ADRIANA RIBEIRO LEITE

REGULAÇÃO DAS JUNÇÕES COMUNICANTES (GAP JUNCTIONS) EM CULTURA DE ILHOTAS PANCREÁTICAS DE RATOS RECÉM - NASCIDOS

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ORIENTADORA: Profa. Dra. Carla Beatriz Collares-Buzato
CO-ORIENTADOR: Prof. Dr. Antonio Carlos Boschero

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BANCA EXAMINADORA

Profa. Dra. Carla Beatriz Collares-Buzato (orientadora) _____

Prof. Dr. Hernandes Faustino Carvalho _____

Profa. Dra. Carla Roberta de Oliveira Carvalho _____

Profa. Dra. Helena Coutinho Franco de Oliveira. _____

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RESUMO

As junções comunicantes (JCs) ou *gap junctions* são canais intercelulares formados pela união de dois hemicanais ou *conexons*, os quais são formados por proteínas integrais pertencentes à família das conexinas (Cx). Estudos em ilhotas pancreáticas têm demonstrado que a comunicação intercelular, via junções comunicantes, é fundamental para adequada biossíntese, estoque e liberação de insulina pelas células β pancreáticas. Condições que promovem a formação de JCs aumentam a secreção e biossíntese deste hormônio. Por outro lado, o bloqueio dos canais ou ruptura das JCs na célula β resultam em comprometimento do processo secretório. Ilhotas pancreáticas de fetos e recém-nascidos de ratos exibem uma resposta secretória de insulina reduzida em comparação às ilhotas de animais adultos. Cultivo prolongado de ilhotas pancreáticas, bem como o tratamento *in vitro* com hormônios somatotróficos, como a prolactina, induzem maturação deste processo de acoplamento estímulo-secreção. Esta tese teve como objetivo investigar os possíveis mecanismos intracelulares de regulação das JCs pela cultura prolongada e pelo tratamento *in vitro* com prolactina em ilhotas pancreáticas de ratos recém-nascidos. Para tal, foi avaliada a localização, o grau de expressão gênica e o conteúdo celular das proteínas integrantes das JCs, as conexinas 43 e 36, bem como o grau de adesão celular pela expressão da β-catenina nas ilhotas nestas condições experimentais. Foram executados os seguintes protocolos: 1) tratamento *in vitro* com prolactina (2μg/mL/dia) durante 7 dias, 2) cultivo das ilhotas por períodos variando de 1 a 7 dias e 3) cultivo por 3 dias em concentrações variáveis de glicose no meio de cultura. Foi detectado um aumento significativo da secreção de insulina após o tratamento com prolactina, tempo prolongado de cultivo e concentração crescente de glicose no meio. Este resultado indica que tais condições experimentais induzem maturação do processo de secreção de insulina em ilhotas pancreáticas de ratos recém-nascidos. O tratamento crônico com prolactina também induziu um aumento na expressão de Cx43 e β-catenina, como demonstrado por Western Blot. Ambas proteínas juncionais foram detectadas por imunocitoquímica na região de contato intercelular nas células das ilhotas. Quanto ao efeito da cultura *per se*, foi observada

uma correlação entre o tempo de cultivo e o aumento na expressão celular de Cx43, Cx36 e β-catenina. No caso das conexinas, a cultura prolongada também resultou em aumento da transcrição gênica, como detectado pelo método de RT-PCR. Quando foram analisadas as variações de expressão das conexinas em resposta à glicose, somente a Cx36 pareceu ser regulada por concentrações crescentes deste secretagogo no meio de cultura. Esses resultados, tomados em conjunto, sugerem que a regulação das conexinas nas ilhotas pancreáticas pela prolactina, glicose e outros fatores contidos no meio de cultura podem ser importantes no processo de maturação das células β em condições de cultivo.

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1.1 INTRODUÇÃO

Comunicação intercelular é um fenômeno de grande importância em todos organismos multicelulares, que visa coordenar as várias atividades celulares, tais como o metabolismo, a diferenciação e o crescimento celular. Existem duas formas de comunicação intercelular. A primeira envolve a liberação no meio extracelular de substâncias, tais como neurotransmissores, hormônios e outros fatores ativos. Nesta forma de comunicação intercelular, células adjacentes, ou localizadas distantes do sítio de liberação, contém receptores específicos, em sua membrana plasmática, que são capazes de reconhecer tais substâncias e responderem a estímulos. A segunda forma de comunicação intercelular opera em grupos de células que estão acopladas através de estruturas especializadas da membrana plasmática denominadas de junções comunicantes (JCs). A junção comunicante, também conhecida como *Gap Junction* (GJ), constitui um dos componentes das junções intercelulares, a qual permite uma difusão direta de íons e moléculas, de baixo peso molecular, de uma célula para outra.

As junções intercelulares (JIs) são especializações da membrana plasmática que interconectam as células organizadas em tecidos e/ou órgãos (Figura 1). Além das junções comunicantes, as JIs são formadas por 3 outros tipos de estruturas: (1) a junção de oclusão, ou *tight junction*, que corresponde a uma região de estreita proximidade das membranas plasmáticas de células adjacentes, devido a uma aparente fusão do folheto externo destas membranas; (2) a *zonula* ou *fasciae adherens*, uma região da membrana de ancoragem dos microfilamentos do citoesqueleto; e (3) os desmossomos, o sítio da membrana de ancoragem dos filamentos intermediários do citoesqueleto (Collares-Buzato 2001, Farquhar e Palade 1963). Estas três últimas junções representam dispositivos de adesão intercelular, enquanto a JC é caracterizada por uma região de contato intercelular reconhecida pela aposição singular das membranas de células vizinhas e presença de canais intercelulares, que estão envolvidos na comunicação intercelular (Collares-Buzato 2001, Musil 1994) (Figura 2).

As junções comunicantes foram, inicialmente, descritas nos anos 60, através de microscopia eletrônica, como uma região, de área variável, das membranas plasmáticas de células vizinhas separadas por uma fenda (do inglês, *gap*) de aproximadamente 2-4 nm. Anos mais tarde, através da técnica de criofratura e observação ao microscópio eletrônico, demonstrou-se que esta estrutura constitui uma região diferenciada da membrana plasmática contendo um arranjo denso de partículas inseridas na membrana, podendo formar uma placa (Beyer e Goodenough 1990). Estas partículas foram, posteriormente, caracterizadas bioquimicamente como sendo de natureza protéica, correspondendo aos canais que interconectam as duas células vizinhas acopladas.

Estrutura e Composição Bioquímica das Junções Comunicantes

As JCs são formadas pela união de dois hemicanais protéicos denominados de *conexons*, que ficam conectados no espaço extracelular. Cada hemicanal é constituído de seis proteínas integrais de membrana denominadas de conexinas (Cx), as quais estão dispostas em um arranjo hexagonal no conexon (Figura 2). Até o momento, foram descritos mais de 20 tipos de conexinas (Harris 2001). Cada uma destas é codificada por um gene específico e são denominadas de acordo com o seu peso molecular, em quilodaltons (kDa), ou segundo a nomenclatura α/β (Tabela I). As conexinas estão classificadas em três subfamílias de acordo com suas homologias: grupo I, também chamado de β-conexinas, grupo II, também chamado de α-conexinas (Spray 1996) e o, recentemente descoberto, grupo III ou δ-conexinas (Belluardo et al. 2000, Serre-Bernier et al. 2000). Cada conexina apresenta uma distribuição tecido-específica e cada célula, na maioria dos tecidos, expressa mais de um tipo de conexina.

As moléculas de conexinas isoladas ficam inseridas na bicamada lipídica de acordo com o modelo representado na Figura 3. O polipeptídeo possui quatro domínios hidrofóbicos transmembrana denominados de M1, M2, M3 e M4, com as extremidades carboxi - (C) e amino - (N) terminais voltadas para o citoplasma (Harris 2001). Além disso, cada conexina apresenta um domínio intracelular denominado de E3 e dois domínios extracelulares denominados de E1 e E2. Essas regiões da molécula das conexinas, E1 e E2, parecem estar envolvidas na interação entre conexons de células adjacentes. As regiões

entre os domínios transmembrana M2 e M3 (E3), assim como a extremidade C-terminal dessas proteínas, são bastante variáveis entre as diferentes conexinas e parecem ser importantes na regulação das junções comunicantes, já que são locais de sítios de fosforilação por quinases (Sáez et al. 1998). Em adição, a porção C-terminal das conexinas é primordial para a manutenção da função das JCs nos tecidos. Quando esta região da molécula sofre mutação em condições experimentais, como no caso do oócito mutante de *Xenopus*, ou em condições patológicas como na doença Charcot-Marie-Tooth, os canais apresentam permeabilidade alterada e função comprometida (Fichman et al. 1991, Spray e Dermietzel 1995).

Os diferentes tipos de conexinas podem estar associados entre si em combinações diferentes. Tal fato pode, significativamente, influenciar as propriedades de permeabilidade dos canais formadores das junções comunicantes. Assumindo-se que diferentes conexinas formam canais com propriedades de permeabilidade diferentes, a possibilidade de diferentes conexinas poderem se combinar, para formar os conexons, pode ter uma relevância fisiológica importante. Assim, o grau e a forma de regulação do acoplamento mediado pelas JCs entre duas células vizinhas dependerão do(s) tipo(s) de conexinas expressas pelas mesmas, e da combinação destas proteínas dentro do mesmo conexon e/ou entre dois conexons formadores do canal. Teoricamente, o canal presente entre duas células adjacentes pode conter dois hemicanais idênticos, constituídos dos mesmos tipos de conexinas (canal denominado homotípico) ou pode conter conexons diferentes composto de subtipos de conexinas distintos (canal denominado heterotípico) (Figura 4) (Rozental et al. 2000). Entretanto, não há evidência direta da existência de canais heterotípicos formadores de JCs *in vivo*. Em geral, os conexons constituem-se de apenas um tipo de conexina (homomérico), porém, podem existir conexons constituídos de diferentes tipos de conexinas (heteromérico) (Figura 4).

FUNÇÃO E REGULAÇÃO DAS JUNÇÕES COMUNICANTES

As junções comunicantes permitem a passagem de íons e moléculas com peso molecular inferior a 1000 Da; dentre essas moléculas estão os monossacarídeos, os aminoácidos, os nucleotídeos e mensageiros intracelulares, tais como o Ca^{+2}, o inositol

trifosfato (IP_3) e o monofosfato cíclico de adenosina (AMPc). Entretanto, estes canais não permitem a passagem de proteínas, DNA, RNA e organelas celulares. Portanto, células conectadas pelas junções comunicantes são acopladas elétrica e metabolicamente, mas mantém sua individualidade genética e estrutural.

As junções comunicantes não são estruturas estáticas, ou poros passivos permanentemente abertos, presentes na membrana plasmática, e, sim, constituem canais passíveis de regulação, permitindo uma maior ou menor passagem de íons e moléculas dependendo do seu estado funcional. Tem sido proposto um modelo para explicar a mudança no estado fechado/aberto destes canais. Como ilustrado na figura 5, a alteração do estado funcional dos canais das junções comunicantes envolve, inicialmente, uma mudança conformacional na estrutura das conexinas e, conseqüentemente, uma alteração na interação intermolecular entre estas proteínas dentro do conexon. Isto resulta em um "deslizamento" das conexinas e, conseqüentemente, fechamento ou abertura do poro, à semelhança de um diafragma.

É sabido que, em condições experimentais, a permeabilidade dos canais das junções comunicantes é sensível a vários parâmetros fisicoquímicos. Os primeiros estudos, evidenciando a regulação destes canais, foram executados em meados da década de 80 e demonstraram a importância da voltagem transjuncional e da concentração de íons hidrogênio no controle da permeabilidade da junção comunicante (Spray e Bennett 1985). Estes estudos, utilizando células embrionárias de anfíbios e de peixes, mostraram que a condutância juncional diminui proporcionalmente ao aumento da voltagem juncional e à diminuição no pH intracelular (Spray e Bennett 1985).

O íon Ca^{+2} constitui um outro componente capaz de regular a comunicação intercelular mediada pelas junções comunicantes. Um aumento na concentração intracelular de cálcio ($[Ca^{+2}]_i$), para níveis altos, está relacionado a uma redução significativa da permeabilidade juncional (Giaume e Venance 1998, Sáez et al. 1993). O papel do Ca^{+2} tem sido estudado em diversos tipos celulares com o objetivo de se determinar qual o nível necessário de $[Ca^{+2}]_i$ capaz de promover o fechamento dos canais formadores das junções comunicantes e como o íon é capaz de agir no canal (Sáez et al. 1993). Foi estabelecido que concentrações não fisiológicas de cálcio intracelular, correspondendo a concentrações superiores a 5 - 10 μM, promovem o fechamento dos canais, uma propriedade importante

em se tratando de condições patológicas, pois esta isolaria as células "doentes", ou em processo de morte celular, das células vizinhas sadias (Giaume e Venance 1998). O íon Ca^{+2} parece não agir sozinho na regulação da permeabilidade celular e, sim, sinergicamente, ou através da ativação de proteínas quinases, já que as conexinas não possuem sítios de ligação para o cálcio em suas seqüências (Sáez et al. 1993).

Outra forma de regulação da comunicação intercelular é através da fosforilação das conexinas. A maioria das conexinas, até então investigadas, constitui-se em fosfoproteínas e pode ser alvo de proteínas quinases endógenas, tais como: a proteína quinase dependente de AMPc (PKA), a proteína quinase dependente de cálcio (PKC), a proteína quinase ativada por mitógeno (MAPK) e a proteína tirosina quinase. Essas proteínas fosforilam as conexinas, essencialmente, na sua região C-terminal, que está localizada no citoplasma (Lampe e Lau 2000). A fosforilação tem sido implicada em vários processos de regulação das junções comunicantes, tais como no transporte das conexinas recém-sintetizadas para a membrana, na formação/ruptura dos conexons, na degradação das conexinas e na regulação da abertura/fechamento dos canais (Lampe e Lau 2000). Tem sido proposto que a fosforilação pós-traducional, no caso de algumas conexinas, está envolvida no transporte destas proteínas ("trafficking") do complexo de Golgi para a membrana. Por sua vez, a ativação de quinases e posterior fosforilação das conexinas nos conexons estão relacionadas, principalmente, com a regulação da permeabilidade dos canais das junções comunicantes (Lampe e Lau 2000).

Estudos, sobre a ação das quinases nas junções comunicantes, têm empregado ativadores e inibidores farmacológicos destas enzimas. Reagentes que promovem aumento do AMPc celular tem sido descritos induzirem um aumento da comunicação intercelular mediada pelas junções comunicantes. Tal aumento está relacionado, possivelmente, a um aumento na permeabilidade dos canais (Spray et al. 1991) ou na biossíntese e "trafficking" das conexinas (Atkinson et al. 1995). O modelo propondo que o AMPc afeta a permeabilidade juncional via PKA tem como base os estudos de Wiener e Loewenstein (1983). Eles observaram um aumento na comunicação intercelular, várias horas após a adição da subunidade catalítica de PKA em uma cultura de células mutantes deficientes em junções comunicantes funcionais e que não expressam a isoenzima I da PKA.

Ésteres de forbol e outros reagentes que ativam a proteína quinase C (PKC), na

grande maioria dos casos, inibem a comunicação intercelular mediada pelas junções comunicantes (Kanemitsu e Lau 1983, Kolb e Somogyi 1991, Lampe e Lau 2000, Moreno et al. 1994, Reynhout et al. 1992). A PKC é capaz de fosforilar resíduos de serina/treonina em sítios específicos da porção C-terminal das conexinas (Harris 2001, Rivedal e Opsahl 2001). Por outro lado, o tratamento com inibidor de proteína quinase, a estaurosporina, induz aumento na condutância dos canais juncionais (Moreno et al. 1994).

Ativação de tirosina quinases também resulta em inibição da permeabilidade das junções comunicantes. A fosforilação nos resíduos tirosina das conexinas ocorre quando tirosina quinases não ligadas a receptores (como por exemplo, a v-Src e a $pp60^{src}$) são ativadas (Atkinson et al. 1981, Loo et al. 1995). Por outro lado, a diminuição na condutância dos canais juncionais após ativação de tirosina quinases ligada a receptor, pelo tratamento com EGF ou PDGF, não envolve fosforilação dos resíduos tirosina, mas, sim, nos resíduos de serina das conexinas, mediada pela MAPK (Kanemitsu e Lou 1993, Lampe e Lau 2000).

Em resumo, a permeabilidade das junções comunicantes pode ser regulada por vários agentes através do controle da abertura e fechamento ("gating") dos canais, o que freqüentemente envolve a fosforilação das conexinas nos seus resíduos de tirosina ou serina/treonina.

