à região promotora de um determinado gene para o início da transcrição. O NFκB é um fator de transcrição que regula a expressão de indutores e efetores da resposta celular imune/inflamatória (Ghosh *et al.,* 1998), além de regular divisão celular (Hayden and Ghosh, 2004) e apoptose (Kuntzen *et al.,* 2007). A família do NFκB é composta por 5 subunidades denominadas p50, p52, p65, c-Rel e RelB, que compartilham o domínio homólogo Rel localizado na região N-terminal da molécula. O domínio Rel é responsável tanto pela ligação do fator de transcrição na fita de DNA quanto pela homo e heterodimerização das subunidades (Hayden and Ghosh, 2008).

Na sua forma inativa, os dímeros de NFκB encontram-se no citosol ligados à proteínas inibitórias IκB ou à proteínas precursoras p100 e p105. São conhecidos 3 subtipos principais de proteínas IκB: IκB alfa, IκB beta e IκB épsilon. Membro atípico da família dos IκBs, a proteína BCL3 localiza-se no núcleo e age como co-ativador transcricional do homodímero p50/p50 (Perkins, 2007). A inibição da translocação do NFκB para o núcleo e subseqüente ligação ao DNA é feita através do bloqueio da seqüencia de localização nuclear de alguma subunidade do NFκB pelo IκB (Ghosh and Karin, 2002). Estimuladores como citocinas pró-inflamatórias (TNF alfa, IL-1, IFN gamma) (Royuela *et al.,* 2008), ésteres de forbol (PMA) (Kim *et al.,* 2000), lipopolissacarídeos (Wang *et al.,* 2000), dentre outros, ativam proteínas IKK (IκB cinases) responsáveis pela fosforilação do IκB em resíduos de serina e treonina. Os IκBs fosforilados sofrem rápida degradação dependente de ubiquitinação pelo proteasomo 26S, resultando no desligamento do complexo IκB-NFκB. A seqüencia de localização nuclear do NFκB exposta, leva à translocação do dímero para o núcleo, promovendo a transcrição de genes específicos geralmente relacionados à resposta imune (Hayden and Ghosh, 2008).

Dependendo do tipo celular, do estímulo utilizado e do grau de ativação do fator de transcrição, o NFκB pode desempenhar funções anti ou pró-apoptóticas. O papel do NFκB nas

células beta pancreáticas é controverso (Naamane *et al.,* 2007). Muitos trabalhos apontam para um efeito pró-apoptótico do NFκB. Foi demonstrado que linhagens de células beta INS-1, quando expostas tanto a altas concentrações de IFN gamma e TNF alfa (Sekine *et al.,* 2000) quanto a agentes quimioterápicos como daunorubicina/doxorubicina (Campbell *et al.,* 2004), apresentaram aumento na expressão de iNOS e na taxa de apoptose, através da ativação do NFκB. O aumento na expressão de iNOS e na taxa de apoptose parece estar relacionado com a ativação de proteínas mediadoras como MEKK-1 e JNK (Mokhtari *et al.,* 2008). Por outro lado, existem algumas evidencias sobre a função anti-apoptótica do NFκB em células beta. Ilhotas de camundongos transgênicos, expressando forma mutante não degradável de IκB alfa que induz inativação permanente do NFκB, apresentaram maior taxa de morte celular após exposição ao TNF alfa (Kim *et al.,* 2007). Ainda, hepatócitos de camundongos transgênicos IKK beta -/- exibem maior taxa de apoptose, levando esses animais à morte durante o estágio embrionário (Li *et al.,* 1999).

Existem poucas informações a respeito dos mecanismos intracelulares desencadeados em células beta durante a exposição ao polipeptídeo INGAP. Assim, neste trabalho estudou-se os mecanismos de ação do INGAP-PP em 2 linhagens de células beta pancreáticas produtoras de insulina, denominadas RINm5F e MIN6. Para isso, foi realizado um *screening* dos elementos responsivos a alguns fatores de transcrição e possíveis vias de sinalização. Os resultados encontrados sugerem que o INGAP-PP sinaliza para as células beta pancreáticas através da ativação do c-Myc, SRE e NFκB, assim como aumenta a proliferação/viabilidade celular e a expressão das proteínas PCNA e receptor muscarínico M3. Foi demonstrado que a ativação do NFκB é de importância fundamental para o aumento na expressão do receptor muscarínico M3. Propõe-se que o aumento na expressão de PCNA e do receptor M3 e a ativação do c-Myc pelo INGAP-PP estão relacionados com o aumento na proliferação/viabilidade celular.

OBJETIVOS

Objetivos

Os objetivos deste trabalho foram avaliar, *in vitro*, os mecanismos de ação do INGAP-PP em linhagens de células beta produtoras de insulina RINm5F e MIN6 na:

1. secreção de insulina e mobilização de cálcio intracelular induzidas por glicose;
2. proliferação celular, através de ensaios de viabilidade celular e expressão da proteína PCNA por *Western Blot*;
3. expressão das proteínas PI3K (p85), AKT total, $p70^{S6k}$, receptor muscarínico M3 e p65 medida por *Western Blot*;
4. ativação dos fatores de transcrição AP-1, c-Myc, NFAT, NFκB, CRE e SRE através do método de gene repórter.

MATERIAIS E MÉTODOS

Materiais e Métodos

Cultura Celular

Linhagens permanentes de células beta produtoras de insulina RINm5F e MIN6 foram mantidas em placas de cultura estéril (TPP, Trasadingen, Switzerland) contendo meio RPMI 1640 (Sigma, St. Louis, USA), acrescido de 10mM de glicose, 10% de soro fetal bovino (FCS) (Nutricell, Campinas, Brasil), 100 U/ml de Penicilina (Sigma, St Louis, USA) e 100 ug/ml de Estreptomicina (Sigma, St. Louis, USA), em atmosfera humedecida contendo 5% CO_2, a temperatura constante de 37ºC. Para os estudos de expressão protéica, 5 x 10^5 células foram cultivadas em placas de 5 cm de diâmetro até atingirem 90% de confluência. Para os estudos de viabilidade celular e gene repórter, 5 x 10^4 e 1.5 x 10^5 células respectivamente foram cultivadas em placa de 96 poços. A contagem de células foi realizada em câmara de Neubauer. Posteriormente, as células foram expostas ao INGAP-PP (10μg/ml) por períodos de tempo variáveis e ao Ditiocarbamato de Pirrolidina (PDTC), inibidor da translocação da subunidade p65 do NFκB para o núcleo.

Geração de Pseudo-ilhotas

Células beta produtoras de insulina MIN6 foram mantidas em placas de vidro não aderentes por 7 dias em meio DMEM acrescido de 10% de FCS. Após este período, formaram-se naturalmente estruturas circulares semelhantes à ilhotas pancreáticas, compostas somente por células beta.

Oscilações de Cálcio

Células beta produtoras de insulina MIN6, dispostas em monocamada, foram incubadas em meio Krebs-Ringer contendo 25 mM de Hepes, 10 mM de glicose, 3% de albumina e 5 μM de

fura-2 por 30 min à 37°C. Após a exposição ao INGAP-PP, a lamínula contendo as células foi transferida para uma câmara aberta regulada termostaticamente (37°C), colocada em um microscópio invertido (Nikon UK, Kingston, UK) e perfundida com tampão Krebs-Ringer em um fluxo de 1.5 ml/min. As ondas excitatórias de 340 e 380 nm foram selecionadas por fonte de luz de xenônio e a emissão foi de 510 nm. A mudança no cálcio citoplasmático foi detectada como uma mudança na proporção F340/F380 (Lenzen *et al.,* 2000).

Secreção de Insulina

A insulina secretada pelas pseudo-ilhotas de células MIN6 expostas ao INGAP-PP (10μg/ml) foi quantificada por radioimunoensaio (Luther *et al*., 2006).

Quantificação Protéica - Western Blot

Células Rim5F e MIN6 controles e expostas ao polipeptídeo INGAP-PP foram coletadas com o auxílio de um "Scraper" (TPP, Trasadingen, Switzerland), homogeneizadas em coquetel anti-protease (EDTA 10 mM, Trisma Base 100 mM, Pirofosfato de Sódio 10 mM, Fluoreto de Sódio 100 mM, Ortovanadato de Sódio 1mM, PMSF 2 mM, Aprotinina 0,1 mg/ml) e centrifugadas por 10 min. a 12000g a 4°C. Foi adicionado ao sobrenadante Triton X100 10%, o equivalente a 10% do volume inicial. A determinação do conteúdo protéico total foi realizada em equipamento ELISA utilizando-se o método de Bradford (BioAgency, São Paulo, Brasil). Alíquotas de proteína total, acrescidas de 30% do volume em tampão Laemmili/DTT (Sigma, St. Louis, USA) foram incubadas em banho seco (100°C) por 5 min e aplicadas em gel 10% SDS-PAGE (Tris-Acrilamida) em paralelo com marcador de peso molecular conhecido (Bio-Rad, Califórnia, USA). Foram utilizados 50 μg de proteína total para a análise das proteínas p85, AKT e $p70^{S6k}$, 30μg para o PCNA e 35 μg para o receptor muscarínico M3 e p65. Após eletroforese, as proteínas foram transferidas para membrana de Nitrocelulose (Bio-Rad, Califórnia, USA) e coradas com Ponceau S (Sigma, St. Louis, USA) para verificação da eficiência da transferência

das proteínas do gel para a membrana. As membranas foram incubadas *overnight* em solução bloqueadora contendo 5% de leite desnatado. Posteriormente, foram incubadas por 6 h em solução de anticorpo primário específico monoclonal anti-p70S6 cinase α (H-9) (1:500), policlonal anti-Akt1/2/3 (H-136) (1:500), policlonal anti-PI3 cinase p85α (Z-8) (1:500), monoclonal anti-PCNA (PC10) (1:1000), e policlonal anti-p65 (C-20) (1:1000) da Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) e policlonal anti-receptor muscarínico M3 (1:2000) da Sigma Chemicals Co. (St. Louis, MO, USA). Anticorpo anti-beta actina (Abcam plc. Cambridge, UK) (1:10000) foi usado como controle interno. Solução de anticorpo secundário conjugado com HRP (InVitrogen, USA) (150ng/ml) foi adicionada as membranas por 2 h a temperatura ambiente. Para visualização das bandas as membranas foram incubadas em solução quimioluminescente (SuperSignal, Pierce Biotechnology Inc., Rockford, IL, USA) e expostas ao filme radiográfico (Kodak, Manaus, Brasil). A densitometria das bandas foi analisada por programa específico (Scion Corp., Frederick, MD, USA).

Isolamento de Frações Citoplasmática e Nuclear

O isolamento nuclear foi realizado de acordo com as instruções fornecidas pelo fabricante (Nuclei EZ Prep, Sigma Chemicals Co.). As células foram lavadas com solução de PBS estéril (NaCl 140 mM, KCl 2,6 mM, KH_2PO_4 1,4 mM, $Na_2HPO_4.2H_2O$ 8,1 mM), retiradas da placa de cultura com o auxilio de um "Scraper" e resuspendidas em 200µl de tampão de extração celular (Hepes 10 mM, $MgCl_2$ 2 mM, KCl 25 mM). Posteriormente, foram mantidas em gelo por 10 min, misturadas utilizando-se *vortex* por 10 seg e centrifugadas à 14000 g por 30 seg a 4 °C. O sobrenadante foi coletado e utilizado como amostra de fração citoplasmática. O *pellet* foi resuspendido em 30 µl de tampão de extração nuclear (Hepes 10 mM, $MgCl_2$ 2 mM, KCl 25 mM e 250 mM de sacarose), mantido em gelo por 20 min e centrifugado por 2 min a 14000 g a 4°C. O sobrenadante foi coletado e utilizado como amostra de fração nuclear. Tanto o tampão de extração celular quanto o de extração nuclear continham o coquetel de anti-proteases descrito acima.

Viabilidade Celular - MTS

As células RINm5F e MIN6 foram expostas ao INGAP-PP por 72 h em placas de cultura de 96 poços. Após esse período, as células foram lavadas com solução de PBS estéril (NaCl 140 mM, KCl 2,6 mM, KH_2PO_4 1,4 mM, $Na_2HPO_4.2H_2O$ 8,1 mM) e incubadas por 4 h em solução de MTS. A viabilidade celular, representada pela produção de NAD(P)H das células controle e expostas ao INGAP-PP, foi realizada através do método espectrofotométrico de redução do sal tetrazólio para formazana solúvel (Promega, Madison, USA). A mistura PMS/MTS foi preparada segundo as indicações do fabricante e diluída para 10% em tampão Krebs (NaCl 109 mM, KCl 4,7 mM, $CaCl_2.2H_2O$ 1,9 mM, $MgSO_4$ 1,2 mM, K_2HPO_4 1,03 mM, $NaHCO_3$ 25 mM, Hepes 20 mM, $C_6H_{12}O_6$ 11,1 mM).

Ativação de Fatores de Transcrição – Ensaio de Gene Repórter

Obtenção dos Vetores

Foram utilizados vetores de transfecção do tipo "*Mercury*" propagados em bactérias *heat shock* competentes (*E. coli,* TOP-10, Invitrogen GmbH, Karlsruhe, Alemanha). Através de choque térmico (4 - 42°C), as bactérias foram transformadas, plaqueadas e selecionadas em ágar contendo Ampicilina (Sigma Chemicals Co., St. Louis, MO, USA). Algumas colônias foram escolhidas e crescidas em meio líquido Luria-Bertani (LB) (BD, Le Pont de Claix, França). Para obtenção dos plasmídeos, as bactérias foram lisadas e os plasmídeos purificados em colunas de sílica (PureLink™ Quick Plasmid Miniprep Kit, InVitrogen, USA). Foram propagados vetores responsivos ao c-Myc, AP-1, NFAT, NFκB, CRE e SRE para transfecção transiente (Lipofectamina 2000, Invitrogen, Carlsbad, USA).

Transfecção

Após 24 h de plaqueamento em meio RPMI 1640, células Rim5F e MIN6 foram lavadas com solução estéril de PBS para a retirada completa de soro e antibióticos. Em seqüência, as células foram mantidas em meio de cultura livre de antibiótico e soro e transfectadas separadamente com os diferentes vetores por 4 h (Lipofectamina 2000, Invitrogen, Carlsbad, USA). Posteriormente, as células foram cultivadas por 18 h em meio RPMI 1640 acrescido de 1% de soro e 2% de antibióticos (solução de 100U/ml de penicilina e 100 µg/ml de estreptomicina). Após este período, as células foram mantidas em meio livre de soro e expostas ao INGAP-PP ou a substâncias utilizadas como controles positivos (para AP-1 e c-Myc, 10% de FCS; para CRE, 25 µM de Forskolin; para NFAT e NFκB, 50 ng/ml de TNF alfa e para SRE, 2 ng/ml de IL-1 beta). Foram coletadas alíquotas do meio após 6 e 24 h de exposição à essas substâncias e a fosfatase alcalina secretada pelas células como sinal do repórter foi medida utilizando-se kit comercial luminescente.

Ensaio do Gene Repórter SEAP

A fosfatase alcalina secretada no meio foi medida por quimioluminescência utilizando-se kit específico (Phospha-Light, Applied Biosystems, São Paulo, Brasil). Alíquotas da amostra foram misturadas em tampão de diluição Phospha-Light e mantidas a 65°C por 30 min para inativação das fosfatases alcalinas endógenas. Posteriormente, foi adicionado tampão contendo L-homoarginina, inibidor de fosfatase alcalina endógena, para garantir que apenas a fosfatase alcalina utilizada como sinal do ensaio de gene repórter fosse medida. Após 20 min de incubação em temperatura ambiente, o sinal quimioluminescente foi medido em luminômetro.

Análise Estatística

Os resultados foram expressos como média ± erro padrão da média. A análise estatistica foi realizada utilizando-se *Student's t-test* ou ANOVA de uma via seguida de pós teste Bonferroni ou Dunnet. $p < 0,05$ foi considerado estatisticamente diferente.

RESULTADOS E DISCUSSÃO

Resultados e Discussão

Os resultados obtidos durante a realização deste trabalho estão apresentados a seguir sob a forma de artigo.

Requirement of NFκB signalling pathway for modulation of cholinergic M3 receptor expression by INGAP-PP in insulin-producing cells

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ABSTRACT

The pentadecapeptide comprising the 104-118 aminoacid sequence of the ilotropin-derived Reg3-related islet neogenesis-associated protein (INGAP-PP) has been implicated in pancreatic beta cell neogenesis and enhancement of insulin secretion in pancreatic islets. Since there is little information regarding the mechanism of action of this peptide, the aim of this study was to investigate intracellular pathways by which INGAP-PP signals in insulin producing-cells. Treatment with INGAP-PP increased insulin secretion and intracellular calcium levels in MIN6 cells. INGAP-PP exposure activated c-Myc, serum (SRE) and particularly NFκB response elements in both MIN6 and RINm5F cells. There was an increase in the proliferation rate of viable cells that was accompanied by an increase in proliferating cell nuclear antigen (PCNA) protein expression following INGAP-PP treatment. Additionally, INGAP-PP increased the expression of the muscarinic M3 receptor subtype. Cells incubated in the presence of foetal calf serum, which activates multiple serum response elements, also showed increased M3 receptor expression. The blockade of NFκB signalling pathway strongly decreased M3 receptor expression in response to both stimuli. In summary, a network of intracellular signals that includes activation of c-Myc signalling pathway and increased PCNA expression might be related to the increased proliferation rate of insulin-producing cells following incubation with INGAP-PP. NFκB signalling plays an essential role in controlling the expression of the acetylcholine M3 receptor.