Funções e Disfunções das Junções Comunicantes nos Tecidos

Evidências têm apontado um importante papel das JCs em vários processos celulares. Nos diferentes tecidos que integram um organismo, as JCs que interconectam as células podem funcionar como canais de íons, metabólitos e/ou de moléculas sinalizadoras intracelulares. O comprometimento dessas funções das JCs nos tecidos tem sido associado a certas doenças humanas, sendo várias de origem genética (Castro et al. 1999, Spray e Dermietzel 1995). Em tecidos excitáveis, como as células nervosas e células musculares cardíacas, os canais formadores da junção comunicante funcionam como canais de íons, principalmente de K^+. No sistema nervoso central, a conexão direta entre neurônios via junções comunicantes forma a base morfo-funcional das sinapses elétricas, encontradas desde animais inferiores até em mamíferos (Haefliger e Meda 2000, Jongsma e Wilders

2000, Rozental et al. 2000). A ativação de circuitos neuronais via sinapses elétricas tem sido associada a comportamentos de fuga e defesa em várias espécies animais (Rozental et al. 2000). As junções comunicantes interligando neurônios permitem a passagem direta de íons de uma célula para outra conduzindo, assim, a onda de despolarização de forma mais rápida e bidirecional (Rozental et al. 2000). Em células musculares cardíacas, os canais formadores de junção comunicante, funcionando como canais de íons, permitem a propagação de potenciais de ação essencial para a perfeita sincronia e ritmicidade do batimento cardíaco. As junções comunicantes estão envolvidas em algumas doenças cardiovasculares. Numerosas evidências sugerem que alterações na distribuição, densidade e propriedades das JCs podem estar envolvidas em arritmias (Jongsma e Wilders 2000). Outro exemplo é o que ocorre na doença de Chagas. A infecção de células cardíacas pelo hemoflagelado *T. cruzi* é acompanhada por marcantes distúrbios na comunicação intercelular. Ocorre uma redução do acoplamento intercelular e condutância entre as células infectadas (Campos de Carvalho et al. 1991). Além disso, a sincronia e ritmicidade das contrações cardíacas são completamente alteradas e perturbadas. Tais alterações funcionais estão associadas a uma diminuição da expressão da proteína juncional presente nestes tipos de canais, a Cx43. Em algumas células infectadas pode ocorrer, ainda, um desaparecimento de Cx43 em algumas regiões da membrana celular, promovendo um comprometimento na comunicação intercelular e, até mesmo, fechamento dos canais (Campos de Carvalho et al. 1991).

Nas células do cristalino, as JCs funcionam como canais, onde metabólitos são livremente trocados entre células acopladas. Como a vascularização da lente é pobre, o acoplamento metabólico entre as suas células permite um fornecimento de nutrientes por toda a extensão do cristalino (Baruch et al. 2001). Foram descritos três tipos de conexinas expressas em células do cristalino: a Cx43, a Cx46 e a Cx50. Perturbações da comunicação intercelular nas células do cristalino estão associadas a alterações nos níveis de cálcio intracelular e aumento da proteólise das cristalinas. Cristalinas são proteínas expressas nas células diferenciadas do cristalino e lhe conferem a propriedade de transparência. Alteração da estrutura molecular das cristalinas, como a que ocorre durante o comprometimento das JCs, tem sido associada à formação de catarata, doença caracterizada pela perda da transparência do cristalino ou da sua cápsula (Baruch et al. 2001).

Nas células de Schwann, as JCs também funcionam como canais de metabólitos. Estes canais agem como um local de rápida propagação e passagem de pequenos metabólitos e mensageiros intracelulares entre os compartimentos citoplasmáticos das células de Schwann (Castro et al. 1999, Collares-Buzato, 2001). A Cx32 é a responsável por formar os canais das JCs nas bainhas de mielina das células de Schwann e mutações no gene codificador desta proteína têm sido associadas à doença humana de Charcot-Marie-Tooth (Castro et al. 1999, Spray e Dermietzel 1995). Esta doença, uma neuropatia congênita, foi a primeira descrição de uma disfunção diretamente resultante de um comprometimento das JCs numa célula. A doença de Charcot-Marie-Tooth é conseqüência de uma desmielinização progressiva das fibras nervosas periféricas resultante desta disfunção nas células de Schwann, os seus sinais clínicos são: uma baixa condutividade nervosa, fraqueza muscular crônica e uma progressiva atrofia muscular, com perda sensorial dos membros inferiores e, também, superiores (Castro et al. 1999, Spray e Dermietzel 1995).

Muitos estudos têm sido desenvolvidos para investigar também a participação do acoplamento celular via junções comunicantes na biossíntese, estocagem e na secreção de alguns hormônios e outros produtos de secreção. Tem sido observado que a secreção glandular é fortemente alterada após o desacoplamento destas células. Ainda, quando o contato intercelular é reestabelecido, a secreção também é reestabelecida e, até mesmo, melhorada. Nas glândulas endócrinas e exócrinas, as células secretoras se comunicam via junções comunicantes, podendo estas funcionar como canais de Ca^{+2} e/ou de mensageiros intracelulares. Em algumas glândulas, como as glândulas tireóides e adrenais, o aumento do acoplamento intercelular está relacionado com o aumento da secreção hormonal (Munari-Silem e Rousset 1996). Inúmeras linhas de pesquisas indicam, também, que o acoplamento intercelular está envolvido no controle da secreção de amilase pelas células acinares do pâncreas exócrino. Células acinares pancreáticas estão acopladas por numerosas junções comunicantes, as quais estão constituídas pelas conexinas Cx32 e Cx26 (Meda 1996). Células acinares, quando dispersas, apresentam uma secreção diminuída desta enzima sob condições basais e mesmo após estímulo colinérgico. Entretanto, apresentam melhora neste quadro, após reestabelecimento dos contatos intercelulares (Meda 1996).

JUNÇÕES COMUNICANTES NO PÂNCREAS ENDÓCRINO

Estudos recentes têm demonstrado também a importância das junções comunicantes (JCs) na função secretora do pâncreas endócrino. As ilhotas de Langerhans constituem a unidade morfológica e funcional da porção endócrina do pâncreas. As ilhotas pancreáticas são estruturas esféricas, imersas na porção exócrina do pâncreas e formadas por cordões celulares, entremeados por capilares, cujas células são interconectadas por junções intercelulares, incluindo as JCs. Estes cordões são constituídos por quatro tipos celulares: células β ou B, responsáveis pela síntese e secreção de insulina; células α ou A, responsáveis pela síntese e secreção de glucagon; células δ ou D, secretoras de somatostatina e células PP, responsáveis pela secreção de polipeptídeo pancreático. As células, no interior das ilhotas, estão organizadas de forma não aleatória. As células α localizam-se preferencialmente na periferia das ilhotas; as células δ e PP podem ser encontradas tanto na periferia como no interior das ilhotas, já as células β localizam-se na região central das ilhotas, formando agregados celulares.

As células β das ilhotas de Langerhans estão conectadas umas com as outras por numerosas e diminutas junções comunicantes formadas pelas conexinas, Cx43 (Meda et al. 1983; 1991, Vozzi et al. 1995), Cx45 (Charollais et al. 1999) e a Cx36 (Serre-Bernier et al. 2000). Dentre essas conexinas, a Cx43 tem sido a mais bem caracterizada e estudada no pâncreas endócrino, enquanto que a Cx36 é a mais nova conexina identificada nesta glândula, que parece ser expressa exclusivamente pelas células β (Serre-Bernier et al. 2000). Em uma célula β, estão presentes de 800 a 2000 canais comunicantes que juntos preenchem menos de 1 μm^2 da membrana plasmática (Meda 1996). Esses canais permitem que células β estejam eletricamente acopladas e que, entre elas, haja um transporte de íons e/ou moléculas sinalizadoras.

Inúmeras linhas de pesquisas indicam uma contribuição importante do acoplamento entre células β no controle da secreção de insulina. Glicose, o principal secretagogo das células produtoras de insulina, induz um aumento intracelular da razão ATP/ADP, despolarização da membrana das células β e, conseqüentemente, aumento intracelular de

Ca^{+2} (Deeney et al. 2000). Esse sinal elétrico/metabólico pode ser passado de uma célula para outra, através de comunicação intercelular via JCs (Kanno et al. 2002). Sendo assim, quando as células β estão acopladas pelas JCs, a secreção de insulina é otimizada, pois frente a um secretagogo, um maior número de células β é estimulada dentro da ilhota, simultaneamente, resultando numa secreção maior e sincronizada (Deeney et al. 2000). Prova disto, é que células β, quando isoladas, apresentam uma secreção basal de insulina aumentada, uma baixa sensibilidade à glicose, uma baixa resposta a outros secretagogos e uma expressão do gene da insulina diminuída (Bosco et al. 1989, Halban et al. 1981, Meda et al. 1979; 1990; 1991, Pipellers et al. 1982, Vozzi et al. 1995). No entanto, com o reestabelecimento desse contato intercelular há uma rápida reversão desses efeitos (Bosco 1989, Halban et al 1982).

Algumas condições experimentais *in vivo*, assim como em *in vitro*, que estimulam a secreção de insulina, estão associadas a um aumento no acoplamento de células β devido, pelo menos em alguns casos, ao aumento do número das junções comunicantes e da expressão das Cx43 (Kohen et al. 1979, Meda et al. 1979; 1983; 1991) e Cx36 (Serre-Bernier et al. 2000). Tem sido sugerido que o acoplamento entre células β pode ser aumentado durante a estimulação com glicose, sendo tal fato detectado pelo aumento sincronizado nas oscilações de $[Ca^{+2}]_i$ em grupos de células acopladas (Charollais et al. 2000). Além disso, condições que inibem a secreção de insulina diminuem ou bloqueiam o acoplamento célula-célula. Um bloqueio farmacológico das JCs altera marcantemente a função dessas células, gerando um aumento da secreção basal de insulina e a perda do estímulo para secreção, tanto em ilhotas de Langerhans isoladas, como no pâncreas intacto (Meda et al. 1990).

Embora o papel das JCs no pâncreas endócrino pareça bem estabelecido, estudos sobre os mecanismos de regulação destas junções nos vários modelos experimentais de disfunção das células β ainda continua pouco explorado.

Tem sido demonstrado que ilhotas de fetos e recém-nascidos de roedores exibem uma reduzida resposta secretória à glicose e a outros secretagogos (como aminoácidos, carbacol e éster de forbol) em comparação às ilhotas de animais adultos (Boschero et al. 1988; 1993). A prolactina, um hormônio somatotrófico, parece desempenhar importante papel na maturação dos mecanismos intracelulares de secreção de insulina e nos

processos de diferenciação celular no pâncreas endócrino (Boschero et al. 1993, Crepaldi et al. 1997, Sorenson et al. 1987). Estudos *in vitro* têm demonstrado que o tratamento com prolactina das ilhotas de ratos recém-nascidos, bem como, de ilhotas de adultos e linhagem de células β (RIN cells) tem efeito mitogênico (Nilsen 1982), promovendo também um aumento na biossíntese de insulina (Brelje et al. 1989), uma diminuição do limiar de estimulação pela glicose (Sorenson et al. 1987), um aumento da expressão do transportador de glicose (GLUT2) na ilhota (Crepaldi et al. 1997) e uma potencialização do efeito de vários secretagogos sobre a secreção de insulina (Boschero et al. 1993, Crepaldi et al. 1997).

Experimentos sugerem que cultura prolongada e/ou fatores contidos no meio de cultura promovem também uma melhora na secreção de insulina em resposta à glicose em ilhotas de ratos recém-nascidos (Anderson 1978, Boschero et al. 1988; 1993, Dudek et al. 1980, McEvoy e Leung 1982). Tais efeitos podem estar relacionados a uma regulação da comunicação intercelular mediada pelas JCs. Há um crescente interesse em desenvolver adequadas condições de cultivo e manter os aspectos funcionais das ilhotas pancreáticas em cultura, devido sua potencial utilização em transplantes (Dufayet de la Tour et al. 2001, Itkin-Ansari et al. 2000).

Os mecanismos intracelulares envolvidos no possível aumento da comunicação intercelular induzido pela prolactina e cultura *per se* permanecem ainda desconhecidos. Estes mecanismos podem estar gerando um aumento do número de canais formadores de JCs por indução da transcrição gênica da conexina, ou pela diminuição da degradação desta proteína, e/ou pelo aumento da permeabilidade dos canais através da modificação funcional desta proteína por fosforilação (Laird et al. 1991, Musil et al. 1990, Vozzi et al. 1995).

A regulação da comunicação intercelular pode ocorrer, potencialmente, em vários níveis durante a formação das junções comunicantes: na transcrição do gene da conexina, na tradução do RNA da conexina, na formação ou "arranjo" das conexinas em um conexon, no transporte para a membrana plasmática, no "arranjo" dos conexons para formar os canais das junções comunicantes, na remoção desses canais da membrana plasmática e na degradação das conexinas (Laird 1996). A adesão célula-célula, mediada pela junção aderente via o complexo caderinas-cateninas, constitui uma outra forma de regulação da comunicação intercelular (Fujimoto et al. 1997). Existem várias evidências que mostram

que as JCs dependem funcionalmente da junção aderente em vários tipos celulares: 1) o comprometimento da adesão celular resulta em ruptura das JCs e desacoplamento intercelular (Kanno et al. 1984, Mayer et al. 1992); 2) a indução de adesão celular através da transfecção com caderinas leva a um aumento da comunicação intercelular (Mege et al. 1988, Musil et al. 1990) e 3) a β-catenina está envolvida na transcrição gênica da Cx43 em cardiomiócitos (Ai et al. 2000).

1.2 Objetivos da Dissertação

Esta dissertação tem como objetivo investigar os possíveis mecanismos intracelulares de regulação das JCs pela cultura prolongada e pelo tratamento *in vitro* com prolactina em ilhotas pancreáticas de ratos recém-nascidos. Para tal, foi avaliada a localização, o grau de expressão gênica e o conteúdo celular das proteínas integrantes das JCs, bem como o grau de adesão celular pela expressão da β-catenina nas ilhotas nestas condições experimentais. Este trabalho concentrou-se no estudo da conexina 43, a primeira a ser identificada e a mais estudada no pâncreas endócrino. Entretanto, no decorrer do desenvolvimento desta tese, foi identificada, também neste tecido, a conexina 36, a qual é expressa, principalmente, pelas células secretoras de insulina, as células β (Serre-Bernier et al 2000). Em função disto, procuramos também avaliar a possível regulação *in vitro* desta proteína em alguns protocolos experimentais.

1.3 Estrutura da Dissertação

Esta dissertação contém quatro capítulos. Os resultados estão apresentados nos capítulos 2 e 3 na forma de artigos científicos. O capítulo 2, que consiste em um artigo já publicado na revista Pancreas (Collares-Buzato et al. 2001), apresenta os dados sobre o efeito da cultura *per se* e o tratamento com prolactina na expressão e localização celular de Cx43 e β-catenina em ilhotas pancreáticas de ratos recém-nascidos. O capítulo 3 apresenta os resultados do efeito das condições de cultura, como o tempo de cultivo e concentração de glicose no meio, sobre a expressão gênica e conteúdo celular de Cx43, Cx36 e β-catenina nas ilhotas pancreáticas. O capítulo 4 apresenta as conclusões gerais dos resultados obtidos. Finalmente, o apêndice A descreve, com detalhes, as metodologias empregadas neste estudo.

Tabela I: A Super-Família das Conexinas

Subfamília	Nomenclatura Grega	Nomenclatura baseada no Peso Molecular	Localização	Referências Bibliográficas
α	α1	Cx43	Coração, ilhotas pancreáticas, músculo liso.	Beyer et al.. 1987.
	α2	Cx38	Embrião.	
	α3	Cx46	Cristalino, rim.	Paul et al. 1991.
	α4	Cx37	Estômago, testículo.	Willecke et al. 1991.
	α5	Cx40	Endotélio, Fibras de Purkinje.	Haefliger et al. 1992.
	α6	Cx45	Coração, rim, intestino.	Hennemann et al. 1992b.
	α7	Cx33	Testículo.	Haefliger et al. 1992.
	α8	Cx50	Cristalino, coração.	White et al. 1992.
β	β1	Cx32	Células acinares, células de Shwann, pancreáticas, hepatócito.	Paul 1986.
	β2	Cx26	Células acinares pancreáticas, rim, hepatócito, intestino.	Zhang et al. 1989.
	β3	Cx31	Queratinócitos, rim.	Hoh et al. 1991.
	β4	Cx31.1	Queratinócitos.	Haefliger et al. 1992.
	β5	Cx30.3	Pele, rim.	Hennemann et al. 1992a.
δ	δ	Cx36	Células β pancreáticas, cérebro.	Serre-Bernier et al. 2000; Belluardo et al. 2000.

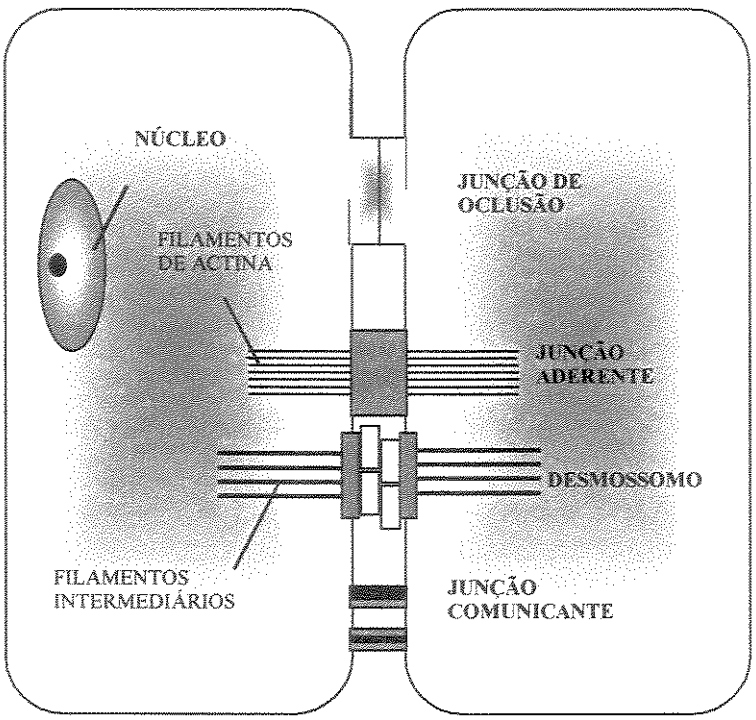


Figura 1: Diagrama mostrando as Junções Intercelulares interconectando duas células vizinhas hipotéticas.