Keywords: diabetes; pseudoislets; mAChR; calcium metabolism; transcription factors; gene expression regulation.

Abbreviations: Reg, regenerating proteins; RIA, radioimmunoassay; Pdx-1, pancreatic duodenal homeobox; AKT, thymona viral oncogene homolog 1; PI3K, phosphatidylinositol 3 kinase; p70S6K, protein 70 S6 kinase; AP1, activator protein 1; CRE, cAMP response element; NFAT,

nuclear factor of activated T-cells; SEAP, secreted alkaline phosphatase; PMSF, phenylmethylsulphonyl fluoride; EDTA, ethylenediamine tetraacetic acid; TNF-alpha, tumour necrosis factor alpha; IL-1 beta, interleukin 1 beta.

1. INTRODUCTION

Loss of pancreatic beta cell mass induces diabetes, and restoration of insulin-secreting cells is a feasible strategy for the treatment of both Type 1 (T1DM) and Type 2 (T2DM) diabetes [1]. INGAP (islet neogenesis associated protein) was first identified in hamster pancreas following hypoxia/mechanical stress, conditions that induce proliferation of insular cells [2]. It has been proposed that pancreatic cells positive for Pdx-1/INGAP represents a sub-population of pancreatic islet precursors in an early stage of development [3], which suggests that INGAP is involved in the neogenesis of beta cells. Interestingly, it has been demonstrated that a pentadecapeptide containing the aminoacid sequence in the interval 104 to 118 (INGAP pentadecapeptide or INGAP-PP) possesses the same properties and induces the same effects as the whole molecule in many target cells [4]. Therefore, INGAP-PP might be an interesting pharmacological agent in the treatment of diabetes.

There is little information available on the mechanisms by which INGAP-PP exerts its effects on pancreatic beta cells. Recently, it has been shown that acute exposure of neonatal rat islets to INGAP-PP stimulates insulin secretion [5] and it is possible that INGAP-PP-induced increases in beta cell mass are secondary to the autocrine insulin signalling cascade that has been previously found to be important in maintaining pancreatic beta cell mass [6]. Loss of beta cells in either T1DM or T2DM cannot be counteracted by the normal proliferation rate. In the particular case of T2DM, a chronic process of impairment of beta cell function leads ultimately to cell death and consequently decreases beta cell mass [7]. Consequently, strategies aiming at increasing beta cell mass may be of great value in the attempts to find a cure or novel therapies for diabetes.

Glucose oxidation, and the subsequent production of ATP, is the main stimulator of insulin secretion in beta cells [8]. The fine tuning of secretion is achieved by several pathways, including the autonomic nervous system that plays a major role in controlling insulin release [9]. While sympathetic activation of adrenergic alpha-2 receptors diminishes, parasympathetic activation of cholinergic M3 receptors enhances insulin release [9-11].

The muscarinic acetylcholine receptors are members of the G-protein coupled superfamily [10]. The INGAP-PP-induced increase in cholinergic M3 receptor expression has been recently described in neonatal pancreatic islets, and up-regulation of these receptors may be one of the factors responsible for the improvement of insulin secretion after INGAP-PP treatment [5]. Control of M3 receptor expression is a complex and not completely understood process, and various regulatory sequences upstream to the M3 receptor gene, comprising proximal and distal elements, might take part in this process [12].

In this study, we investigated cell signalling pathways activated by the INGAP-PP using two models of pancreatic beta cells, namely the RINm5F and the MIN6 insulin-producing cells. For this purpose, screening of transcription factor-responsive element activation was carried out by means of reporter gene assays. Our results provide evidence that INGAP-PP signals in beta cell through activation of c-Myc, SRE and, in particular, NFκB response elements. Stimulation of the NFκB transcription factor is primarily responsible for the increase in the cholinergic M3 receptor expression following INGAP-PP treatment.

2. MATERIALS and METHODS

2.1. Materials

INGAP-PP, a pentadecapeptide with the 104–118 aminoacid sequence of INGAP (NH-Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser-COOH), was provided by GenScript Corp. (Scotch Plains, NJ, USA). Quality control of the peptide (aminoacid analysis and mass

spectrometry) indicated >95% purity and a molecular weight of 1501.63. All SDS-PAGE and immunoblotting equipment were from Bio-Rad systems (Richmond, CA, USA). Anti-NFκB p65 (C-20), a kind gift from Prof. L. A. Velloso (Campinas, Brazil), anti-p70 S6 kinase α (H-9) anti-Akt1/2/3 (H-136), anti-PI 3-kinase p85α (Z-8), and anti-PCNA (PC10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-beta actin antibody was from Abcam plc. (Cambridge, UK). Recombinant rat IL-1beta and TNF-alpha, Lipofectamine 2000, Top10 competent bacteria, and the Phospha-Light Secreted Alkaline Phosphatase Reporter Gene Assay System were from Invitrogen Corp. (Carlsbad, CA, USA). Reporter gene vectors were from Clontech (Mercury Pathway Profiling Systems, Clontech Laboratories, Inc., Mountain View, CA, USA). Nitrocellulose membranes (Hybond N, 0.45 μm) were from Amersham (Little Chalfont, Buckinghamshire, UK). The CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay was from, Promega Corp. (Madison, WI, USA). Anti-muscarinic acetylcholine receptor M3 antibody, RPMI 1640 and DMEM medium, ammonium pyrrolidinedithiocarbamate (PDTC), and all other reagents were from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2. Cell culture

RINm5F (passages 75-85) and MIN6 (passages 25-35) insulin-producing cells were cultured in RPMI 1640 or DMEM medium, respectively, supplemented with penicillin, and streptomycin in a humidified atmosphere at 37°C and 5 % CO_2. Unless otherwise indicated, FCS-free medium was used. Cells were plated at a density of 5 x 10^5 per 50-mm plastic dish and allowed to grow until reaching 90 % confluence. When using 96-well plates, 5 x 10^4 for MTS or 1.5 x 10^5 starting cells for reporter gene analysis were used. Subsequently, cells were exposed to 10 μg/ml of INGAP-PP for the desired period. This concentration was selected on the basis of previous in vitro studies with pancreatic islets [5].

2.3. Pseudoislet generation

MIN6 cells were grown in non-adherent Petri plates for 1 week in DMEM medium supplemented with 10 % FCS. Thereafter, naturally-formed pseudoislets were hand-picked using a micropipette and immediately used for the experiments.

2.4. Insulin secretion.

The effects of INGAP-PP on insulin secretion from MIN6 pseudoislets was measured by radioimmunoassay (RIA) as previously described [5, 13].

2.5. Calcium measurements

MIN6 cells attached to glass coverslips were incubated in a physiological salt solution containing 10 mmol/l Hepes, 2 mmol/l glucose, 2mmol/l calcium chloride, and supplemented with 5 µmol/l fura-2 acetoxymethyl ester for 30 min at 37°C. Measurements of changes in intracellular free Ca2+ were performed as previously described [14].

2.6. MTS cell proliferation assay

The number of proliferating viable cells was determined by the colorimetric method of reduction of tetrazolium salt into soluble formazan, according to the manufacturer's instructions.

2.7. Western blotting analyses

Control and INGAP-PP-incubated cells were homogenised by sonication in ice-cold medium containing protease inhibitors and centrifuged at 12,000 g and 4°C for 10 min. Either 30 µg of the total protein for PCNA, 35 µg for the cholinergic M3 receptor and p65 NFκB subunit, or 50 µg for all other protein expression analysis from the supernatants were resolved by electrophoresis in 10 % SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. After blocking in 5 % non-fat milk solution overnight, immunodetection was performed after an incubation period of 6 h with either a rabbit polyclonal p85 alpha antibody (1:500 dilution), a rabbit polyclonal Akt antibody (1:500 dilution), a mouse monoclonal p70S6k antibody (1:500 dilution), a mouse monoclonal PCNA antibody (1:1,000 dilution), a rabbit polyclonal cholinergic M3 receptor antibody (1:2,000 dilution), a rabbit polyclonal p65 antibody (1:1,000 dilution). A rabbit polyclonal beta actin

antibody (1:10,000 dilution) was used as a housekeeping antibody. Membranes were then exposed to 150 ng/ml specific secondary peroxidase-conjugated antibody (anti IgG (H+L)–HRP, Invitrogen) for 2 h at 22ºC, and visualized by chemiluminescence (SuperSignal, Pierce Biotechnology Inc., Rockford, IL, USA). The bands were quantified using the Scion Image software (Scion Corp., Frederick, MD, USA).