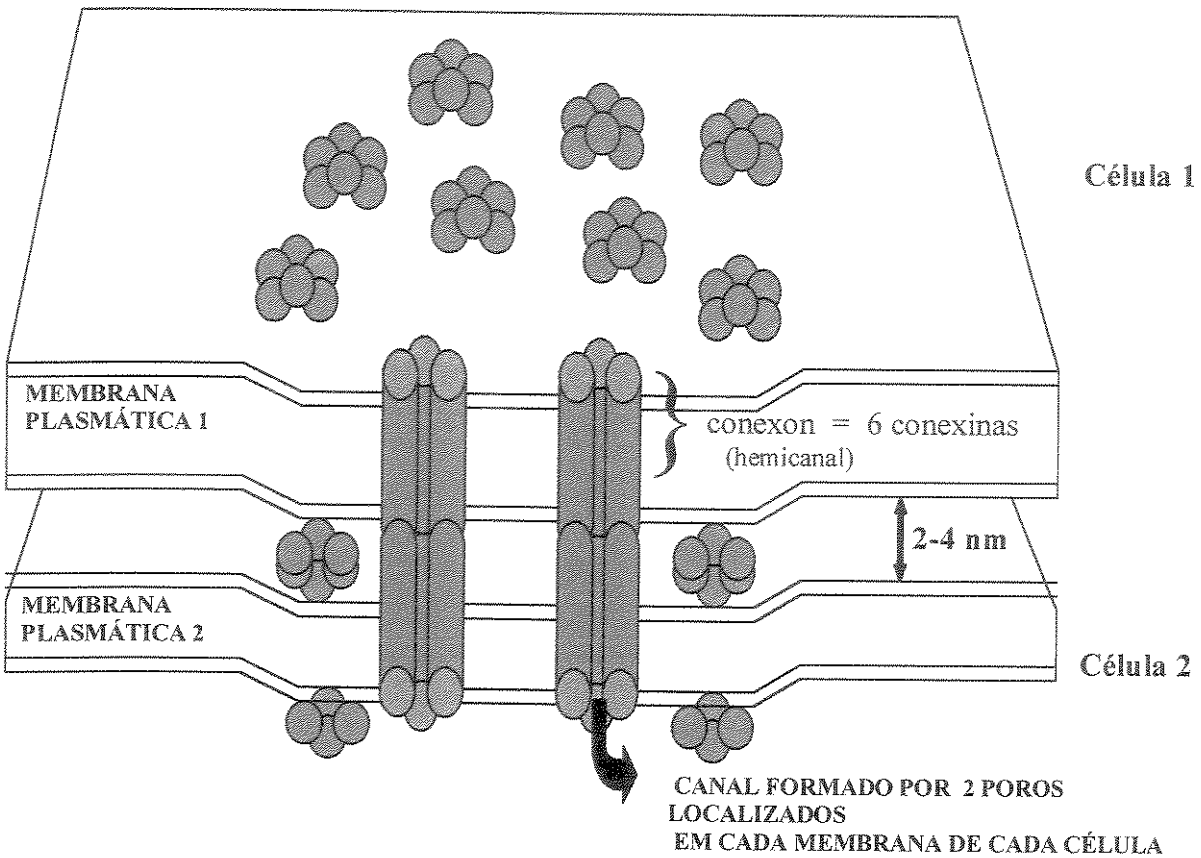


Figura 2: Desenho esquemático das Junções Comunicantes.

A junção comunicante é caracterizada por uma região de contato intercelular reconhecida pela aposição singular das membranas de células vizinhas e presença de canais intercelulares que estão envolvidos na comunicação intercelular, permitindo a passagem de íons e moléculas de baixo peso molecular.

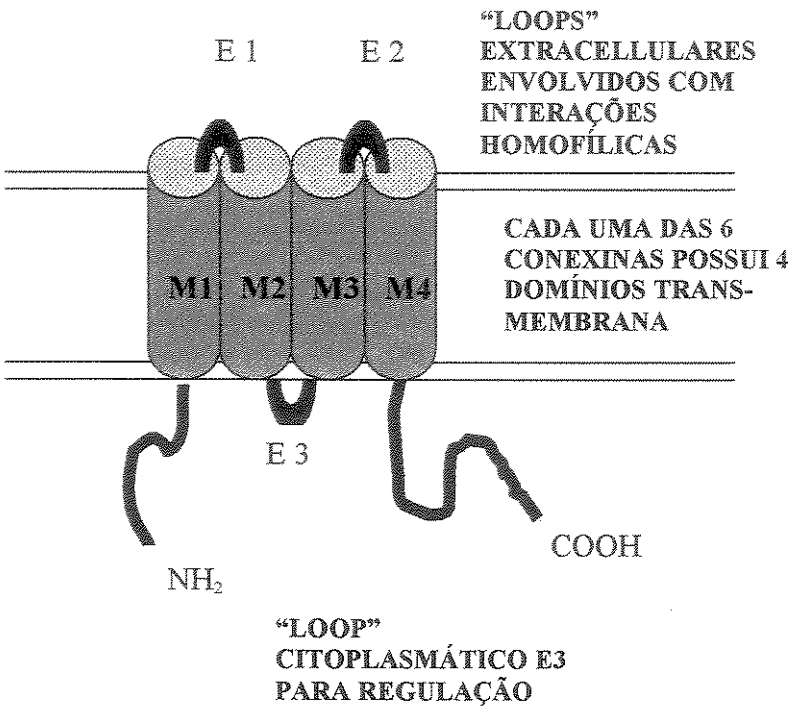


Figura 3. Modelo da estrutura molecular das conexinas

Modelo típico da molécula das conexinas. Os cilindros representam os domínios transmembrana (M1-M4). Os loops entre o primeiro e segundo, assim como entre o terceiro e quarto domínio transmembrana, são extracelulares (E1 e E2, respectivamente).

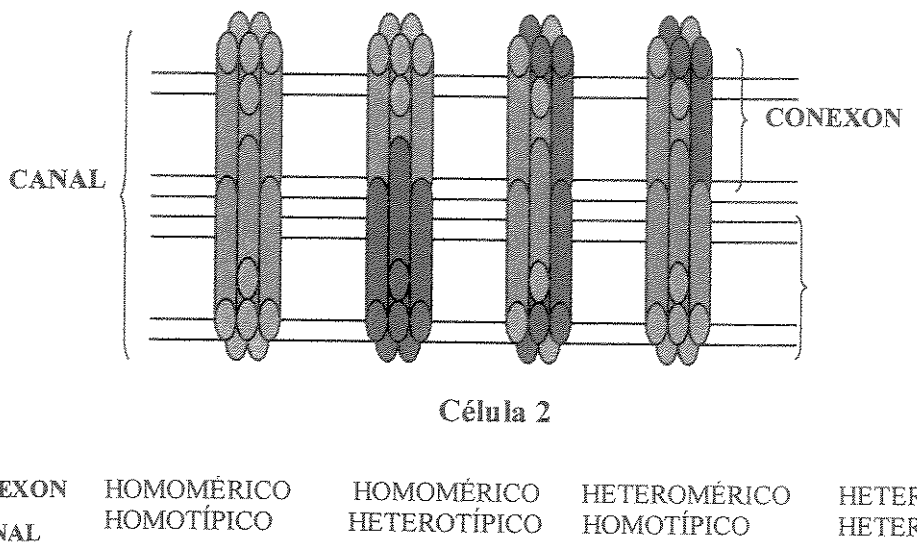


Figura 4. Esquema ilustrando os possíveis arranjos dos conexons para formar os canais das Junções Comunicantes.

Os conexons são constituídos de 6 subunidades de conexinas. As cores vermelho e verde, representam tipos de conexinas diferentes que estão ilustradas em várias configurações. Os conexons podem ser homoméricos (constituídos de 6 subunidades idênticas de conexina) ou heteroméricos (constituídos de mais de um tipo de conexina). Dois conexons quando associados, formam um canal de Junção Comunicante. O canal pode ser considerado homotípico (se os dois conexons forem idênticos) ou heterotípicos (se os dois conexons forem diferentes).

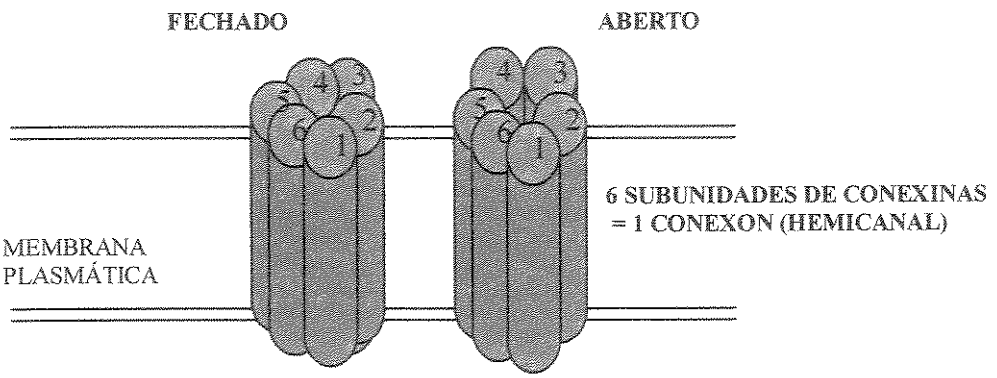


Figura 5: Desenho esquemático da alteração do estado funcional, aberto ou fechado, dos canais das junções comunicantes.

A alteração do estado funcional das JCs envolve uma mudança conformacional na estrutura das conexinas e, conseqüentemente, na interação intermolecular entre essas proteínas dentro do conexon permitindo um "deslizamento" dessas proteínas e gerando a abertura ou fechamento desses canais.

CAPÍTULO 2º

RESULTADOS: 1º ARTIGO

MODULATION OF GAP AND ADHERENS JUNCTIONAL PROTEINS IN CULTURED NEONATAL PANCREATIC ISLETS.

Abstract

It is well known that fetal and neonatal pancreatic islets present lower insulin secretory response as compared to adult islets. In culture conditions and following treatment with mammosomatotropic hormones, neonatal islets undergo maturation of the secretory machinery that might involve regulation of cell-cell contacts within the islet. This study is an investigation of the effect of prolonged culturing and *in vitro* treatment with prolactin on the expression of the gap junction-associated connexin 43 and the adherens junction-associated β-catenin in cultured neonatal rat islets. Pancreatic islets from neonatal Wistar rats were cultured for 24h or 7 days and the treated group was exposed to 2μg/ml prolactin daily for 7 days. Connexin 43 and β-catenin were barely detected at the cell-cell contacts in 24h-cultured islets as revealed by immunocytochemistry. Nevertheless, both junctional proteins were well expressed at the junctional region in islet cells cultured for 7d and showed even higher staining in islets following chronic prolactin treatment. In accordance with the morphological data, neonatal islets cultured for 24h displayed relatively low level of connexin 43, as determined by Western Blot. Culturing for 7d *per se* or combined with prolactin treatment induced a significant increase in Cx 43 expression; this was 40% higher in the prolactin-treated group than in the control group. Moreover, an enhancement of the expression of β-catenin and translocation of this protein to the cell-cell contact site was also observed in neonatal islets cultured for 7d as compared to those cultured for 24h. Prolactin *in vitro* treatment induced even higher expression of β-catenin in islet cells. A correlation was observed between the increased expression of these junctional proteins and an increase in insulin secretion in cultured neonatal islets. In conclusion, prolonged culturing and *in vitro* treatment with prolactin induce modulation of gap and adherens junctional proteins in pancreatic islets, which may be an important event for the *in vitro* maturation process of neonatal islet cells.

1. Introduction

Cell-to-cell contacts are crucial for the proper secretory response within the pancreas (Bosco et al. 1989). These interactions depend on membrane specializations known as intercellular junctions (IJs) that include the tight junction, adherens junction, desmosome and gap junction. All these junctions have been demonstrated to be dynamic and regulated membrane structures in several different tissues (Collares-Buzato et al.1994, Musil et al. 1994). Among the IJs, gap junctions (GJ) have been the most studied in the pancreas. Gap junctions (GJs) are characterized by the presence of intercellular channels that are involved in the cell-to-cell communication (Loewenstein et al.1981, Musil et al. 1994). These channels are composed of members of a family of closely related integral membrane proteins known as connexins, that are assembled in a hexagonal pattern within the membrane delineating a central hydrophilic pore (Dahl et al. 1995, Musil et al. 1994, Spray et al 1994). Evidence has indicated the importance of the GJ-mediated intercellular communication for the insulin secretion by B-cells. *In vivo* and *in vitro* studies have demonstrated that stimulated insulin secretion by glucose and other secretagogues are associated to increased GJ-mediated coupling between B-cells (Meda et al. 1979; 1991). Experimental conditions that inhibit the insulin secretion result in reduction or blockage of coupling between B-cells, while the pharmacological inhibition of the GJ channels induces impairment of the secretory function of these islet cells (Meda et al. 1983; 1990). The observation that both synthesis and release of insulin are markedly altered after dispersion of B-cells *in vitro* conditions, and rapidly improved after cell reaggregation, further suggests that the secretory mechanisms depend on cell-to-cell contacts (Bosco et al. 1989, Halban et al. 1982).

Although the importance of GJ-mediated intercellular communication in endocrine pancreas is well established, the mechanisms underlying the regulation of these junctions in experimental models for B-cell disfunction are still unexplored. One of these models is the fetal and neonatal rat islets that show in relation to the adult rat islets reduced secretory response of insulin to glucose and the other secretagogues such as amino acids, muscarinic agonists and phorbol ester (Boschero et al. 1988; 1989). Prolactin (PRL) and other lactogenic substances appear to play important role in the maturation of the secretory

machinery ocurring in the B-cells. *In vitro* studies have reported that the treatment with PRL of neonatal rat islets increases insulin secretion, decreases the glucose stimulation threshold, enhances GLUT2 glucose transporter expression, and intracellular glucose oxidation (Brelje et al. 1989, Sorenson et al. 1987). It has been reported that under prolonged culture conditions, neonatal islet also shows improvement of the insulin secretion response to glucose and amino acids, indicating a possible role for a culture medium-containing factor in the B-cell maturation process (Boschero et al. 1993, Freinkel et al. 1984, McEvoy et al. 1982). In the case of the PRL treatment, this hormone also increases GJ-mediated coupling between B-cells as revealed by the intercellular transference of intracellular microinjected Lucifer Yellow dye between adjacent cells (Michaels et al. 1987, Sorenson et al. 1987). This observation indicates that cell-cell contacts mediated by GJ may play an important role in the hormone-induced neonatal B-cell maturation. Meanwhile, a possible GJ upregulation during prolonged culture of fetal and neonatal islets has not been assessed.

The intracellular mechanisms involved in this increase of cellular communication induced by PRL are still unknown and might involve one or several possible levels of intercellular gate control. The regulation of gap junctional communication can occur potentially at multiple levels: transcription of the connexin gene, translation of connexin transcripts, assembly of connexins into connexons and transport to the plasma membrane, assembly of connexons into GJ intercellular channels, gating of functional GJ channels and disassembly and degradation of the connexin proteins (Musil et al. 1994,Spray et al. 1994). Cell-to-cell adhesion mediated by the cadherin-catenin complexes within the adherens junction (AJ) may also affect intercellular communication by modulating some of these GJ channel formation steps (Ai et al 2000, Mege et al. 1988). The aim of this study was to investigate the effect of prolonged culturing and *in vitro* treatment with prolactin on the cellular expression and localization of the GJ-associated Cx43 and the AJ-associated β-catenin in neonatal rat islets. We report here that those experimental conditions induce upregulation of the expression of both junctional proteins. A correlation was observed between the increased expression of Cx43 and β-catenin and an increase in insulin secretion followed by 7d-culture *per se* or combined with chronic PRL treatment in neonatal islets. We suggest that the regulation of gap and adherens junctional proteins by

prolactin and culture medium-containing factors may be an important event for the maturation process of B-cells observed at *in vitro* conditions.

2. Materials and Methods

Materials

All chemicals, cell culture media and supplements were purchased from Sigma (St Louis, MO, USA). All sterile plastics (Petri dishes and Multiwell plates) were purchased from Falcon. Radiolabelled insulinand protein A were obtained from Amersham Life Science (Cleveland, Ohio, USA). FITC-conjugated secondary antibodies were purchased from Sigma. Anti- Cx43 and anti-β-catenin monoclonal antibodies were supplied by Zymed Laboratories (San Francisco, CA, USA).