2.8. Plasmid transfection and SEAP activity quantification for the reporter gene assay

Well characterized plasmids containing responsive elements to NFκB, c-Myc, AP1, serum (SRE), cAMP (CRE), and NFAT were propagated in bacteria and purified using minipreps (PureLink Quick Plasmid Miniprep Kit, Invitrogen). Insulin-producing cells were plated in appropriate medium containing 10 % FCS and allowed to attach for 24 h. The medium was then replaced by FCS-free antibiotic-free medium and cells were separately transfected with each vector construct (Lipofectamine 2000, Invitrogen). Four hours after transfection, FCS (final concentration of 1 %) and antibiotics were added to the medium and cells were allowed to grow for an additional 18 h. Thereafter, medium was replaced again by FCS-free phenol red-free medium, which was used until the end of the protocols. After 24 h, either INGAP-PP or specific compounds for positive controls were added to the medium and samples were collected after 6 and 24 h incubation. The reporter signalling of the secreted alkaline phosphatase (SEAP) was measured using a commercial luminescent kit, according to the manufacturer's instructions (Phospha-Light Secreted Alkaline Phosphatase Reporter Gene Assay System, Invitrogen).

2.9. Fractionation of cytosolic and nuclear fractions

Nuclei were isolated according to the instructions contained in the "Nuclei Isolation Kit: Nuclei EZ Prep" (Sigma Chemicals Co.). The composition of the hypotonic lysis buffer, from which cytosolic fraction is obtained from the supernatant, was: Hepes (10 mmol/l), MgCl2 (2 mmol/l) and KCl (25 mmol/l). Nuclei-containing pellets were washed twice in the nuclei resuspension buffer, composed of hypotonic lysis buffer added of 250 mmol/l of sucrose. Finally, nuclear proteins were obtained by incubating and vortexing the nuclei pellets with the nuclei extraction buffer, composed of the

hypotonic lysis buffer with the addition of 1 % Triton X100. All buffers contained a protease inhibitor cocktail, composed of PMSF (2 mmol/l), sodium orthovanadate (10 mmol/l), sodium fluoride (100 mmol/l), aprotinin (0.1 mg/ml) and EDTA (200 mmol/l).

2.10. Statistics

All data are expressed as means ± SE. Statistical analyses were performed using Student's t test or one-way ANOVA followed by Bonferroni's or Dunnett's test, as required. $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Validation of insulin-producing cells as a model to study INGAP-PP signalling

We first tested whether MIN6 cell-derived islet-like structures (pseudoislets), which are well-known to respond to glucose and other nutrient and non-nutrient stimuli [13], show stimulated insulin secretion in response to acute exposure to INGAP-PP as do neonatal rat islets [5]. Exposure of MIN6 pseudoislets to INGAP-PP caused an acute, reversible increase in insulin secretion that was nearly half of the amplitude of the increase observed in response to 20 mmol/l glucose, which is the main physiological signal for insulin secretion (Fig. 1A). This observation confirmed that INGAP-PP acutely stimulates insulin secretion from MIN6 beta-cells, as it does in primary islets [5]. To reinforce further the validation of insulin-producing cells as a model and to provide molecular insights into the effects of INGAP-PP that may have functional relevance for insulin secretion, we measured the intracellular calcium handling by MIN6 cells upon INGAP-PP stimulation. Figure 1B shows that 10 μg/ml INGAP-PP stimulated a reversible increase in intracellular calcium at 2 mmol/l glucose in fura 2-loaded MIN6 cells, and that the cells showed a similar magnitude increase in response to 20 mmol/l glucose. INGAP-PP also further increased MIN6 cell intracellular calcium levels when it was administered in the presence of 20 mmol/l glucose (Fig. 1B, starting at 17 min

incubation). The cells remained viable after exposure to INGAP-PP as they were able to mount an appropriate elevation in calcium in response to the purinergic agonist, ATP.

3.2. Effect of INGAP-PP on proliferation of insulin-producing cells

Since insulin-producing cells comprise very pure populations [15, 16], making them very useful for proliferation studies, we next explored a possible pro-proliferative effect of INGAP-PP using a well-validated MTS cell proliferation kinetic assay [17]. Exposure of both RINm5F and MIN6 insulin-producing cells to 10 µg/ml of the INGAP-PP for 72 h increased the number of proliferating viable cells as shown by the MTS test (Fig. 2). This effect was observed in both the presence (Fig. 2A) and absence (Fig. 2A and 2B) of 10 % foetal calf serum. In the presence of INGAP-PP alone, MTS reduction increased to 162±17 % and 155±13 % of control values for RINm5F and MIN6 insulin-producing cells, respectively, compared to cells grown in the absence of the peptide (Fig. 2B).

3.3. Effect of INGAP-PP on the expression of the p85, Akt1/2/3, p70^{S6k}, PCNA and muscarinic M3 receptor in insulin-producing cells

Incubation of insulin-producing cells with INGAP-PP for 72 h did not change the expression of the downstream insulin signalling pathway proteins p85, Akt1/2/3 and p70^{S6k}. However, there were increases of 187±19 % and 170±8 % in the expression of PCNA, as a direct indicator of proliferation [18], and of 169±4 % and 222±20 % in the expression of the muscarinic M3 receptor subtype, for RINm5F and MIN6 cells, respectively (Fig. 3).

3.4 Effect of INGAP-PP on intracellular signalling pathways in insulin-producing cells

A gene reporter screening analysis of RINm5F and MIN6 cells in response to INGAP-PP exposure is shown in Table 1. INGAP-PP (10 µg/ml) exposure increased c-Myc, as well as SRE response element activation, in both cell types. A strong activation of NFκB signalling in both cells starting already at 6 h incubation was also observed. At 24 h incubation NFAT was activated by INGAP-PP only in RINm5F cells while AP1 was activated only in MIN6 cells.

3.5. Effect of INGAP-PP on NFκB translocation to the nucleus in insulin-producing cells

Translocation of NFκB was measured using a specific antibody against the p65 subunit in both cytosolic and nuclear fractions. A time course for incubation of insulin-producing cells with INGAP-PP is shown in Fig. 4. There was an increase in the nucleus/cytosol optical density ratio for p65 expression at 6 h, which was enhanced at 24 h in both RINm5F and MIN6 cells. No significant differences were found at the 72 h exposure time though activation was still greater than controls. These results confirm that activation of the NFκB signalling pathway in insulin-producing cells occurs as early as 6 h after exposure to INGAP-PP.

3.6. Contribution of NFκB signalling to INGAP-PP-induced upregulation of M3 receptor expression in insulin-producing cells

There was an increase in the acetylcholine M3 receptor expression in insulin-producing cells exposed to INGAP-PP for 24 h (Fig. 5), similar in magnitude to that obtained after 72 h incubation of RINm5F and MIN6 cells with INGAP-PP, as shown in Figure 3. Blocking NFκB translocation by incubation of cells with the specific inhibitor PDTC (pyrrolidine dithiocarbamate) caused a significant ($p < 0.05$) decrease of M3 expression in both control and INGAP-PP-treated cells. Thus, RINm5F cells showed a decrease of 49 % and 62 % and MIN6 cells showed a reduction of 52 % and 57 % after NFκB inhibition for control and INGAP-PP-treated cells, respectively.

3.7. Differential contribution of serum response elements and the NFκB response element for INGAP-PP-stimulated expression of the acetylcholine M3 receptor in insulin-producing cells

There was an increase in the acetylcholine M3 receptor expression in both RINm5F and MIN6 insulin-producing cells incubated in the presence of 10 % foetal calf serum (FCS) for 24 h compared to cells grown in FCS-free medium (Fig. 6). This effect was further enhanced in cells incubated with both FCS and INGAP-PP. Blocking NFκB translocation with PDTC caused a strong decrease of M3 expression in both tested situations, especially in the MIN6 cells incubated with FCS alone, which were very sensitive to the NFκB inhibition. While RINm5F cells presented a

decrease of 60 % and 61 %, MIN6 cells presented a decrease of 85 % and 61 % after NFκB blockade for cells treated with FCS alone or FCS plus INGAP-PP, respectively.

4. DISCUSSION

INGAP-PP modifies the expression of several genes that control different metabolic and secretory functions of pancreatic beta cells [19], and short-term exposure to this polypeptide is associated with increased phosphorylation of proteins involved in pancreatic islet PI3K and MAPK signalling pathways [5]. However, no information is available on the early intracellular signalling pathways by which INGAP-PP signals in beta cells and which lead to long term changes. To address this question, we treated two insulin-producing cell lines (RINm5F and MIN6 cells) with INGAP-PP and analysed cellular cascades transducing extracellular INGAP-PP signals into molecular events. Our results indicate that INGAP-PP activates c-Myc, SRE and especially NFκB signalling cascades, increases the cellular proliferation rate and induces an NFκB-dependent increase in the expression of the cholinergic M3 receptor. Thus, it can be concluded that INGAP-PP might increase replication of pre-existing cells and that NFκB plays a critical role for the appropriate M3 receptor expression in insulin-producing cells. Additionally, under our experimental conditions, INGAP-PP is recognized by the insulin-producing MIN6 cells in the same way as occurs in rat islets, affecting simultaneously and significantly not only the secretion capacity, as previously observed, but also the calcium levels within cells.

The INGAP-PP has been formerly associated with neogenesis of pancreatic islets from ductal precursors [20], including those from animal models of diabetes [1, 3]. However, no clear evidence for INGAP-PP-induced replication of beta cells has been observed so-far. Therefore, it is intriguing that in the current study INGAP-PP increased proliferation of insulin-producing cells since insulin is the major hormone expressed by RINm5F cells [15], and the only hormone secreted by MIN6 cells [16], indicating their appropriate use as pancreatic beta cell models. Hence, due to the lack of ductal precursors in the insulin-producing cell lineages, our results indicate that INGAP-PP-

dependent increase in cell mass might also occur through increased replication of pre-existing beta cells in addition to neogenesis. This effect is similar to the Reg1-associated replication of pre-existing beta cells in pancreatomized animals [21], and strengthens the concept of using INGAP-PP for restoration of beta cell mass, since pancreatic beta cells from the adult have a very limited capacity for proliferation [22]. Alternatively, INGAP-PP may also decrease the basal apoptosis rate, therefore increasing cell number through increased survival rate. However, INGAP-PP also present the same effects in cells cultivated in the absence or presence of FCS. The latter condition shows very low levels of basal apoptosis (K Souza, unpublished observation), and therefore this hypothesis is less likely to explain the increase in the number of viable cells after INGAP-PP treatment. Notably, according to a previous in vivo binding study, labelled-INGAP-PP is specifically bound to liver, small intestine and particularly to pancreatic islets [23], which suggests that INGAP-PP may selective and specifically act upon the pancreas, stimulating beta cell growth without affecting various other tissues functions.

The activation of the transcription factor c-Myc may be involved in proliferation of beta cells following INGAP-PP treatment. The pro-proliferative action of c-Myc has also been previously proposed for other cell types [24]. Interestingly, activation of c-Myc may render pro-apoptotic or pro-proliferative effects in pancreatic beta cells depending on the period [25] and level of activation [26]. Relatively lower c-Myc activation increases beta cell proliferation, whereas higher activation causes death of beta cells [26]. Mild activation of c-Myc by INGAP-PP, as compared to the greater activation triggered by FCS (Table 1), is therefore an additional indication of pro-proliferative effects of the polypeptide. The interrelation of c-Myc and PCNA is another evidence for INGAP-PP-dependent induction of proliferation in beta cells, as PCNA is also a cell cycle-related gene playing an important role in replication, cell cycle progression and DNA repair [27]. Supporting our interpretation, similar simultaneous induction of c-Myc activation and PCNA expression has been proposed to explain the stimulatory effect induced by other agents in proliferating epithelial cells [28]. Taken together, these results indicate that c-Myc activation and PCNA expression may be

ascribed, at least partly, as part of the mechanism by which INGAP-PP exerts its effect upon cell proliferation.

Recently, the crucial importance of the M3 muscarinic acetylcholine receptor for regulated insulin secretion and glucose homeostasis has been elegantly demonstrated [10]. Additionally, it has already been shown that the M3 receptor stimulation activates MAP kinases [29, 30] and may have potential survival and anti-apoptotic effects [31] in other cell types. Together with the well-characterized effect of the M3 receptor on the induction of early growth genes [32] and with the fact that the INGAP-PP not only triggers insulin-release and increase intracellular calcium levels but also both induces proliferation and increases M3 receptor expression, these results allow us to hypothesize that insulin-producing cells might transduce, at least partly, the INGAP-PP signals into intracellular pro-proliferative events through the M3 receptors. An M3 receptor-dependent increase in PCNA expression has also been observed for other cell types exposed to muscarinic agonists [33]. PCNA is also increased in INGAP-PP-treated insulin-producing cells, and this provides additional evidence for an interdependent role for the combined action of c-Myc, PCNA, and M3 receptor on the progression of the cell cycle.

Our results also demonstrate, to our knowledge for the first time, a direct correlation between activation of NFκB and expression of cholinergic M3 receptors in insulin-producing cells. Regarding such a relationship, it has previously been shown that glucocorticoids both decrease NFκB activity in the brain [34] and decrease the expression of the cholinergic M3 receptor in smooth muscle [35]. Notably, in the current study there was a relatively small activation of NFκB by INGAP-PP, compared to the larger activation triggered by TNF-alpha (Table 1). NFκB may play a dual role in pancreatic beta cells, namely either pro-apoptotic [36] or pro-survival [37]. Exposure of pancreatic beta cells to pro-inflammatory compounds in vitro, in particular IL-1beta, leads to a strong activation of the NFκB response element, NFκB expression and ultimately cell death by apoptosis [36, 38]. On the other hand, NOD mice expressing a constitutive form of IκB, which blocks NFκB translocation to the nucleus, are more susceptible to develop diabetes than wild type

controls [37]. These differences might be due to the degree of NFκB activation and, hence, our results may provide a possible explanation for the opposing findings regarding the nature of the NFκB effects on pancreatic beta cells, in the same fashion as that proposed for activation of c-Myc [26].

Interestingly, NFκB responsive elements are underrepresented in the human cholinergic M3 receptor gene [12]. Conversely, NFAT-resembling responsive elements are overrepresented. Our results clearly suggest an essential role of NFκB, whereas NFAT elements might function as secondary players in the modulation of M3 receptor expression (Table 1). This is an interesting finding, as an important role has been shown for other responsive elements and sequences, which are present in low number in the genome, including that for constitutive expression [39, 40].

Modulation of the M3 receptor expression through FCS-dependent activation of multiple serum elements also depends on NFκB activation, as demonstrated in the present work. Regulatory elements may alter gene expression in several ways. An interesting proposal that fits the importance of the underrepresented NFκB response element in the M3 receptor gene is the formation of enhanceosomes. For instance, the expression of the human interferon-beta gene is modulated by a cooperative action of NFκB, IRF6, HMG-I, and ATF-2/c-Jun complex [41]. Absence of any of the transcription factors or co-activators or alterations in the recognizing sequences can render the enhanceosome inactive. NFκB may also control the M3 receptor expression through an indirect mechanism, modulating other genes that might be able to operate directly or indirectly in the proper control of M3 receptor expression. NFκB can take part in the modulation of a multitude of genes, including other transcription factors [42] that cannot be excluded by the current data as possible modulators of M3 receptor expression. Nevertheless, our results allow us to conclude that NFκB has an essential role in controlling M3 receptor expression in insulin-producing cells, although the precise regulatory pathways must still be defined.

5. CONCLUSIONS

INGAP-PP induces enhancement of glucose induced-insulin secretion and increases intracellular calcium levels in MIN6 insulin-producing cell line. Significant changes in a network of intracellular signals that include c-Myc signalling pathway activation, increased PCNA expression and NFκB-dependent cholinergic M3 receptor expression accompanies functional changes. INGAP-PP-dependent activation of the c-Myc signalling pathway and increased PCNA expression might be involved in proliferation of insulin-producing cells. Activation of NFκB signalling pathway is critical for the modulation of cholinergic M3 receptor expression. A cooperative action of direct effects of INGAP-PP on beta cell replication and increased M3-dependent proliferation might occur in in vivo situations.

ACKNOWLEDGEMENTS

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LEGENDS TO FIGURES

Figure 1. Kinetics of INGAP-PP-evoked stimulation of insulin secretion and intracellular calcium handling. A) MIN6 pseudoislets were continuously perifused with a physiological salt solution at 37ºC. After a pre-perifusion period of 1 h (2 mmol/l glucose) the effluent was collected every 2 min for 70 min. INGAP-PP (10 µg/ml) was applied, as indicated, in the presence of 2 mmol/l glucose. Results are expressed as % of mean values calculated for min 0 to 10 (2 mmol/l glucose), and normalized against total insulin content. Insulin secretion rates are means ± SE of three different experiments. Peak of INGAP-PP-induced insulin release was significantly increased ($p < 0.05$; t-test) compared to basal insulin secretion (2 mmol/l glucose). B) Fura 2-loaded MIN6 beta cells responded to INGAP-PP (10 µg/ml) at 2 mmol/l and 20 mmol/l glucose with a reversible increase in intracellular calcium. Peak of intracellular calcium increase was 86±2 % of 100 µmol/l ATP response. Results are means ± SE, n=36 separate cells.