Culture of islets of Langerhans

Islets from neonatal (2 to 36h) Wistar rats were obtained as previously described (14). They were mantained in culture within sterile Petri dishes at 37°C in a humidified 5% CO_2/air atmosphere. The culture medium consisted of RPMI-1640 containing 10 mM glucose and supplemented with 5% fetal calf serum (heat inactivated) and 100 IU penicillin/ml and 100 µg streptomycin/ml.The dishes were divided in three experimental groups: neonatal islets cultured for 24h (group N24), neonatal islets cultured for 7 days (group NC) and neonatal islets cultured for 7 days receiving a dose of prolactin (2µg/ml) daily (group NPRL). The culture medium was changed every second day up to 7 days. At the end of the culture period, the islets were individually collected under a dissecting microscope using a micropipette. Part of these islets were used to determine the insulin secretion levels and the remaining were aldehyde-fixed for immunocytochemistry or frozen down to -70 °C in RPMI-1640 medium containing 2mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% glycerol for immunoblot.

Immunocytochemistry

The cell location of Cx43 and β-catenin proteins was determined by a standard indirect immunofluorescence technique (Collares-Buzato et al. 1994; 1998). A pool of at least 1500 islets were pelleted within an eppendorf, fixed in 2% paraformaldehyde (in phosphate buffer saline, pH 7.4, PBS) for 30 min at room temperature (RT). After then, they were included in increasing concentrations of gelatin solutions (5, 10 and 25%) at 37 °C. After

incubation, the gelatin blocks were embedded in OCT compound and frozen in n-hexane with liquid nitrogen. Cryostat sections (6μm thick) were picked up on poly-L-lysine-coated glass slides, air dried and treated with 0.1% Triton X-100 (in PBS) for 10 min at RT. Sections were, then, washed three times with TTBS (10mM Tris, 150mM NaCl, pH 7.4, 0.1% Tween 20) and incubated with TTBS containing 5% bovine serum albumin (BSA) for 1h at RT. For immunofluorescence labelling of Cx43, these islet sections were firstly incubated with anti-Cx43 or anti-β-catenin monoclonal antibodies (dilution 1:50 in TBS plus 1% BSA) overnight at 4°C and, then, incubated with the specific secondary antibody conjugated with fluorescein (FITC-labelled anti-mouse IgG, dilution 1:75 in TBS plus 1% BSA) for 2h at RT. Following final wash, the sections were mounted in a commercial antifading agent (Vectashield, Vector Laboratories, Burlingame, CA, USA) and visualised by confocal laser scanning microscopy (CLSM; BioRad MRC 1024UV; Bio-Rad, Hercules, CA, USA) using an inverted fluorescence microscope.

Western Blot

A pool of at least 1000 islets from each experimental group was homogenized by sonication in an anti-protease cocktail (10mM imidazole (pH 8.0), 4mM EDTA, 1mM EGTA, 0.5μg/ml pepstatin A, 200KIU/ml aprotinin, 2.5μg/ml leupeptin, 30 μg/ml trypsin inhibitor, 200μM DL-Dithiothreitol (DTT), 200μM PMSF). After a 10-min centrifugation of the sonicate at 3000g and 4°C, the supernatant was collected and its total protein content was determined by the DC protein assay kit (Bio-Rad Laboratories). Sample of crude membrane preparation of each experimental group containing 70 μg total protein was incubated for 60min at 37 °C with 20% of total volume with 5x concentrated Laemmli sample buffer (1M sodium phosphate pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% mercaptoethanol). These samples were fractionated by electrophoresis in a 8% polyacrylamide gel. Electrotransfer of proteins from gel to nitrocellulose (Bio-Rad) or PVDF (Millipore,Bedford, MA, USA) membranes was performed for 60min at 20V constant in the absence of methanol and SDS. After checking for efficient transfer by Ponceau S staining, the membranes were saturated with 5% dry skimmed milk in TTBS (10mM Tris, 150 mM NaCl, 0.5% Tween 20) overnight at 4°C. Cx43 was detected in the membrane after 2h-incubation at room temperature (RT) with a mouse monoclonal

antibody against heart Cx43 (dilution 1:1000 in TTBS plus 3% dry skimmed milk) or anti-β-catenin (dilution 1:1000 inTTBS plus 3% dry skimmed milk). The membrane was, then, incubated with a rabbit anti-mouse IgG (dilution 1:1500 in TTBS plus 3% dry skimmed milk) followed by further 2h-incubation at RT with I^{125} -labelled protein A (2000 cpm). Radiolabelled protein bound to the antibodies was detected by autoradiography. Band intensities were quantified by optical densitometry of the developed autoradiogram, using the Scion Image Beta sofware.

Insulin secretion

After culture, the islets were gently detached from the plates under a dissecting microscope. Clean islets were washed with a bicarbonate-buffered solution (pH 7.4) (composition: 115mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$ and 24mM $NaHCO_3$ supplemented with 3mg/ml bovine serum albumin and 10mM glucose and equilibrated with a mixture of 95% O_2 – 5% CO_2) and then transfered to a 24-multiwell plates. Groups of 8 to 10 islets from the different experimental groups were placed within each well of 24-multiwell plates and incubated with 1ml supplemented RPMI-1640 medium containing 10 mM glucose for 2h. After the incubation period, aliquots of the supernatant were taken and stored at -20°C. The insulin content of these samples was determined by radioimmunoassay and was expressed as nanograms per islet/hour (13).

Statistical Analysis

All the results are expressed as means ± SEM together with the number of individual experiments (n). The statistical significance of differences among more than two experimental groups was assessed by ANOVA and for multiple comparison between pairs of groups was used the Bonferroni's Test. The significance level was set at $P < 0.05$.

3. Results

Islets of Langerhans from different experimental groups showed distinct insulin secretory response to 10mM glucose (Table I). Neonatal islets treated with PRL for 7d displayed an insulin secretion level of 1.6 ($P<0.02$), 3.5 times ($P<0.02$) higher than those untreated neonatal islets cultured for 7d (NC group) or for only 24h (N24 group), respectively. Moreover, the NC group showed significantly higher secretion as compared to the N24 group ($p<0.05$) (Table I).

Figure 1 depicts the increase in Cx43 expression observed following chronic treatment with PRL, as revealed by Western blot of homogenized islets samples. PRL treatment (2μg/ml/day) for 7d *in vitro* resulted in approximately 40% increase in the expression of this GJ-associated protein as compared to untreated islets cultured for 7d belonging to the NC group. Islets from this latter group showed higher Cx43 expression in relationto neonatal islets cultured for only 24h, which displayed relatively low amount of this protein (Figure 1a).

This increased Cx43 expression was correlated with an increase in GJ channel number located at the cell membrane as revealed by immunocytochemistry (Figure 2 a-c). Immunolabelling for Cx43 protein showed a punctate staining pattern at the intercellular membrane region in 7d cultured neonatal islets (Figure 2b,c); this Cx43 labelling was not observed in all cells. The immunorreaction for Cx43 was significantly stronger at regions of cell-cell contact in 7d-cultured neonatal islets treated with PRL (Figure 2c) than in untreated neonatal islets (Figure 2b). A higher number of cells seemed to be labelled in PRL-treated islets (Figure 2c) as compared to those in untreated islets (Figure 2b). On the other hand, neonatal islets cells cultured for only 24h showed a very faint labelling at the cell-cell contact region (Figure 2a).

Besides the Cx43, prolonged culture *per se* or 7d-treatment with PRL also induced changes in expression of another junctional protein, namely β-catenin, known to be associated to the adherens junctions in several epithelial cells (Figure 3). As revealed by Western blotting, we observed relatively high expression of β-catenin in the NC and NPRL groups. Prolonged culture *per se* or PRL treatment induced, respectively, a 9-fold

($P<0.001$) and 11-fold ($P<0.001$) increase in β-catenin expression in neonatal islets as compared to those cultured for only 24h.

In agreement with the immunoblotting data, 7d-culturing *per se* and followed by PRL treatment induces increase in junctional expression of β-catenin in islet cells (Figure 4 b,c). In both groups, a bright labelling at the perijunctional region of all islet cells was observed (Figure 4 b,c). In contrast, islets cultured for only 24h showed very few labelled cells, mainly located at the islet periphery, probably representing non-B cells (Figure 4a), following immunostaining for β-catenin. The labelling in these cells was observed throughout the cell cytoplasm (Figure 4a).

FIGURES

Table I : Chronic treatment with PRL or prolonged culture *per se* induced increase in the insulin secretion by islets of Langerhans *in vitro*.

Groups[a]	Insulin secretion (ng/islets.h)[b]
N24	0.20±0.02 (16)
NC	0.44±0.05 (21)*
NPRL	0.70±0.07 (26)**δ

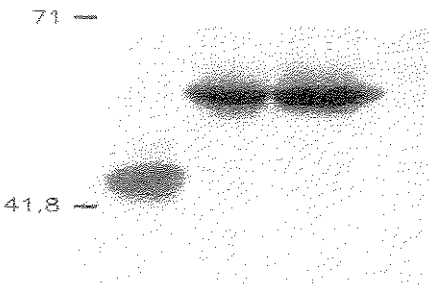
[a] The experimental groups were as followed: N24, neonatal islets cultured for 24h; NC, neonatal islets cultured for 7d; NPRL, neonatal islets cultured for 7d and treated daily with PRL (2μg/ml/day).

[b] Islets were stimulated for 2h with 10mM glucose added to the culture media. Insulin released within the medium was measured by radioimmunoassay. The results were expressed as means ± SEM (number of islets)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ in relation to N24 group; $\delta p<0.02$ in relation to NC group (t-Student test)

FIGURE 1

a)


71
41,8
N24
NC
NPRL

b)

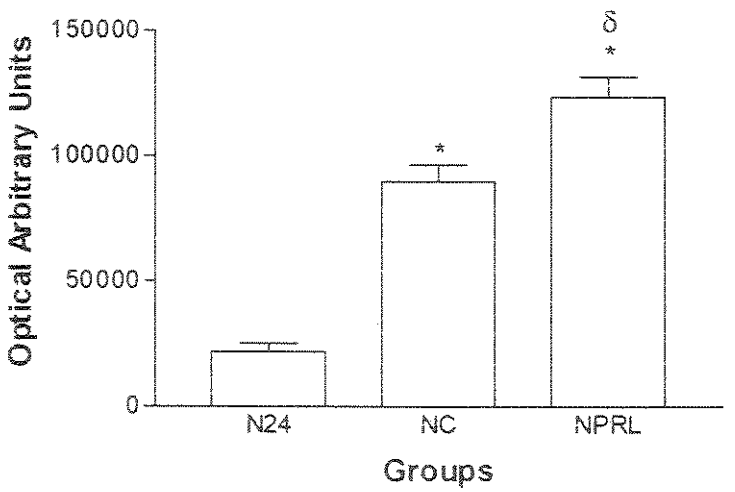

Optical Arbitrary Units
150000
100000
50000
0
δ
*
*
N24
NC
NPRL
Groups

Figure 1 - Increase in Cx43 expression in neonatal pancreatic islets induced by *in vitro* treatment with prolactin or prolonged culture *per se*.

The level of Cx43 was detected by Western Blot in islet sonicate. As compared to culturing for 24h (N24, lane 1), culture of islets for 7d *per se* (NC, lane 2) or 7d-treatment with prolactin (2μg/ml/day) (NPRL, lane 3) induced significant increase in the expression of Cx43 and a slower gel mobility of this protein (a). Expression of Cx43 after prolactin treatment was 40% higher than the 7d-cultured control as measured by densitometry (b). Values of densitometry for each experimental group are shown in b and expressed as means ± standard errors. The result shown in a is representative of 5 independent experiments. Legends: **N24**, neonatal islets cultured for 24h; **NC**, neonatal islets cultured for 7d; **NPRL**, neonatal islets cultured for 7d and treated daily with PRL (2μg/ml/day).

$^{*}p<0.001$ in relation to N24 group; $^{\delta}p<0.05$ in relation to NC group. (Bonferroni's test.)

FIGURE 2

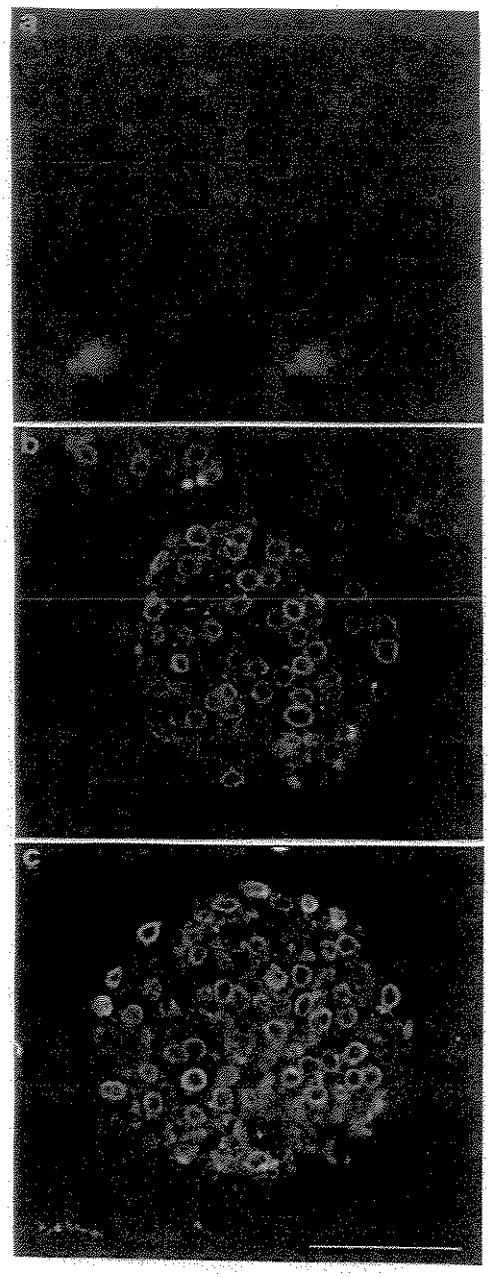
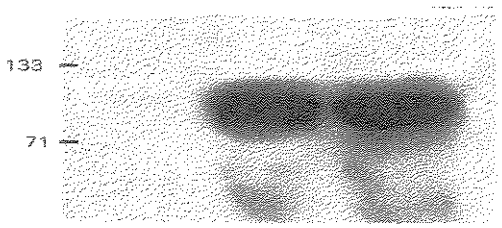


Figure 2 - Higher junctional content of Cx43 followed by *in vitro* treatment with prolactin (PRL) or prolonged culture *per se.*

a-c "En face" (X-Z) confocal images showing indirect immunoflorescence staining of Cx43 in neonatal islets cultured for 24h (a), in neonatal islets cultured for 7d (b) and in neonatal islets cultured for 7d and treated daily with PRL (2μg/ml/day)(c). Images were taken at the same sensitivity of the confocal laser scanning microscope. Note that Cx43 labelling showed a bright punctate pattern on the plasma membrane and was significantly stronger at regions of cell-cell contact in 7d-cultured neonatal islets treated with PRL (c, arrowhead) than in untreated neonatal islets cultured for 7d (b, arrowhead). Neonatal islets cultured for only 24h showed a very faint labelling for Cx43 (a). Bar, 50μm.

FIGURE 3

a)


133
71
N24
NC
NPRL

b)

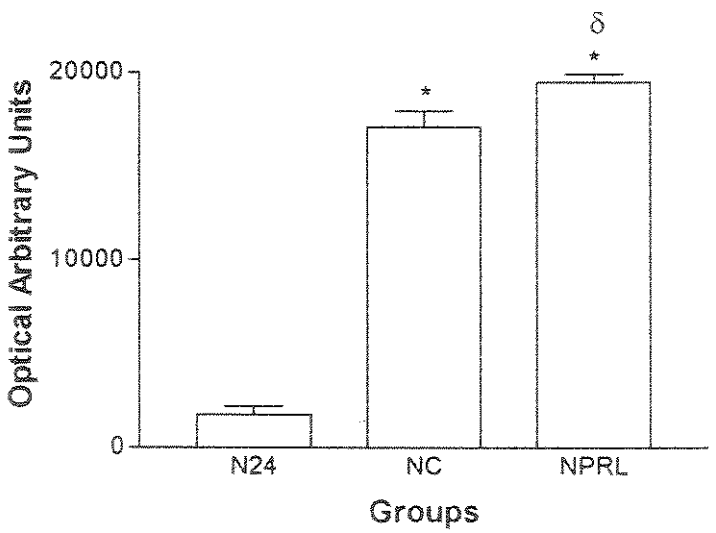

δ
*
*
20000
10000
0
Optical Arbitrary Units
N24
NC
NPRL
Groups

Figure 3 - Increase in β-catenin expression in neonatal pancreatic islets induced by *in vitro* treatment with prolactin (PRL) or prolonged culture *per se*.

The level of β-catenin was detected by Western Blot in islet sonicate. Note that NC (lane 2) and NPRL (lane 3) show significant higher expression of this junctional protein than the N24 group (lane 1) (in a and b). Values of densitometry for each experimental group are shown in b and expressed as means ± standard errors. The result shown in a is representative of 4 independent experiments. Legends: **N24**, neonatal islets cultured for 24h; **NC**, neonatal islets cultured for 7d; **NPRL**, neonatal islets cultured for 7d and treated daily with PRL (2μg/ml/day).

$^{*}p<0.001$ in relation to N24 group; $^{\delta}p<0.05$ in relation to NC group. (Bonferroni's test.)

FIGURE 4

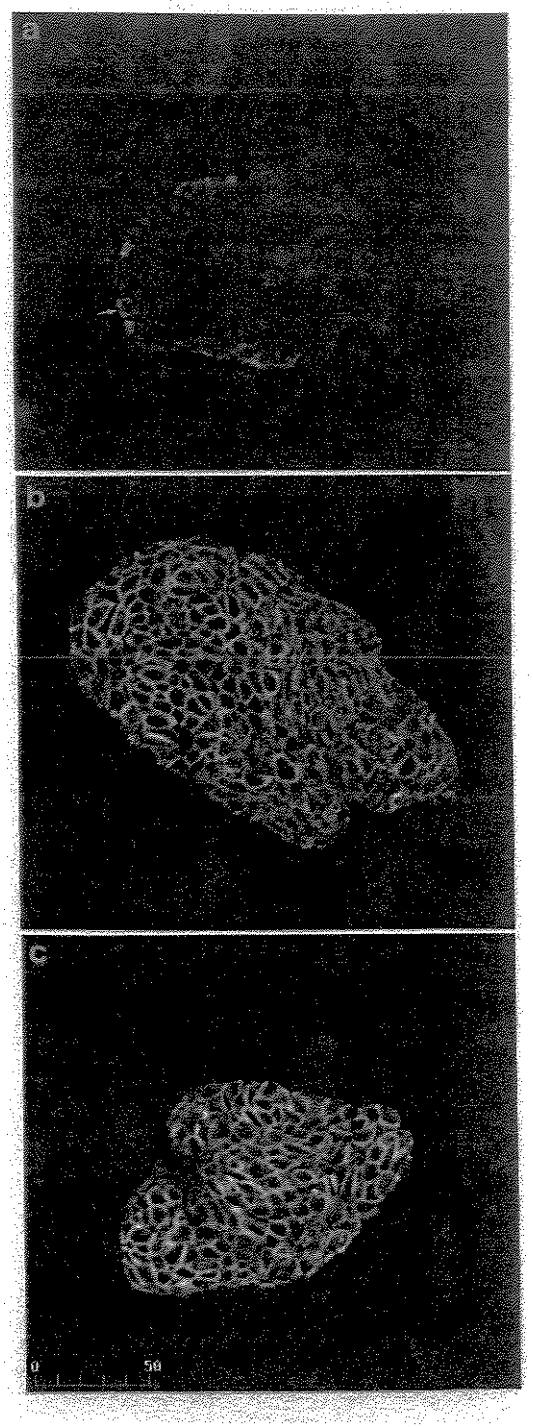


Figure 4 - Higher junctional content of β-catenin followed by *in vitro* treatment with prolactin (PRL) or prolonged culturing *per se*.

a-c "En face" (X-Z) confocal images showing indirect immunoflorescence staining of β-catenin in neonatal islets cultured for 24h (a), in neonatal islets cultured for 7d (b) and in neonatal islets cultured for 7d and treated daily with PRL (2μg/ml/day) (c). Images were taken at the same sensitivity of the confocal laser scanning microscope. Islets cultured for only 24h showed few labelled cells following immunostaining for β-catenin; this labelling was observed throughout the cell cytoplasm (a, arrowhead). Note that 7d-culturing *per se* or in combination with PRL treatment induced overexpression of this junctional protein at the cell-cell contact region (b,c). The intensity of the junctional staning was slightly higher in the islets treated with PRL as compaired with its control Bar, 50μm.

4. Discussion

Gap junctions are cell surface especializations that mediate direct exchange of ions and small molecules between adjacent cells. In exocrine and endocrine glands, GJ can function primarily as channels for Ca^{2+} or secondary messengers (i.e. cAMP and IP_3) (Musil et al. 1994, Spray et al. 1994). The intercellular exchange of theses elements allows amplification of the secretory gland response since ensures a rapid recruitment of secreting cells located distantly from the site of signalling. The importance of the GJ-mediated cell coupling has been particularly well demonstrated in the endocrine pancreas; increased intercellular communication is directly correlated to an increase in secretory insulin response in B-cells (Meda et al. 1979; 1991). The means of upregulating gap junction function can include an increase in the number of GJ channels resulted of an overexpression of the connexin type found in the islets of Langerhans, the Cx43, and/or an enhancement of GJ channel permeability (Musil et al. 1994, Spray et al. 1994). Moreover, another level of regulation for gap junctional intercellular communication may be through cell-cell adhesion, which are mediated by another type of intercellular junction, the adherens junction (AJ) in several tissue types. There are now strong evidence that gap junction depends functionally on the adherens junction. Impairment of the intercellular adhesion with antibodies against AJ-associated cadherins results in GJ disassembly or intercellular uncoupling (Behrens et al. 1985, Kanno et al. 1984). On the other hand, the transfection of junctional communication-deficient cell line with DNA encoding cadherins increases cell coupling (Mege et al. 1988).

In this study, we have demonstrated that 7d-culture *per se* or combined with a chronic treatment with PRL induce an enhancement in the expression of Cx43 in pancreatic islets from neonatal rats. PRL treatment *in vitro* resulted in further increase in the junctional content of this GJ protein as compared to prolonged culturing of islets in the absence of this hormone. In parallel, both experimental conditions led to an increase in the glucose-evoked insulin secretion by neonatal islets as compared to 24h-culturing. The increase in Cx43 expression as revealed by Western Blot was confirmed by immunocytochemistry. Neonatal islets cultured for 7d and treated with PRL show greater levels of Cx43 staining at the cell-cell junctional region as compared to untreated neonatal islets cultured for only 24h. This

observation indicates that the increase in cellular Cx43 expression was accompanied by an increase in number of connexin-formed channels at the cellular membrane site.

The elevated Cx43 expression following treatment with PRL is in accordance with previous observation showing an increase in cell coupling in neonatal islets with this hormone as demonstrated by intercellular transference of microinjected Lucifer Yellow to neighbouring cells (Michaels et al. 1987, Sorenson et al. 1987). Moreover, the present data suggest that this prolactin-induced increase in intercellular communication may be result of an increased number of functional GJ channels at the membrane rather than due to only upregulation of the gating function of these channels. Prolactin and other mammosomatotropic hormones have major upregulatory effects on pancreatic islet B-cell function, including the enhancement of intercellular GJ coupling, that seem to be involved in islet adaptation to pregnancy and maturation of B-cell glucose sensing mechanism during the perinatal period of life (Boschero et al. 1993, Brelje et al. 1991, Crepaldi-Alves et al. 1997, Sekine et al. 1998). The mechanism of PRL actions is depended on the trigger of several intracellular signalling pathways (e.g. JAK/STAT and MAP kinases pathways (Bole-Feysot et al. 1998, Ling et al 1994) leading to activation of nuclear promoter elements on PRL-responsive genes. It is a matter of further investigation whether these pathways also mediate the PRL effect on GJ function.

Concerning the stimulatory effect of prolonged islet culture (7d) on Cx43 expression and insulin secretion, it is plausible to suggest that lactogenic factors and/or hormones present within the serum or even other culture medium components may be accounted for these effects. It is well documented that the presence of serum and certain nutrients at relatively high concentrations (such as glucose and metabolizable amino acids) within the medium are essential for survival, growth and differentiation of islet B-cells *in vitro* conditions (Ling et al. 1994, Sekine et al. 1997). In accordance with our data, previous works have shown a partial maturation of the insulin response to glucose in neonatal islets during a 7 day-culture period using a serum-containing media compositionally similar to that employed here (Boschero et al. 1988, Freinkel et al. 1984, McEvoy et al. 1982). Although the cell-cell coupling has not been directly assessed in this 7d-cultured islets, we can hypothesized based on the Cx43 expression data that this maturation of the stimulus-

secretion coupling process observed following culturing *per se* may also involve an increase in GJ-mediated intercellular communication.

In addition, we have demonstrated in our study that 7d-culture accompanied or not by PRL treatment also induced increase in the expression of β-catenin, a protein associated to another intercellular junction, the adherens junction (AJ). This protein play a crucial role on cell adhesion in several epithelial cell types by modulating the linkage of cadherins to α-catenin which in turn interacts with the actin cytoskeleton (Adams et al. 1996, Hinck et al. 1994). β-catenin is not only an essential structural and regulatory component of adhesion complexes but is also part of the Wnt/Wingless signaling pathway implicated in cell fate decision and pattern formation during development (Barth et al. 1997, Simcha et al. 1998) . It has been reported that the cytoplasmic β-catenin pool binds to certain transcriptional factors (e.g. TCF/LEF-1) within the nucleus and specifies gene expression (Barth et al. 1997, Simcha et al. 1998). The present study is a first description of the expression and regulation of this protein in islet cells. β-catenin is low expressed in poorly insulin-secreting neonatal islets cultured for 24h that show a cytoplasmic localization for this junctional protein in cells located at the islet periphery probably non-B cells. PRL treatment or culturing *per se,* that seem to improve the glucose-induced insulin secretory function, induced upregulation of this protein and its translocation to the cell-cell contact region. Moreover, there seems to be a positive correlation between upregulation on Cx43 and β-catenin expression in islet cells following these experimental conditions. Interestingly, recent work has shown a direct association between β-catenin-associated Wnt signaling and Cx43 expression and GJ function in cardiomyocytes (Ai et al. 2000). Although, functional relationship between AJ and GJ is now extensively documented for several cell types (Ai et al. 2000, Behrens et al. 1985, Kanno et al. 1984, Mege et al. 1988), it is a matter of further investigation whether similar relationship exists in the islet cells, specifically in the case of our experimental model.

In conclusion, we have reported in this study an increase in expression of the gap junction-associated connexin 43 induced by prolonged culturing *per se* and *in vitro* treatment with prolactin in neonatal rat pancreatic islets. A direct correlation was observed between the increased expression of Cx43 and an increase in insulin secretion followed by 7d-culture *per se* or combined with chronic PRL treatment in neonatal islets. In addition,

we first documented the expression of the AJ-associated β-catenin by islet cells and its upregulation following culture *per se* and PRL treatment. The observed regulation of the gap and adherens junctions by PRL and culture medium-containing factors may be involved in the maturation process of B-cells in islets of Langerhans observed at *in vitro* conditions.

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CAPÍTULO 3º

RESULTADOS: 2º ARTIGO

CO-EXPRESSION AND REGULATION OF CONNEXINS 36 AND 43 AND β-CATENIN IN CULTURED NEONATAL PANCREATIC ISLETS.

Abstract

Previous studies have shown that fetal and neonatal pancreatic islets present a lower insulin secretory response as compared to adult islets. Prolonged culturing leads to an improvement of the glucose-induced insulin secretion response in neonatal pancreatic islets that may involve regulation of gap and adherens junction-mediated cell communication. There has been an increasing interest in developing adequate culture conditions for fetal and neonatal pancreatic β-cells and their cell precursors due to their potential use in transplantation for diabetes therapeutics. In this study, we investigated the effect of culturing neonatal islet cells at varying period of time and with different glucose medium concentrations on the cellular expression of the endocrine pancreatic gap junction-associated Cx36 and Cx43 and the adherens junction-associated β-catenin. We report here that prolonged culture (7d) induces upregulation of the expression of these junctional proteins in neonatal islets. The *in vitro* increase in the expression of both connexins, but not of the β-catenin, displayed a time-dependent pattern. A correlation was observed between the increased mRNA and protein expression of Cx36 and Cx43 and an increase in insulin secretion following islet culturing. In addition, increasing glucose concentration within the culture medium induced a concentration-dependent enhancement of Cx36 islet expression but not of Cx43 expression. In conclusion, we suggest that the regulation of gap and adherens junctional proteins by culture medium-containing factors and glucose may be an important event for the maturation process of β cells observed at *in vitro* conditions.

1. Introduction

Gap junctions (GJs) are intercellular channels formed by the linkage of two hemichannels, or connexons, across extracellular space (Kumar & Gilula 1996). Each hemichannel is made of six integral transmembrane protein subunits, named connexins (Cx). These gap junctional proteins form the connexin superfamily composed by, at least, 20 members, each one is encoded by a separate gene and appears to have a cell and tissue-specific distribution. Intercellular channels provide a pathway for direct intercellular exchange of ions and also molecules with a molecular weight below 1000Da, allowing cell-to-cell communication. Gap junctions are thought to coordinate several cellular activities, such as contraction of cardiac and smooth muscle (Barr et al. 1965), transmission of neuronal signals at electric synapses (Rozental et al. 2000), pattern formation during development (Lo 1996) and secretion in exocrine and endocrine glands (Meda 1996).

Insulin - secreting pancreatic β cells are connected by clusters of minute gap junctions observed in intact and isolated islets of Langerhans and in cultured endocrine pancreatic lines (Collares-Buzato et al. 2001, Meda et al. 1991). Several lines of evidence indicate a contribution of the GJ -mediated cell coupling to the control of insulin secretion. Single β cells show a perturbed endocrine functioning, characterized by an increased basal release of insulin, poor secretory responsiveness to secretagogues, decreased hormone biosynthesis, decreased expression of the insulin gene and loss of the normal cAMP-dependent control of insulin secretion (Bosco et al. 1989, Halban et al. 1982, Pipellers 1984). Restoration of β - to β cell contacts is accompanied by a rapid improvement of these alterations in the insulin secretion process. In addition, several insulin-producing cell lines (HIT, RIN, INS-1), that show multiple secretory defects as judged by a markedly low insulin content and loss of responsiveness to the glucose stimulus, have also lost the ability to directly exchange cytoplasmatic molecules by junctional cell coupling (Vozzi et al. 1995). *In vivo* and *in vitro* stimulation of insulin release was found to be associated with an enhancement of β cell coupling due to an increase in the number of gap junctions and Cx43 expression (Collares-Buzato et al. 2001, Kohen et al. 1979, Meda et al. 1979, 1983, 1991).

Recently, Serre-Beinier and colleagues (2000) found that Cx36 is also expressed by insulin-producing β cells but not by other endocrine islet cell types. In comparison to Cx43, the Cx36 has been less studied in the endocrine pancreas. The actual importance of this connexin, as well as the possible mechanism of regulation of its expression in the pancreatic islets, remain to be established.

It is known that fetal and neonatal pancreatic islets present a lower insulin secretory response as compared to adult islets. In culture conditions and following treatment with mammosomatotropic hormones, neonatal islets undergo maturation of the secretory machinery that might involve regulation of cell-cell contacts within the islet (Boschero et al. 1993, Collares_Buzato et al. 2001, Crepaldi et al. 1997). We have recently demonstrated a correlation between the increased expression of junctional proteins, such as Cx43 and β-catenin, and an increase in insulin secretion following culture of neonatal islets (Collares-Buzato et al. 2001). There has been a increasing interest in developing adequate culture conditions for fetal and neonatal pancreatic β-cells and their cell precursors due to their potential use in transplantation for diabetes therapeutics (Demeterco et al. 2000; Itkin-Ansari et al. 2000). Studies on growth, maturation and differentiation of pancreatic fetal β cells will allow to optimizing the *in vitro* maintenance of these cells before transplantation.

In this study we investigated the effect of culturing neonatal islets cells at varying period of time and with different glucose medium concentrations on the cellular expression of the endocrine pancreatic gap junction-associated Cx36 and Cx43 and the adherens junction-associated β-catenin. We report here that prolonged culture of neonatal islets induces upregulation of the expression of all these junctional proteins. A correlation was observed between the increased mRNA and protein expression of Cx36 and Cx43 and an increase in insulin secretion following islet culturing at varying period of time and increasing glucose medium concentration. We suggest that the regulation of gap and adherens junctional proteins by culture medium-containing factors and glucose may be an important event for the maturation process of β cells observed at *in vitro* conditions.

2. Materials and Methods

Materials

All chemicals and cell culture media supplements were supplied by Cultilab or Nutricell (Campinas, SP, Brazil). All sterile plastics (Petri dishes and Multiwell plates) were purchased from Falcon. Radiolabelled insulin – I^{125} and protein A I^{125} were obtained from Amersham Life Science (Cleveland, Ohio, USA). FITC and TRITC-conjugated secondary antibodies were purchased from Sigma. Anti – Cx36 polyclonal antibody and anti – Cx43 monoclonal antibody were supplied by Zymed Laboratories (San Francisco, CA, USA). The Chemiluminecent Substrate Kit was purchased by SuperSignal West Pico (USA).

Prolonged Culture of Islets of Langerhans

Islets from neonatal (24 to 48h) Wistar rats were obtained as previously described (Collares-Buzato et al. 2001; Crepaldi-Alves et al. 1997). They were maintained in culture within sterile Petri dishes at 37°C in a humidified 5% CO_2/air atmosphere. The culture medium consisted of RPMI-1640 containing 10 mM glucose and supplemented with 5% fetal calf serum (heat inactivated) and 100 IU penicillin/ml and 100 μg streptomycin/ml. The dishes were divided in three experimental groups: neonatal islets cultured for 1 day (group N1d), neonatal islets cultured for 3 days (group N3d) and neonatal islets cultured for 7 days (group N7d). The culture medium was changed every second day up to 7 days. At the end of the culture period, the islets were individually collected under a dissecting microscope using a micropipette. Part of these islets was used to determine the insulin secretion levels. The remaining were frozen down to–70 °C in anti-protease cocktail (10 mM imidazole (pH 8.0), 4 mM EDTA, 1 mM EGTA, 0.5 μg/ml pepstatin A, 200 KIU/ml aprotinin, 2.5 μg/ml leupeptin, 30 μg/ml trypsin inhibitor, 200 μM DL-Dithiothreitol (DTT), 200 μM PMSF) for immunoblot, or in Trizol solution (GIBCO BRL Life Technologies) for total RNA extraction.