Figure 2. Cell proliferation after exposure of insulin-producing cells to the INGAP-PP in the presence or absence of 10 % foetal calf serum (FCS). A) RINm5F and MIN6 cells were cultured in the absence (w/o FCS) or presence (+ 10 % FCS) of foetal calf serum and exposed to 10 µg/ml INGAP-PP for 72 h. Control cells were grown in the absence of INGAP-PP. MTS conversion to a soluble formazan product by viable cells was determined by kinetic measurement of absorbance at 490 nm. N=6. Letters above bars represent $p < 0.05$ vs. Control w/o FCS (a), INGAP w/o FCS (b), Control + 10 % FCS (c), INGAP + 10 % FCS (d); ANOVA followed by Bonferroni. B) Percentage values related to exposition of insulin producing cells to INGAP-PP alone. N=6. * $p < 0.05$; t-test against respective controls.

Figure 3. Effect of INGAP-PP on the expression of proteins implicated in cell proliferation and of the cholinergic M3 receptor subtype in insulin-producing cells. RINm5F and MIN6 cells were seeded 24 h before incubation with 10 µg/ml INGAP-PP. After 72h of incubation, the cells were lysed and used for Western blot analyses. Blots on the left are representative of 5 independent experiments. Graphs on the right are mean values for proliferating cell nuclear antigen (upper graphic) or acetylcholine M3 receptor (lower graphic) expression, both normalised to the housekeeping protein beta actin. * $p < 0.05$; t test against respective control.

Figure 4. Effect of INGAP-PP on NFκB p65 subunit translocation into the nucleus in insulin-producing cells. RINm5F and MIN6 cells were exposed to 10µg/ml INGAP-PP for the indicated incubation periods. Thereafter, cells were lysed and cytosolic and nuclear fractions were separated and used for Western blot analyses. Blots on the top are representative of 3 independent experiments. C, cytosolic fraction; N, nuclear fraction. CTL, control; ING, INGAP-PP-treated cells. * $p < 0.05$ against controls; ANOVA followed by Dunnett.

Figure 5. Effect of blockade of NFκB on the INGAP-PP-induced acetylcholine M3 receptor protein expression in insulin-producing cells. RINm5F and MIN6 cells were seeded 24 h before incubation with 10 μg/ml INGAP-PP. After 24 h of INGAP-PP incubation in the presence or absence of the NFκB inhibitor PDTC, the cells were lysed and used for Western blot analyses. Blots on the top are representative of 5 independent experiments, normalised to the housekeeping protein beta actin. Letters above bars represent $p < 0.05$ vs. Control (a), INGAP (b), Control + PDTC (c), INGAP + PDTC (d); ANOVA followed by Bonferroni.

Figure 6. Effect of foetal calf serum (FCS) on acetylcholine M3 receptor protein expression in insulin-producing cells with or without NFκB blockade. RINm5F and MIN6 cells were seeded 24 h before incubation with 10 % FCS or 10 % FCS plus 10 μg/ml INGAP-PP. After 24 h of FCS or FCS+INGAP-PP incubation in the presence or absence of the NFκB inhibitor PDTC, the cells were lysed and used for Western blot analyses. Blots on the top are representative of 4 independent experiments, normalised to the housekeeping protein beta actin. Letters above bars represent $p < 0.05$ vs. Control (a), Control + 10 % FCS (b), INGAP-PP + 10 % FCS (c), PDTC + 10 % FCS (d), INGAP + PDTC + 10 % FCS; ANOVA followed by Bonferroni.

Figure 1. Kinetics of INGAP-PP-evoked stimulation of insulin secretion and intracellular calcium handling

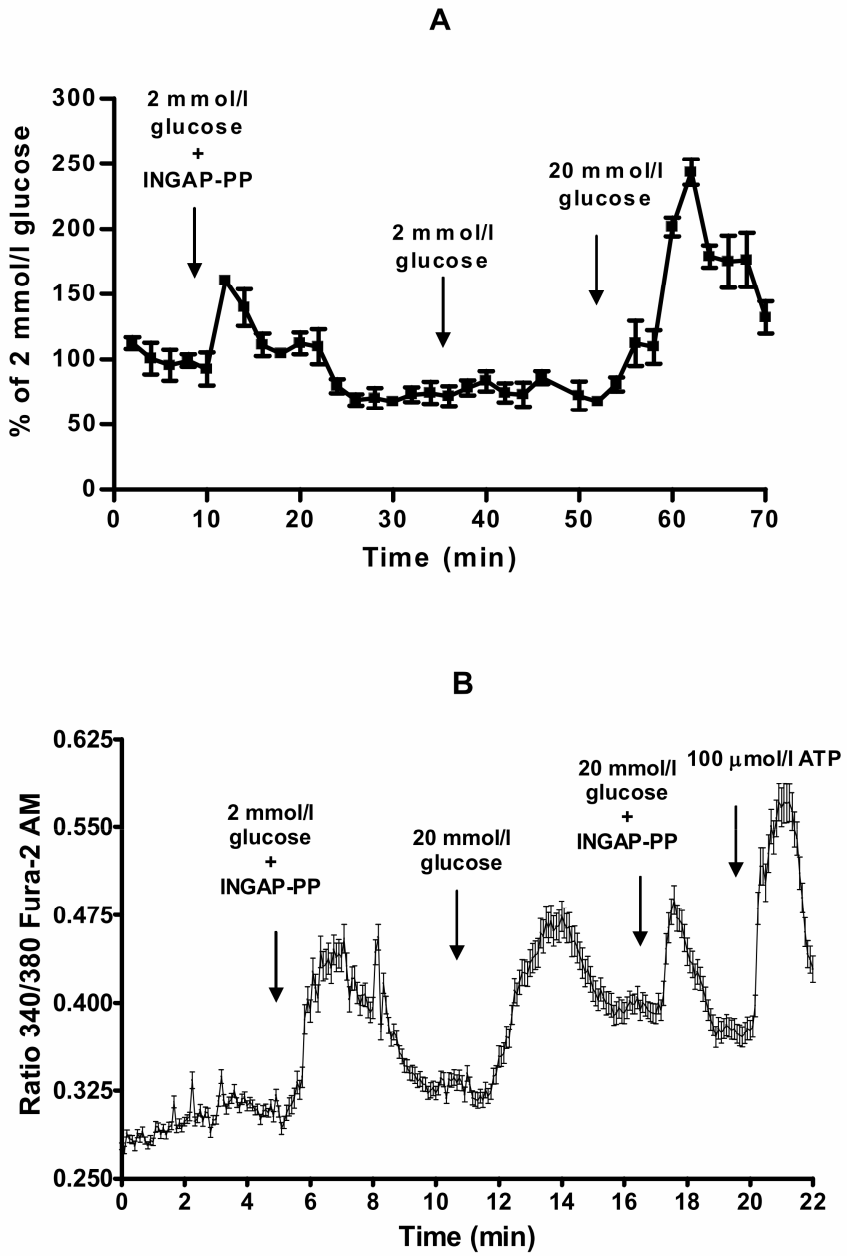

Figure 2. Cell proliferation after exposure of insulin-producing cells to the INGAP-PP in the presence or absence of 10 % foetal calf serum (FCS)

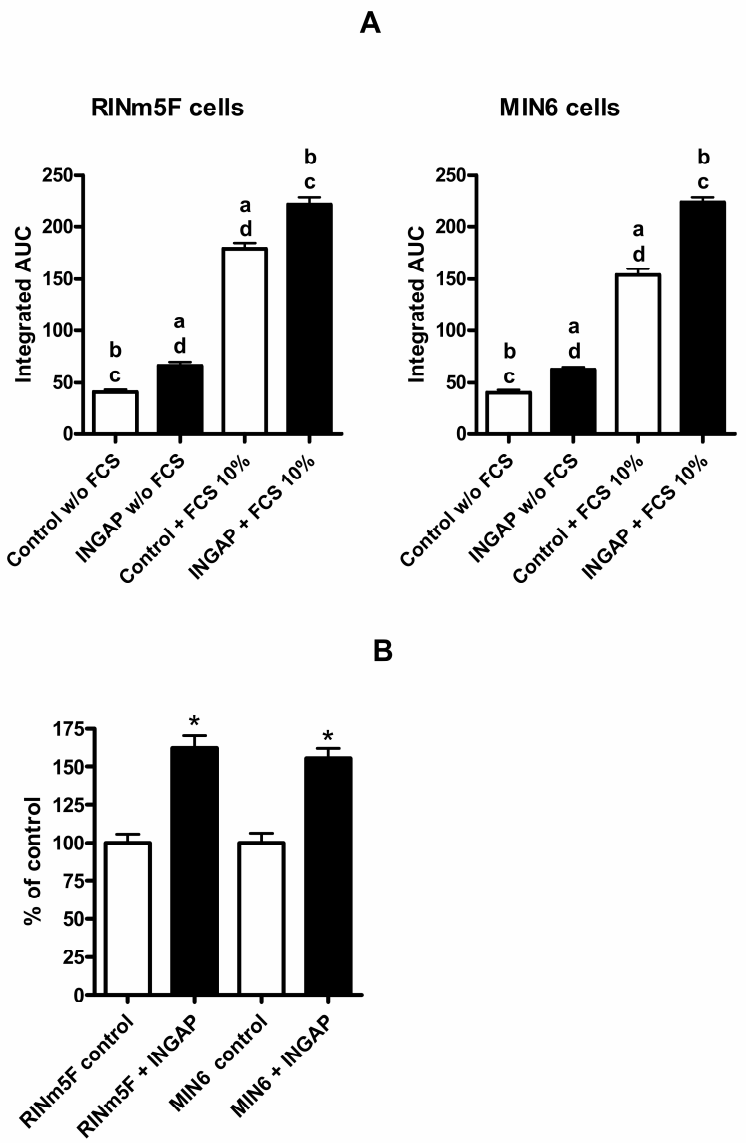

Figure 3. Effect of INGAP-PP on the expression of proteins implicated in cell proliferation and of the cholinergic M3 receptor subtype in insulin-producing cells