Islet Culture in Different Concentrations of Glucose

Isolated neonatal islets were cultured in RPMI supplemented as described above, containing 2.8 mM or 5.6 mM or 10 mM of glucose. The glucose content of each medium was determined previously by the glucose assay kit (Biotrol Diagnostics). The culture medium was changed every day up to 3 days of culture. At the end of the culture period, the islets were individually collected under a dissecting microscope using a micropipette.

Western Blot

A pool of at least 1000 islets from each experimental group was homogenized by 15 seconds of sonication in an anti-protease cocktail. The total protein content was determined by the DC protein assay kit (Bio-Rad Laboratories). For the detection of Cx43 and β-catenin the samples were centrifugated and the supernatant protein content was quantified. Sample of crude membrane preparation of each experimental group containing 70 μg total protein was incubated for 60 min at 37 °C with 30% of total volume with 5x concentrated Laemmli sample buffer (1 M sodium phosphate pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% SDS, 2% mercaptoethanol). These samples were fractionated by electrophoresis in a 8% polyacrylamide gel for detection of Cx43 and β-catenin, or 10% for detection of Cx36. Electrotransfer of Cx36 protein from gel to nitrocellulose (Bio-Rad) membrane was performed for 60 min at 120 V and the electrotransfer of Cx43 and β-catenin to nitrocellulose membrane was performed for 60min at 20 V constant in the absence of methanol and SDS. After checking for an efficient transfer with Ponceau S dye, the membranes were saturated with 5% dry skimmed milk in TTBS (10 mM Tris, 150 mM NaCl, 0.5% Tween 20) overnight at 4°C. Cx36 was detected in the membrane after 4 h-incubation at room temperature (RT) with a rabbit polyclonal antibody against Cx36 (Zymed)(dilution 1:500 in TTBS plus 3% dry skimmed milk) followed by a 2 h-incubation at RT with I^{125} -labelled protein A (dilution 1:1000 in TTBS plus 1% dry skimmed milk). Alternatively, the Cx36 expression was also detected by quimioluminescence after 1 h-incubation at RT with the same primary antibody followed by 1 h-incubation at RT with anti-rabbit IgG - conjugated with peroxidase (dilution 1:1000 in TTBS plus 1% dry skimmed milk). Cx43 and β-catenin were detected in the membrane after 2 h-incubation at room temperature (RT) with a mouse monoclonal antibody against Cx43 and β-catenin

(Zymed) (dilution 1:500). The membrane was, then, incubated with a rabbit anti-mouse IgG (dilution 1:1500 in TTBS plus 3% dry skimmed milk) followed by further 2h-incubation at RT with I^{125} -labelled protein A (dilution 1:1000 in TTBS plus 1% dry skimmed milk). Radiolabelled protein bound to the antibodies was detected by autoradiography. Band intensities were quantified by optical densitometry using the Scion Image Release Beta 4.0.2 Software of the developed autoradiogram.

RT-PCR

A pool of 500 islets was lysed in 1 mL of Trizol solution (GIBCO BRL Life Technologies) by 10 seconds of homogenization in vortex. The total RNA was extracted using the single step acid-guanidium-thiocyanate-phenolchloroform method as described by the manufacturer. RNA concentrations were determined from optical absorbance at A_{260nm}. cDNA was synthesized from 2 μg of islet RNA in a volume of 25 μL using the RNase H⁻ reverse transcriptase SuperScript II and random primers (GIBCO BRL Life Technologies). The cDNA samples were stored at -20^0C.

Primers

The Cx43, Cx36, insulin and β-actin transcripts in neonatal pancreatic islets were determined by PCR using the following primer pairs, showed in table I.

Table I: List of oligonucleotides used in the experiments

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')	Size of cDNA bp
Cx43	5' CCG ACG ACA ACC AGA ATG CC 3'	3' GA ACC CTA TCG ACC CGC TTG 5'	322
Cx36	5' AGT GGT GGG AGC AAG CGA GAA G 3'	3'CCG AAG TCA CAG GGT CCC AAC A 5'	282
Beta-Actin	5'ACC ACA AGC TGA GAG GGA AAT CG 3'	3'CGT TTA CGA AGA TCC GCC TGA C 5'	533

RT-PCR Analysis

PCR was carried out in 25 μL reactions using 1 μL of cDNA as template. The PCRs contained: 2.5 μL of 10X PCR buffer (GIBCO Life Technologies), 0.75 μL of 50 mM $MgCl_2$ (GIBCO Life Technologies), 0.5 μL of 10 mM dNTP Mix (GIBCO Life Technologies), 0.5 μL of 100 pM of each primer and 0.2 μL of Taq DNA Polymerase (GIBCO Life Technologies) The optimal amplification cycle was determined for each primer pairs from a curve using RNA samples of the N1d group. This amplification cycle corresponded to the number of cycles two points below the amplification plateau value.

After a 5 min start at 94^0C, amplification of Cx43 was carried out for 25 cycles, each comprising 30 s at 94^0C, 30 s at 57^0C, and 45 s at 72^0C, using an Perkin Elmer 9600 (Manufacturer). After the last cycle, an elongation step of 4 min at 72^0C was performed. The amplification of Cx36 was carried out for 35 cycles, comprising the same steps described above, and the amplification of insulin was carried out for 23cycles. In all cases, negative controls were provided by submitting a sample of water to amplification. Beta-actin expression was used as an internal control in all PCR reactions.

Insulin secretion

Insulin secretion

After culture, islets were gently detached from the plates under a dissecting microscope. Clean islets were washed with a bicarbonate-buffered solution (pH 7.4) (composition: 115 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 24 mM $NaHCO_3$) supplemented with 3 mg/ml bovine serum albumin and glucose at the same concentration to that used in the culture medium and equilibrated with a mixture of 95% O_2 – 5% CO_2. Groups of 10 islets from the different experimental groups were placed within each well of 24-multiwell plates and incubated with 1ml of the bicarbonate-buffered containing 10 mM glucose for 2 h. After the incubation period, aliquots of the supernatant were taken and stored at -20°C. The insulin content of these samples was determined by radioimmunoassay and was expressed as nanograms per islet x time.

Statistical Analysis

All the results are expressed as means ± standard error (SEM) together with the number of individual experiments (n). The statistical significance of differences among more than two experimental groups was assessed by ANOVA and for comparison between pairs of groups was used the Bonferroni's test. The significance level was set at $P < 0.05$.

3. Results

Figure 1 shows the insulin secretory response in neonatal pancreatic islets when cultured at varying period of time and with a culture media containing different glucose concentrations. As described in figure 1a, group of islets cultured for only 1 day (N1d) showed a significantly lower secretion than groups of islets cultured for 3 days (N3d) ($p<0.01$) and 7 days (N7d) ($p<0.001$). Yet, the N7d group showed a significantly greater secretion when compared with the N3d group ($p<0.01$). Figure 1b shows the insulin secretion by islets cultured for 3 days in different concentrations of glucose within the medium. The groups of islets cultured with 10mM glucose (N3d 10) showed significantly greater secretion when compared with the group of islets cultured with 10mM glucose for only one day (N1d 10) ($p<0.001$). A significant increase in insulin release was also detected in N3d 10 group when compared with the groups of islets cultured with 2.8mM (N3d 2.8; $p<0.001$) and 5.6mM glucose (N3d 5.6; $p<0.001$) for the same period of time.

To assess whether these *in vitro* alterations in insulin secretion were followed by changes in expression of GJ-associated proteins in homogenized islet samples, the expression of connexin 36 and connexin 43 in isolated islet was assessed by Western Blot. As shown in figures 2 and 3, culturing for 7 days resulted in a significant increase in the expression of Cx36 (comparing to N1d, $p<0.001$; comparing to N3d, $p<0.01$) (Figures 2a and 2c) and Cx43 (comparing to N1d, $p<0.001$; comparing to N3d, $p<0.05$) (Figures 3a and 3b) as compared with N1d and N3d experimental groups. A significant higher increase in the expression of both connexins was also verified in the N3d group in comparison to the N1d group ($p<0.05$).

In addition, a prolonged islet culturing also induced an increase in the expression of the adherens junction-associated (AJ) protein, β-catenin as assessed by Western Blot. As shown in figure 4, islet culturing for 7 days resulted in a significant increase in the expression of β-catenin as compared with N1d ($p<0.001$) (Figure 4a and 4b). Nevertheless, no significant difference in the expression of the AJ protein was detected between N3d and N7d groups.

To detect the level of expression of Cx36 and Cx43 transcripts in cultured neonatal islets, an analysis was carried out by RT-PCR of the total RNA, using specific primers expected to amplify the entire coding sequence of Cx36 and Cx43 (Figure 5). As compared to the islet group cultured for only one day, prolonged culturing increased the Cx36 gene expression in 40% and 87% in the groups of islets cultured for 3 and 7 days, respectively (Figures 5a and 5b). Furthermore, prolonged culturing also induced an increase of 49% in the expression of Cx43 in N3d group and an increase of 63% in the N7d group in comparison with the N1d group (Figures 5c and 5d).

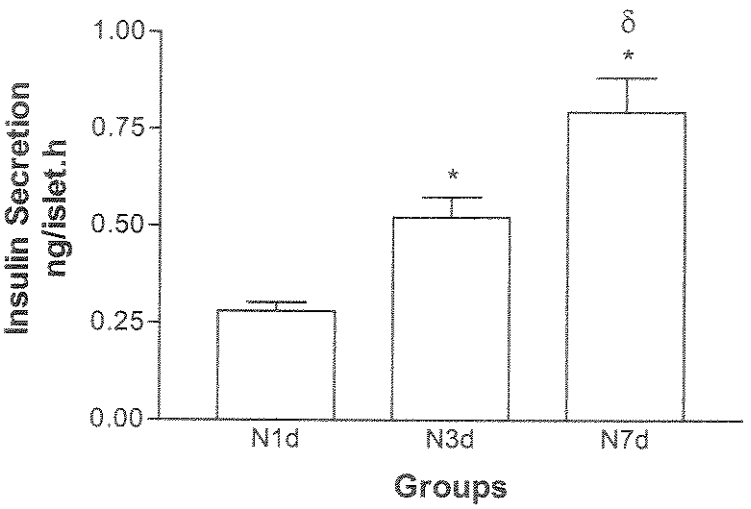
To evaluate whether the glucose concentration within the culture medium alters the expression of GJ-associated proteins, an analysis was carried out by Western Blot using islet samples cultured for 3 days in different concentrations of glucose. Figure 6 shows a glucose-dependent increase in Cx36 protein expression in cultured neonatal islets. As compared to the groups of islets cultured with 2.8mM glucose, culture of islets cultured with 5.6mM ($p<0.05$) and 10mM glucose ($p<0.001$) induced a significant increase in the expression of Cx36. A significant increase was also detected in N3d 10 group as compared to the N3d 5.6 group ($p<0.05$) (Figures 6a and 6b). A relatively low expression level of Cx36 was obtained in neonatal islets cultured for 1 day in 10mM glucose as compared to all above islet groups cultured for 3 days.

Meanwhile, different glucose concentrations in the media did not significantly alter the Cx43 expression in cultured neonatal pancreatic islets (Figure 7). As compared to the N3d 2.8 group, the groups of islets cultured with 5.6 and 10mM glucose showed no significant changes in the expression of the gap junctional protein. Also, no significant increase was detected between the N3d 5.6 and N3d 10 groups regarding the Cx43 expression. Nevertheless, in accordance of previous observation, the expression of this gap junctional protein was higher following 3d-culturing, regardless the glucose medium concentration, in comparison with the islet group cultured for only 1d (Figure 7).

FIGURES

FIGURE 1

a)


Insulin Secretion
ng/islet.h
1.00
0.75
0.50
0.25
0.00
δ
*
*
N1d
N3d
N7d
Groups

b)

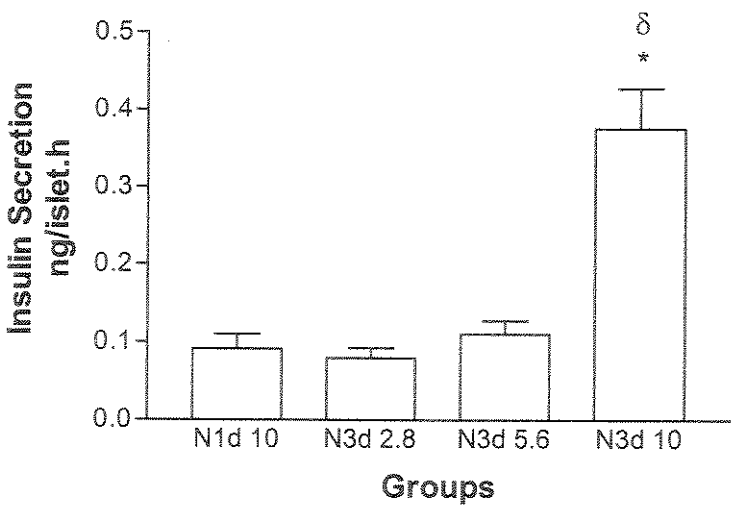

Insulin Secretion
ng/islet.h
0.5
0.4
0.3
0.2
0.1
0.0
δ
*
N1d 10
N3d 2.8
N3d 5.6
N3d 10
Groups

Figure 1: Influence of prolonged culturing and glucose medium concentration on insulin secretion in neonatal islets.

In **a**, neonatal islets were cultured in supplemented RPMI 1640 medium containing 10mM glucose for a period of 1d (N1d), 3d (N3d) or 7d (N7d). In **b**, groups of islets were cultured for 3 days in culture media containing 2.8mM (N3d 2.8), 5.6mM (N3d 5.6) or 10mM (N3d 10) of glucose. Also in **b**, a group of neonatal was cultured for only 1d with 10mM glucose for comparison (N1d 10). After culturing, islets from all groups were isolated and exposed to Krebs buffer containing 10mM glucose for 2 h. Insulin released within the buffer was measured by radioimmunoassay. Results are expressed as means ± SEM. The results are representative of 4 independent experiments.

In **a**, $^{*}P<0.05$ as compared to N1d group, $^{\delta}P< 0.05$ as compared to N3d group (ANOVA followed by Bonferroni´s test).

In **b**, $^{*}P<0.001$ as compared to N1d group, $^{\delta}P< 0.001$ as compared to N3d 2.8 and to N3d 10 groups (ANOVA followed by Bonferroni´s test).

FIGURE 2

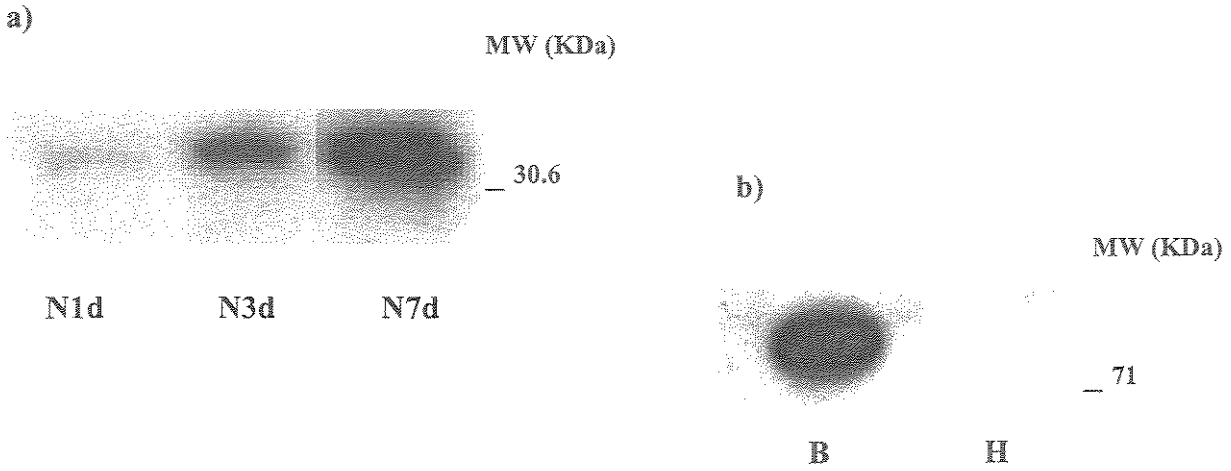

a)
MW (KDa)
30.6
N1d
N3d
N7d
b)
MW (KDa)
71
B
H
c)
Optical Arbitrary Units
5000
4000
3000
2000
1000
0
δ
*
*
N1d
N3d
N7d
Groups

Figure 2: Increase in the Cx36 expression induced by prolonged culture of neonatal islets.