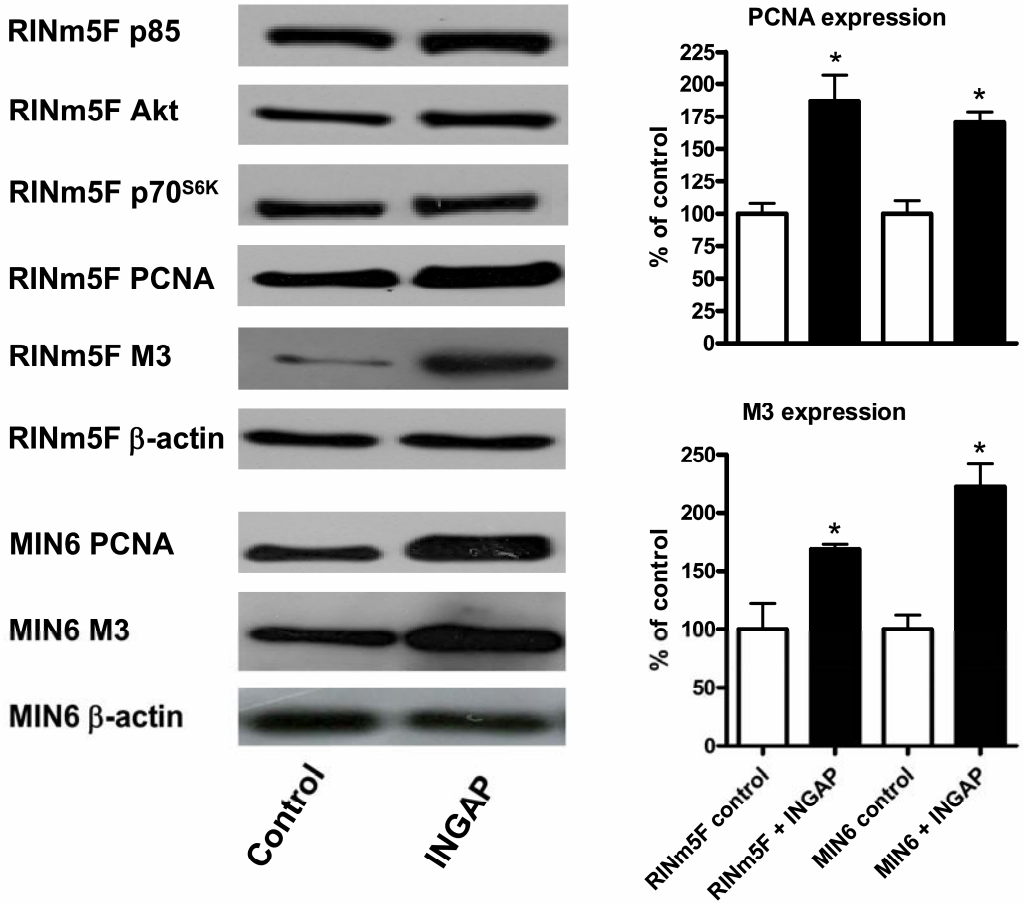

Figure 4. Effect of INGAP-PP on NFκB p65 subunit translocation into the nucleus in insulin-producing cells

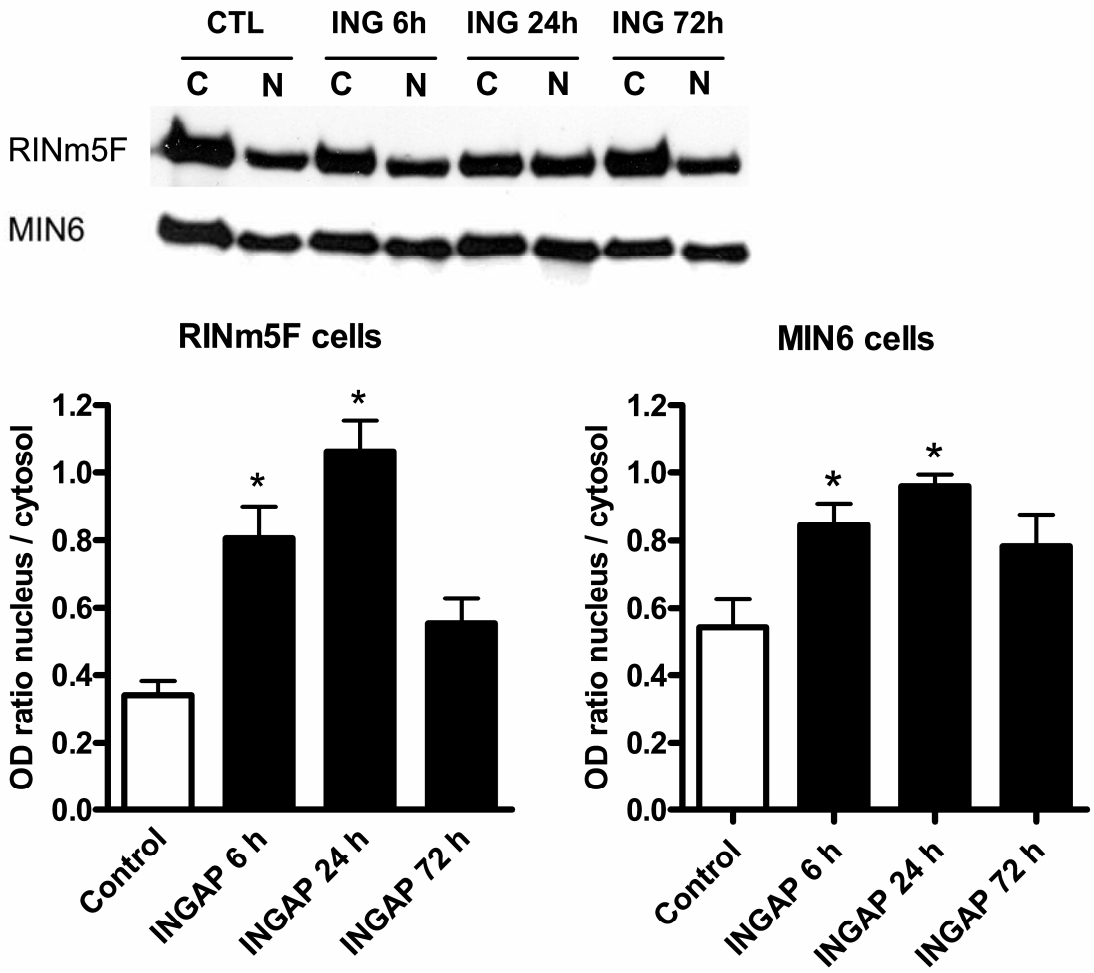

Figure 5. Effect of blockade of NFκB on the INGAP-PP-induced acetylcholine M3 receptor protein expression in insulin-producing cells