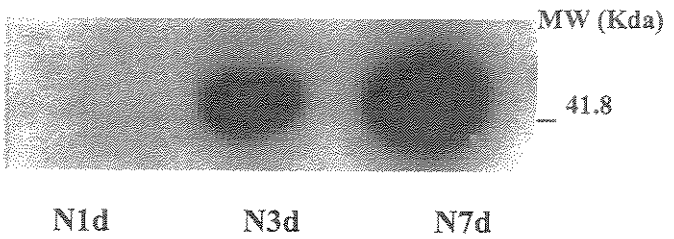
The level of Cx36 was detected by Western Blot in islet sonicate. As compared to islet culturing for 1 and 3 days, culture of islets for 7d induced significant increase in the expression of Cx36 **(a)**. A low expression level of Cx36 was seen in neonatal islets cultured for 1 day **(a)**. Brain and liver were used as positive and negative controls for Cx36, respectively **(b)**. The Cx36 antibody detected a homodimer in the brain. Values of densitometry for each experimental group are shown in **b** and expressed as means ± standard errors **(c)**. The results in **a** is representative of 4 independent experiments.

Legends: N1d, neonatal islets cultured for 1day; N3d neonatal islets cultured for 3 days and N7d, neonatal islets cultured for 7 days.

$^{*}P<0.05$ as compared to N1d group, $^{\delta}P< 0.001$ as compared to N3d group. (ANOVA followed by Bonferroni's test).

FIGURE 3

a)


MW (Kda)
41.8
N1d
N3d
N7d

b)

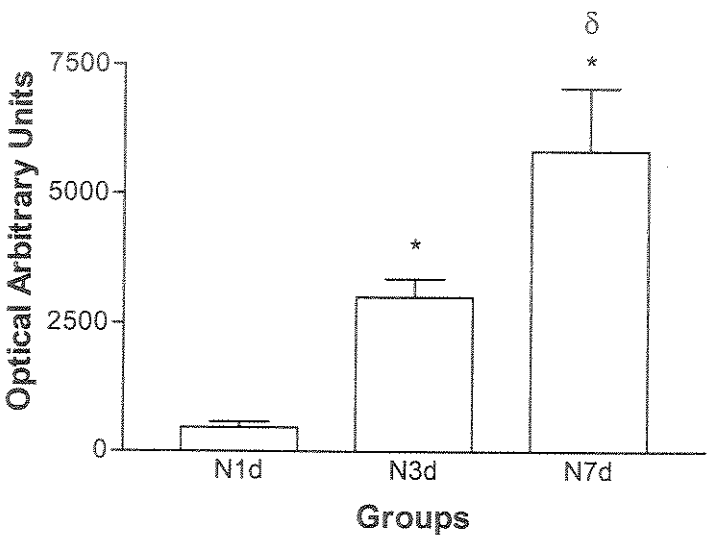

δ
*
*
7500
5000
2500
0
Optical Arbitrary Units
N1d
N3d
N7d
Groups

Figure 3: Increase in Cx43 expression induced by prolonged culture of neonatal pancreatic islets.

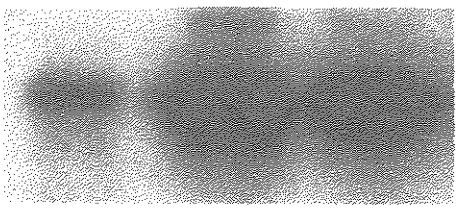
The level of Cx43 was detected by Western Blot in islet sonicate. As compared to culturing for 1 and 3 days, culture of islets for 7d induced significant increase in the expression of Cx43 **(a)**. A significant increase was also detected between groups of islets cultured for 1 and 3 days **(a)**. Values of optical density for each experimental group are shown in **b** and expressed as means ± standard errors **(b)**. The results in **a** are representative of 6 independent experiments.

Legends: N1d, neonatal islets cultured for 1day; N3d neonatal islets cultured for 3 days and N7d, neonatal islets cultured for 7 days.

$^{*}P<0.001$ as compared to N1d group, $^{\delta}P< 0.05$ as compared to N3d group (ANOVA followed by Bonferroni's test).

FIGURE 4

a)


MW (kDa)
133
N1d
N3d
N8d

b)

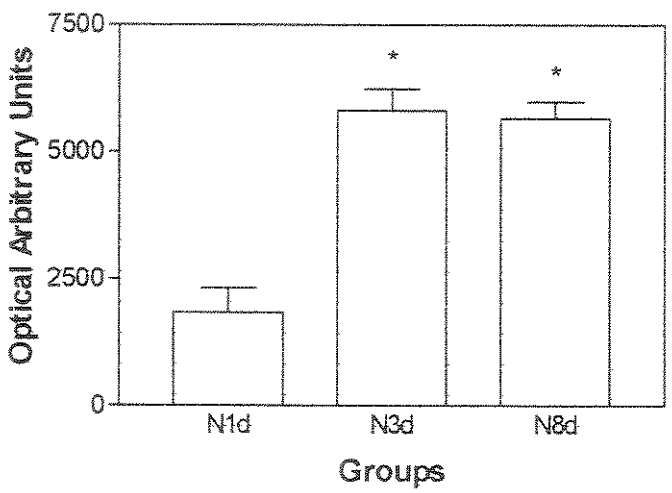

7500
5000
2500
0
Optical Arbitrary Units
*
*
N1d
N3d
N8d
Groups

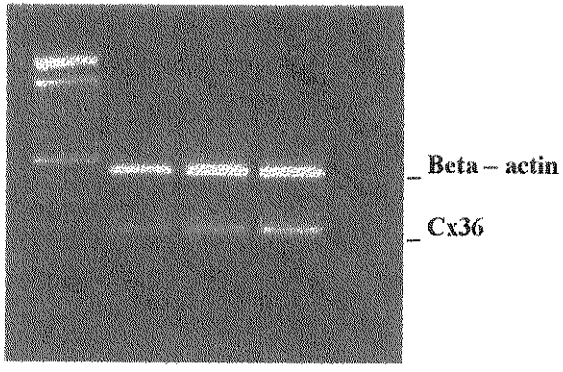
Figure 4 - Increase in β-catenin expression in neonatal pancreatic islets induced by prolonged culture

The level of β-catenin was detected by Western Blot in islet sonicate. Note that N3d and N7d show significant higher expression of this junctional protein than the N1d group. Values of band densitometry for each experimental group are shown in b and expressed as means ± standard errors. The result shown in a is representative of 4 independent experiments.

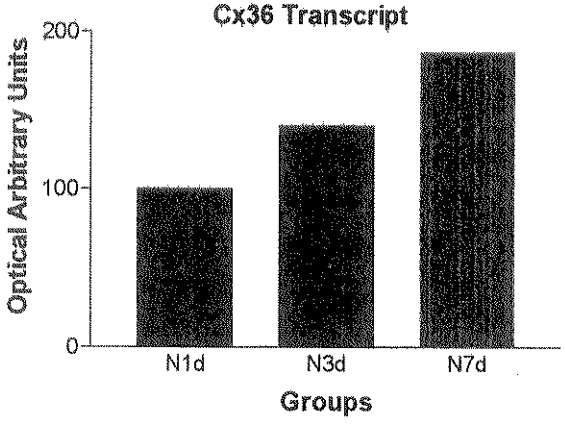
Legends: N1d, neonatal islets cultured for 1 day; N3d, neonatal islets cultured for 3 days; N8d, neonatal islets cultured for 7 or 8 days.

FIGURE 5

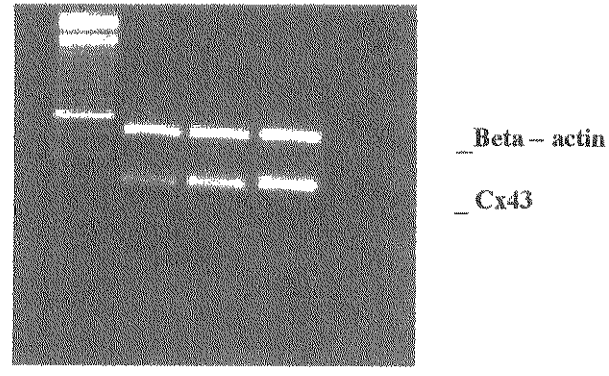
a)



b)



c)



d)

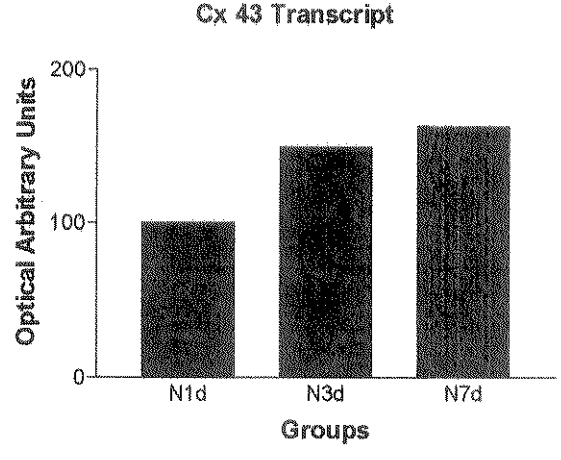
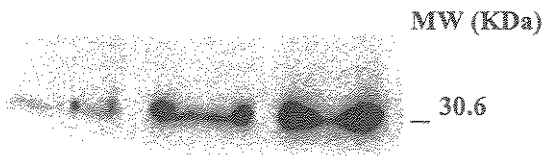


Figure 5: Increase in Cx36 and Cx43 gene expression in neonatal pancreatic islets induced by prolonged culture.

Cellular content of Cx36 **(a, b)** and Cx43 **(c, d)** RNA transcripts in cultured neonatal islets was analyzed by RT-PCR amplification method. As compared to culturing for 1 and 3 days, culture of islets for 7d induced an increase in the expression of both Cx36 **(a, b)** and Cx43 **(c, d)**. In all cases, negative controls were provided by submitting a sample of water **(H_2O)** to amplification. Cx36 and Cx43 samples were submitted to 35 and 25 amplification cycles, respectively. Beta-actin gene expression was used as an internal control.

FIGURE 6

a)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N1d
N3d 2.8
N3d 5.6
N3d 10

b)

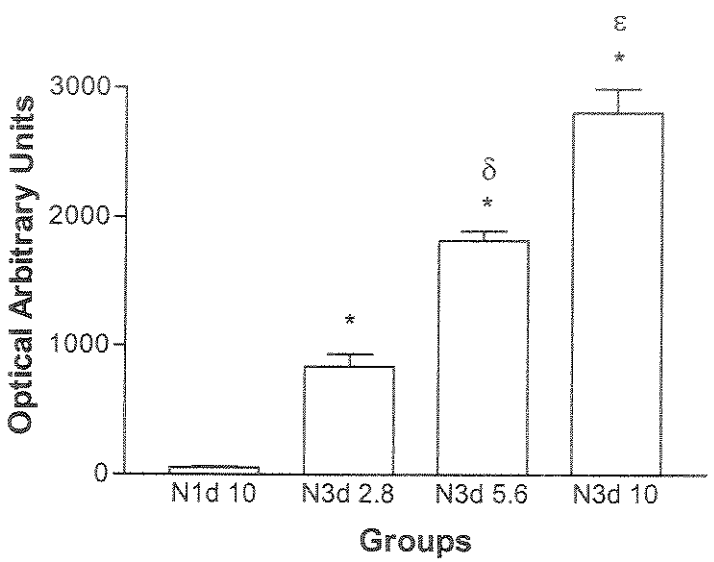

Optical Arbitrary Units
3000
2000
1000
0
ε
*
δ
*
*
N1d 10
N3d 2.8
N3d 5.6
N3d 10
Groups

Figure 6: Glucose-dependent increase in Cx36 expression in cultured neonatal pancreatic islets.

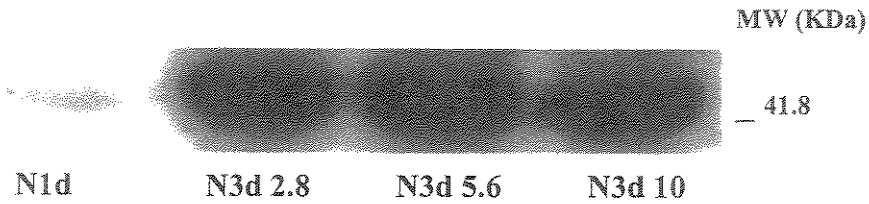
The level of Cx36 was detected by Western Blot in islet sonicate. As compared to the groups of islets cultured for 3d with 2.8mM glucose, culture of islets in 5.6mM and 10mM glucose induced a significant increase in the expression of Cx36. A significant difference was also detected between N3d 5.6 and N3d 10 groups. Meanwhile, a very low expression level of Cx36 was seen on the neonatal islets cultured for only 1 day. Values of optical density of the bands for each experimental group are shown in **b** and expressed as means ± standard errors. The results in **a** is representative of 5 independent experiments.

Legends: N1d, neonatal islets cultured for 1day with 10mM glucose; N3d 2.8, neonatal islets cultured with 2.8mM glucose for 3 days; N3d 5.6, neonatal islets cultured with 5.6mM glucose for 3 days; N3d 10, neonatal islets cultured with 10mM glucose for 3 days.

$^{*}P<0.001$ as compared to N1d group, $^{\delta}P< 0.05$ as compared to N3d 5.6 group and $^{\varepsilon}P<0.05$ as compared to N3d 10 group (ANOVA followed by Bonferroni 's test).

FIGURE 7

a)


MW (KDa)
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N1d
N3d 2.8
N3d 5.6
N3d 10

b)

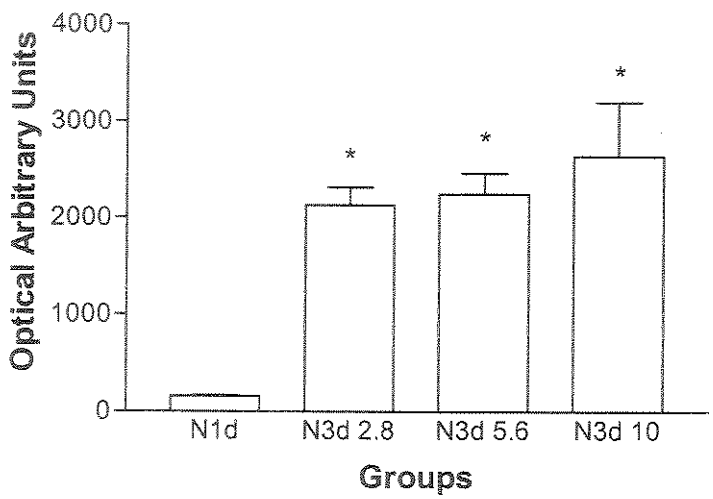
Optical Arbitrary Units
4000
3000
2000
1000
0
*
*
*
N1d
N3d 2.8
N3d 5.6
N3d 10
Groups

Figure 7: Increasing glucose concentrations within the media do not alter the Cx43 expression in cultured neonatal pancreatic islets.

The level of Cx43 was detected by Western Blot in islet sonicate. As compared to those islets cultured for only 1 day, islet culturing for 3d induced increase in Cx43 expression, that was independent of glucose concentrations within the culture media. As compared to the group of islets cultured with 2.8mM glucose, culture of islets in 5.6mM or 10mM glucose-containing media did not significantly change the expression of Cx43. Values of optical density of the bands for each experimental group are shown in **b** and expressed as means ± standard errors. The result in **a** is representative of 4 independent experiments.

Legends: N1d, neonatal islets cultured for 1day; N3d 2.8, neonatal islets cultured with 2.8mM glucose for 3 days; N3d 5.6, neonatal islets cultured with 5.6mM glucose for 3 days; N3d 10, neonatal islets cultured with 10mM glucose for 3 days.

*$P<0.001$ as compared to N1d group (ANOVA followed by Bonferroni's test).

4. Discussion

The pancreatic β-cells that secrete insulin are interconnected by gap junctions (Meda 1996). Previous studies have suggested that, at least in experimental conditions, these channels contribute to regulate insulin biosynthesis and release (Halban et al. 1982, Meda et al. 1979, Vozzi et al. 1995). Thus, single β-cells, which are not coupled by connexin-made channels, show poor expression of the insulin gene and release low amounts of hormone, even after stimulation (Halban et al. 1982, Meda et al. 1979, Vozzi et al. 1995). Also, restoration of β-cells contacts is paralleled by a rapid improvement of both insulin biosynthesis and release (Bosco et al. 1989, Halban et al. 1982, Pipeleers et al. 1982, Meda et al. 1990).

Evidence indicates that insulin secretion in response to glucose and to other secretagogues is diminished during the fetal and neonatal stage of development (Boschero et al. 1988; 1990; 1993, Grill et al. 1981). The inability of glucose to elicit an adult pattern of secretion, despite adequate insulin reserves and biosynthesis capacity of the fetal β-cells, indicates that the glucose signal for secretion is poorly recognized at early stages of the animal development (Bone et al. 1981, Heinze et al. 1975). The maturation of the secretory response is influenced by many factors, including fuel metabolites, neurotransmitters, and hormones (Sorenson et al. 1987). In culture conditions and following treatment with mammosomatotropic hormones, neonatal and adult islets undergo maturation of the secretory machinery that might involve regulation of cell-cell contacts within the islet (Brelje & Sorenson 1991, Collares-Buzato et al. 2001, Michaels et al. 1987, Sorenson et al. 1987).