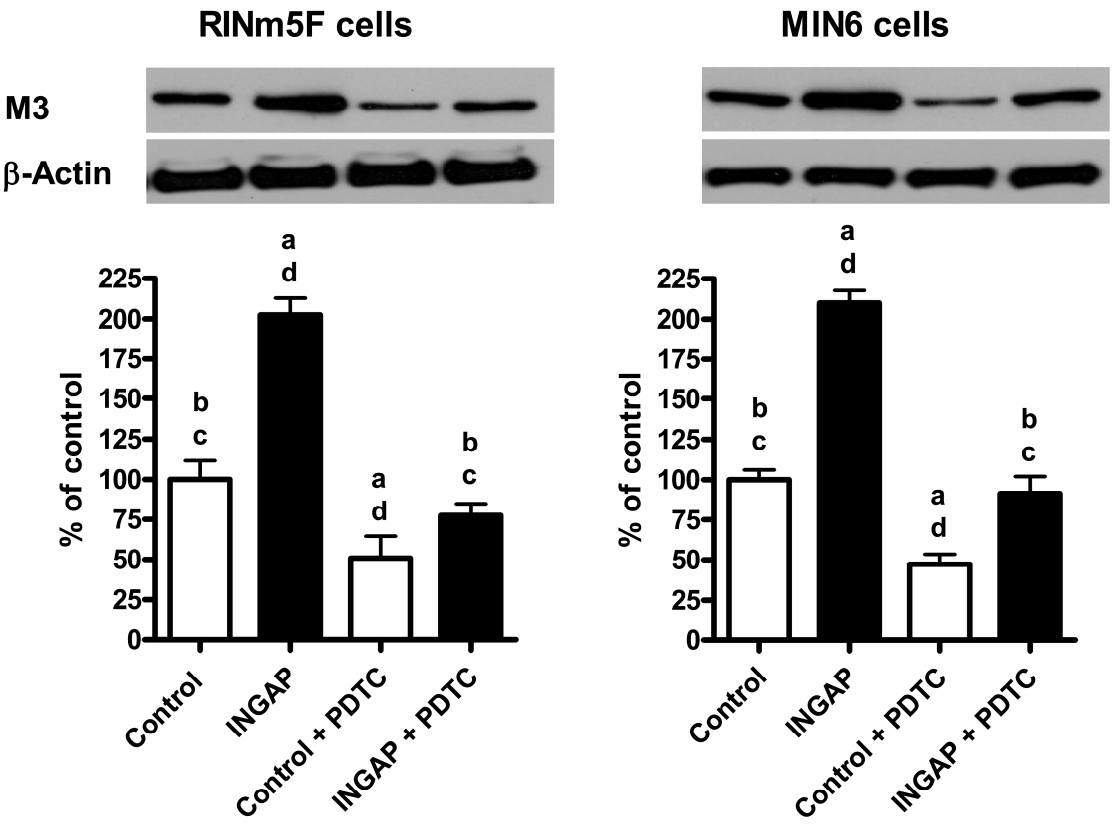

Figure 6. Effect of foetal calf serum (FCS) on acetylcholine M3 receptor protein expression in insulin-producing cells with or without NFκB blockade

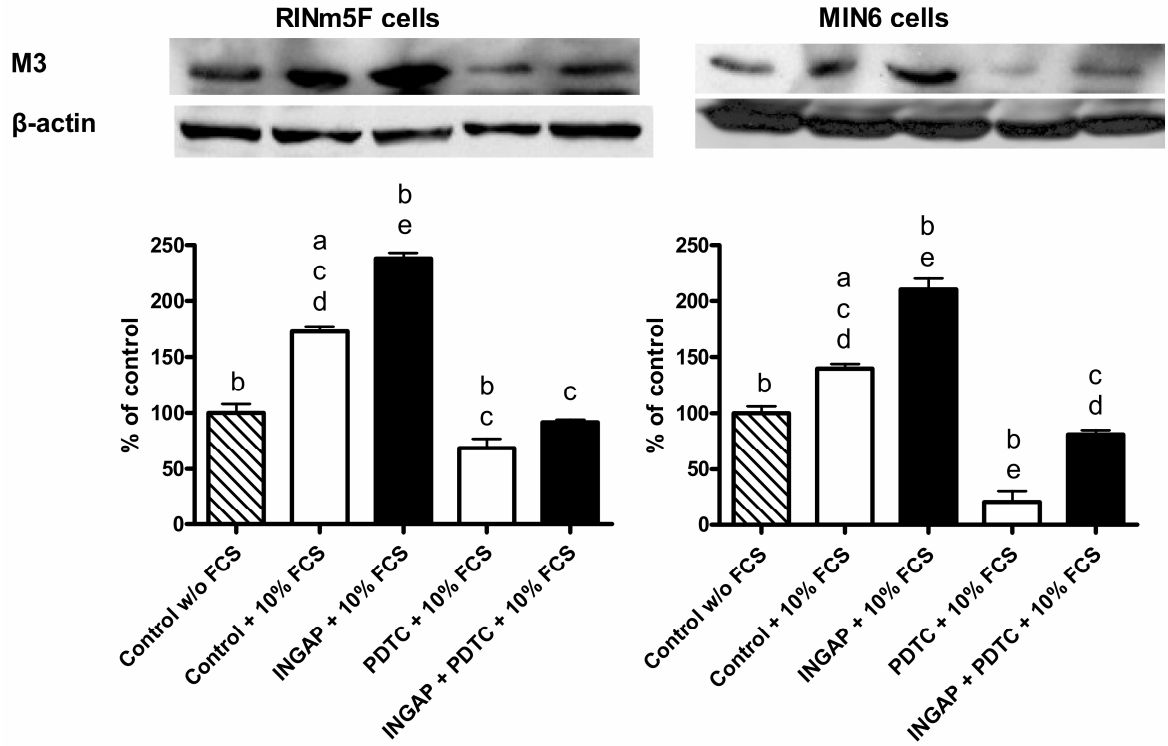

Table 1. Intracellular signalling pathways after exposure of insulin-producing RINm5F and MIN6 cells to INGAP polypeptide

	RINm5F insulin-producing cells					
	6 h			24 h		
	Control	INGAP	Positive control	Control	INGAP	Positive control
AP-1	1.0 ± 0.1	1.2 ± 0.1	3.0 ± 0.4	1.0 ± 0.1	1.2 ± 0.4	3.3 ± 0.4
CRE	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.3	1.0 ± 0.1	1.5 ± 0.4
c-Myc	1.0 ± 0.1	1.4 ± 0.2	2.6 ± 0.2	1.0 ± 0.1	1.7 ± 0.1 *	4.6 ± 0.4
NFAT	1.0 ± 0.2	0.9 ± 0.1	1.5 ± 0.3	1.0 ± 0.1	1.5 ± 0.2 *	2.4 ± 0.2
NFκB	1.0 ± 0.0	1.8 ± 0.4 *	2.8 ± 0.5	1.0 ± 0.2	2.4 ± 0.3 *	5.8 ± 0.3
SRE	1.0 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.0 ± 0.1	1.8 ± 0.1 *	2.4 ± 0.2
	MIN6 insulin-producing cells					
	6 h			24 h		
	Control	INGAP	Positive control	Control	INGAP	Positive control
AP-1	1.0 ± 0.1	1.3 ± 0.1	2.8 ± 0.3	1.0 ± 0.1	1.4 ± 0.1 *	2.5 ± 0.2
CRE	1.0 ± 0.0	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.5 ± 0.1
c-Myc	1.0 ± 0.1	1.5 ± 0.2 *	2.5 ± 0.3	1.0 ± 0.0	1.3 ± 0.1 *	3.2 ± 0.1
NFAT	1.0 ± 0.1	1.0 ± 0.2	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.0	1.7 ± 0.2
NFκB	1.0 ± 0.1	2.2 ± 0.1 *	3.1 ± 0.3	1.0 ± 0.1	1.6 ± 0.1 *	3.2 ± 0.1
SRE	1.0 ± 0.1	1.2 ± 0.0	1.2 ± 0.1	1.0 ± 0.0	1.3 ± 0.1 *	3.0 ± 0.1

Insulin-producing RINm5F and MIN6 cells were exposed for 6 or 24 h to the INGAP polypeptide (10 μg/ml) in serum-free medium. Results are presented as -fold ± SE and compared against control values obtained from cells incubated without INGAP. Positive controls were 10 % FCS (AP-1 and c-Myc responsive elements), 25 μmol/l Forskolin (CRE), 50 ng/ml TNF-α (NFAT and NFκB), 2 ng/ml IL-1β (SRE). N=9. * $p < 0.05$, t test against respective controls.

CONCLUSÕES

Conclusões

Pode-se concluir que a exposição ao INGAP-PP aumenta a secreção de insulina estimulada por glicose bem como as concentrações de cálcio intracelular em células MIN6. O aumento na expressão da proteína PCNA e na ativação do fator de transcrição c-Myc está relacionado ao aumento encontrado na viabilidade das células beta RINm5F e MIN6 após a exposição ao INGAP-PP.

O aumento na expressão protéica do receptor muscarínico M3, induzida pelo INGAP-PP, é modulada direta ou indiretamente pelo fator de transcrição NFκB. Dados da literatura mostram que a expressão do receptor M3 também leva à um aumento na viabilidade e proliferação celular. Desta forma, propõe-se que o aumento na expressão do receptor M3 induzido pelo peptídeo pode atuar sinergicamente no aumento da viabilidade celular. Adicionalmente, o aumento na expressão do receptor M3 indica função importante da sinalização colinérgica na melhora da secreção de insulina, induzida pelo INGAP-PP *in vivo*.

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