To our knowledge, however, no study to date has aimed at elucidating the molecular mechanisms involved in the *in vitro* maturation of the stimulus-secretion coupling in neonatal pancreatic islets β-cells. In the present study, we investigated the possible involvement of the GJs in this process by analyzing the effect of culturing neonatal islet cells, at varying period of time and with different glucose medium concentrations, on the cellular expression of Cx36 and Cx43. We also evaluate whether the alterations in the expression of these GJ-associated proteins, after prolonged culture, were paralleled by the expression of the adherens junction-associated protein, β-catenin.

Our results confirm previous observations that culture of neonatal islets, for a relatively long period, results in an increase in insulin secretion, indicating that islet culturing induces maturation of the secretory process (Collares-Buzato et al 2001, Kohen et al 1979, Meda et al 1979; 1983; 1991). These *in vitro* alterations in insulin secretion were followed by a time-dependent increase in the protein expression of the GJ-associated Cx36 and Cx43, and also in the RNA transcripts of these connexins, as assessed by Western Blot and RT-PCR, respectively. This data is in accordance with our previous work suggesting that the connexin expression is upregulated after islet culturing (Collares-Buzato et al. 2001). *In vivo* and *in vitro* stimulation of insulin release has been found, by others, to be associated with an enhancement of β cell coupling due to an increase in the expression of Cx43 in adult rat islets (Kohen et al. 1979; Meda et al. 1979; 1983; 1991). Recently, Serre-Beinier and colleagues (2000) have described that Cx36 is the connexin species unambiguously detected between insulin-producing cells in adult endocrine pancreas. However, the present work has first demonstrated that the Cx36 is co-expressed with Cx43 and can be regulated in neonatal pancreatic islets.

Besides the connexins, the *in vitro* maintenance of neonatal islets for 7d resulted in enhancement of expression of the adherens junctional protein, β-catenin. Nevertheless, this culture-induced increase in the islet content of β-catenin did not show a time-dependent pattern, which contrasted with Cx36 and Cx43. In previous work, we have demonstrated that the prolactin-induced upregulation of Cx43 was paralleled by increases in cellular expression and junctional content of β-catenin in cultured neonatal islets (Collares-Buzato et al. 2001). β-catenin, as a component of the adherens junctions, can affect gap junctions-mediated intercellular communication in several cell types (Ai et al. 2000, Behrens et al. 1985, Kanno et al. 1984, Mege et al. 1988). Also, an important reciprocal relationship between β-catenin signaling and Cx43 gene expression and gap junctional function has been described in cardiomyocytes (Ai et al. 2000). In the case of endocrine pancreas, however, further study has to be directed toward more fully elucidating the potential contribution of β-catenin signaling to connexin 36 and 43 gene expression.

Concerning the stimulatory effect of prolonged culture of neonatal islets on gap junction-associated proteins, Cx36 and Cx43, and insulin secretion, it is plausible to suggest that hormones present within the serum, or even other medium components, may

be accounted for these effects. The presence of serum and certain nutrients, at relatively high concentrations, such as glucose, within the medium are essential for *in vitro* survival, growth and differentiation of islet β cells *in vitro* (Ling et al. 1994, Sekine et al. 1997). Following this idea, we also evaluated whether glucose concentration within the culture medium altered the expression of GJ-associated proteins. Islet culture with high glucose concentration resulted in maturation of the insulin secretory process. Our data showed a glucose-dependent increase in Cx36 expression in cultured neonatal islets. As compared to the groups of islets cultured with 2.8mM glucose, islet culturing in media containing 5.6mM and 10mM glucose induced a significant increase in the expression of Cx36. Meanwhile, different glucose concentrations did not significantly alter Cx43 expression in cultured neonatal pancreatic islets. In contrast to Cx36, the Cx43 expression has been demonstrated not to be specific for pancreatic β-cells (Serre-Beinier et al. 2000). Yet, a central role of Cx36 in glucose-induced insulin secretion has been suggested by the observation that level of Cx36 expression correlates with the glucose responsiveness of different insulin-producing lines (Serre-Beinier et al. 2000). Altogether, these findings attribute a crucial function to Cx36 made gap-junction channels in intercellular coupling and secretory functioning of β-cells.

In conclusion, we report here that culturing of neonatal pancreatic islets induces upregulation of the expression of the connexins 36 and 43 as well as β-catenin. A correlation was observed between the increased mRNA content and protein expression of Cx36 and Cx43 and increase in insulin secretion induced by prolonged culture and high glucose concentration within the culture medium. We suggest that the regulation of gap and adherens junctional proteins by culture medium-containing factors and glucose may be an important event for the maturation process of β cells observed at *in vitro* conditions.

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CONCLUSÃO

Os resultados obtidos nesta dissertação mostram que:

- O tratamento *in vitro* com prolactina resulta em aumento no número de junções comunicantes na região de contato intercelular das células das ilhotas de ratos recém-nascidos, além de induzir aumento na expressão de conexina 43, como detectado por imunofluorescência indireta e Western Blot, respectivamente.
- Tratamento com prolactina de ilhotas de recém-nascidos também induziu aumento na expressão e imunolocalização na região de contato intercelular da proteína associada à junção aderente, a β-catenina.
- Cultivo prolongado (7 dias) *per se* induziu aumento na expressão das conexinas 36 e 43 e da β-catenina em ilhotas de ratos recém-nascidos, como determinado por *Western Blot*. Em particular, o garu de expressão das conexinas nas ilhotas em cultura pareceu correlacionar com o tempo de cultivo e o grau de secreção de insulina.
- A Cx36 é regulada por diferentes concentrações glicose no meio de cultura, ao contrário do que é observado com relação a expressão de Cx43 nas ilhotas de ratos recém-nascidos.
- Foi possível detectar uma correlação direta entre o aumento da secreção de insulina, aumento do RNAm e da expressão protéica de conexinas 36 e 43 em ilhotas pancreáticas de ratos recém-nascidos mantidos em cultura.
- A modulação *in vitro* das junções comunicantes e junção aderente induzida pela prolactina, cultivo prolongado e glicose, possivelmente, consiste num evento importante no processo de maturação e diferenciação celular nas ilhotas pancreáticas.

CAPÍTULO 5º

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APÊNDICES

APÊNDICE A

MATERIAIS E MÉTODOS

1. Animais

Os animais utilizados para desenvolvimento deste trabalho foram obtidos do Centro de Bioterismo da Unicamp (Campinas, SP).

Foram utilizados ratos Wistar recém-nascidos (1 a 2 dias de vida), de ambos os sexos, com aproximadamente 5g em peso cada.

2.1 Cultura de Ilhotas de Recém-Nascidos

Os animais foram mortos por decaptação com o auxílio de um tesoura e, em seguida, mergulhados em solução de álcool a 70% iodado, para esterilização. Estes animais foram, então, transferidos, para uma placa de Petri estéril, no interior de um fluxo laminar horizontal, local onde procedeu a laparotomia para exposição e remoção do pâncreas, com auxílio de material cirúrgico adequado.

Os pâncreas retirados foram, então, transferidos para um béquer contendo solução de Hanks estéril (composição iônica: 141.5 mM Na^{+}, 5.8 mM K^{+}, 1.3 mM Ca^{+2}, 0.8 mM Mg^{+2}, 143.6 mM Cl^{-}, 4.2 mM HCO^{-3}, 0.3 mM HPO_4^{+2}, 0.3 mM SO_4^{+2}, 0.8 mM SO_4^{+2}, 0.4 mM $H_2PO_4^{-}$). Após serem cortados em pequenos fragmentos com o auxílio de uma tesoura, os órgãos foram transferidos para um tubo centrífuga, com tampa, estéril onde foi feita a digestão parcial do pâncreas com a utilização da enzima colagenase (0.2 mg/órgão) (Sigma) a 37°C, sob agitação constante por aproximadamente 5 min. Sob estas condições, foi possível a digestão, principalmente, da porção exócrina do pâncreas com a preservação das ilhotas pancreáticas. Foram feitas, então, três sucessivas lavagens da suspensão de ilhotas, utilizando solução de Hanks através de rápidas centrifugações para retirada do excesso de colagenase. Na última lavagem, foi utilizado o meio de cultura RPMI 1640 (Sigma) contendo 10mM de glicose, 5% de soro fetal bovino inativo (Sigma ou Nutricel), 1% de uma solução contendo 10.000 IU/ml penicilina e 10 mg/mL de estreptomicina (Nutricel).

Um volume de suspensão de ilhotas foi, então, aliquotado em placas de Petri estéreis (Falcon) contendo 10 mL de meio de cultura RPMI 1640 completo.

As placas contendo as ilhotas pancreáticas foram mantidas a 37 °C sob atmosfera umidificada de 3% CO_2 em ar (modelo CO-24, SANYO). As ilhotas foram mantidas sob esta condição por tempos variados dependendo do protocolo experimental (vide seções 4 e 5), até no máximo 8 dias.

Num dos protocolos experimentais testados na presente dissertação (vide seção 5), as ilhotas foram cultivadas por 3 dias com meio RPMI completo contendo diferentes concentrações de glicose, a saber: 2.8 mM, 5.6 mM ou 10 mM. Previamente a sua utilização, a concentração de glicose no meio foi dosada utilizando-se o kit Biotrol Diagnostics – Glicose (GOD-PAD). A troca do meio de cultura, neste caso, foi feita a cada 24 h.

2.1.1 Coleta das Ilhotas Pancreáticas

As ilhotas isoladas de recém-nascidos, mantidas em cultura, foram coletadas sob lupa, uma a uma, com a utilização de uma pipeta Pauster previamente estirada e umidificada com solução de Hanks e albumina bovina tipo V (3 mg/mL) (Sigma). Durante a coleta, as ilhotas foram transferidas para uma placa de Petri contendo solução de Krebs (composição iônica: 139 mM Na^+, 5 mM K^+, 1 mM Ca^+, 1 mM Mg^{+2}, 123.6 mM Cl^-, 24 mM HCO^{3-}) em pH 7.4, contendo glicose na concentração do meio de cultura onde as ilhotas foram cultivadas. Um grupo de ilhotas foi separado para a determinação da secreção estática de insulina. As demais ilhotas foram transferidas para eppendorfs, devidamente etiquetados, e congeladas a –70 °C em coquetel anti-Protease, (composição: 4 mM EDTA, 5 µg/mL pepstatina A, 10 mM imidazol, 1mM EGTA, 2 µg/mL aproptina, 200µg/mL PMSF, 200 µg/mL DTT, 2.5 µg/mL leuptina, 0.5 µg/mL pepstatina, 30 µg/mL inibidor de tripsina), até o desenvolvimento da técnica de Western Blot.

3. Secreção Estática de Insulina

Grupos de 8 a 10 ilhotas foram incubadas, em placas Multiwells, com 1 mL de solução de Krebs contendo glicose, nas concentrações de 2.8 mM ou 10 mM, por 2 h a 37 °C na incubadora.

Ao término do período de incubação, alíquotas de 500 µL foram coletadas e estocadas a –20 °C até a dosagem de insulina por radioimunoensaio.

4. Radioimunoensaio

A insulina secretada foi detectada de acordo com o método descrito por Scott et al. 1981.

Foram utilizadas duplicatas de alíquotas das amostras contendo insulina, numa diluição prévia de 1:10, para todas as amostras, em Tampão Fosfato (200 mM Na_2HPO_4, 260 mM $NaH_2PO_4.H_2O$).

A curva padrão foi construída em triplicatas, utilizando-se um padrão de insulina (doado gentilmente pelo Dr. Luiz Flávio de Freitas Leite, Novo Nordisk Biolabs), nas concentrações de 0.02 ng/mL, 0.039 ng/mL, 0.078 ng/mL, 0.16 ng/mL, 0.31 ng/mL, 0.63 ng/mL, 1.25 ng/mL, 2.5 ng/mL, 5 ng/mL. Insulina de rato marcada com I^{125} (100 µg/mL) e 20 µL de anticorpo monoclonal anti-insulina (diluição 1:150000) (doado gentilmente pela Dra. Leclerc- Meyer, Universidade Livre de Bruxelas) foram adicionados às amostras diluídas e aos tubos da curva padrão. Para o ensaio, foram efetuados os seguintes tubos de referência em triplicata: Total (T), contendo 200 µL de solução de insulina marcada (5000 cpm/200 µL solução); *Non-specific binding* (NSB), contendo 100 µL de tampão fosfato e 200 µL da solução de insulina marcada; Referência (REF), contendo 100 µL de tampão fosfato, 200 µL da solução de insulina marcada e anticorpo anti-insulina. Feitas todas as pipetagens, os tubos foram agitados em *vortex* e mantidos durante 48 h a 4 °C. Após o período de incubação, os tubos das amostras, da curva e de referência (com exceção do tubo Total) receberam 200 µL de uma solução contendo 2.5% de carvão (Norit A), 0.5% de albumina e 0.23% de dextran T 70. Os tubos foram mantidos por 20 min a 4 °C e

centrifugados por 20 min a 542 g. O sobrenadante foi descartado por aspiração e a radioatividade dos tubos foi medida por um contador *gama* (Beckman Gama 5500). Por intermédio dos valores obtidos na curva padrão, nos tubos contendo concentração fixa de insulina, calculou-se os valores de insulina contidos nas amostras utilizando-se o programa MS-DOS AGC.

5. *Western Blot*

Para detectar o nível de expressão celular das proteínas juncionais Cx36, Cx43 e β-catenina foi empregado a metodologia de *Western Blot*. Todo o procedimento com as ilhotas foi feito em banho de gelo.

Um grupo de aproximadamente 500 ilhotas foi centrifugado rapidamente e o sobrenadante desprezado e, a seguir, foi adicionado 40 μL de coquetel anti-protease. As ilhotas foram, então, sonicadas nesta solução, por aproximadamente 10s e, em seguida, a quantidade de proteína foi dosada, utilizando-se o kit para ensaio de proteína, BioRad Protein Assay-Dye Reagent Concentrate (BioRad). Foi utilizado como referencial a curva padrão de albumina.

Em particular, para detecção de Cx43 e β-catenina, o homogeneizado foi centrifugado por 10min em uma centrífuga refrigerada a 3000 g. O precipitado foi desprezado e a dosagem protéica foi feita, como descrito anteriormente, no sobrenadante obtido.

Amostras contendo 70 μg de proteínas totais foram, então, incubadas a 37 °C por 1h em 20% do volume de Tampão de Laemmli cinco vezes concentrado (composição: azul de bromofenol 0.1%, fosfato de sódio 1M, glicerol 50%, SDS 10%).

Para corrida eletroforética, foi utilizado um gel bifásico constituído por: 1) gel de empilhamento contendo 6% de acrilamida diluído em tampão contendo EDTA 4mM, SDS 2%, Trisma base 50mM, pH 6.7 e 2) gel de resolução contendo 10% de acrilamida para detecção de Cx36 ou 8% de acrilamida para Cx43 e β-catenina, diluído em tampão contendo: EDTA 4mM, SDS 2%, Trisma base 750mM, pH 8.9. A corrida foi efetuada a 200 V por aproximadamente 30 min com Tampão de Corrida (composição: Trisma base

200mM, glicina 1.52M, EDTA 7.18mM e SDS 0.4%). As amostras foram, então, transferidas para uma membrana de nitrocelulose (BioRad).

A transferência foi procedida durante 60 min a 120 V em gelo, banhada com Tampão de Transferência para Cx36 (20% Metanol, 0.02% SDS, Trisma base 25mM, glicina 192mM). Entretanto, a fim de facilitar a detecção de Cx43 e β-catenina na membrana, a transferência foi promovida durante 60 min a 30 V e sem adição de Metanol e SDS na solução tampão. Após transferência, a membrana foi corada com solução Ponceau (Sigma), para certificação de que tanto a dosagem protéica quanto a transferência procederam adequadamente.

Após transferência, a membrana foi bloqueada com 5% de leite desnatado em solução Basal (SB) (composição: Trisma base 0.1M, NaCl 0.5M, Tween 20 0.05%) por 12 h a 4 °C. A proteína Cx36 foi detectada na membrana de nitrocelulose por incubação por 4h, à temperatura ambiente, com anticorpo monoclonal específico anti-Cx36 (Zymed, diluição 1:500 em SB com 3% de leite desnatado). Posteriormente, a membrana foi lavada 3 vezes, por 5 min com solução basal, seguida de exposição por 2h, à temperatura ambiente, com proteína A marcada com I^{125} (2000 com/mL em TTBS com 1% de leite desnatado). Alternativamente, a Cx36 foi detectada na membrana de nitrocelulose, após bloqueio, através de quimioluminescência por incubação por 1 h, à temperatura ambiente, com anticorpo monoclonal específico anti-Cx36 (Zymed, diluição 1:500 em SB com 3% de leite desnatado). A membrana foi, então, lavada 6 vezes por 8 min cada com SB, exposta por 1 h, à temperatura ambiente, com anticorpo anti- IgG de coelho conjugado com peroxidase (diluição 1:1000) (Chemicon), seguida de lavagem final com SB (6 vezes por 8 min cada).

A proteína Cx43 e β-catenina foram detectadas na membrana de nitrocelulose por incubação por 2 h a temperatura ambiente, com anticorpo monoclonal específico anti-Cx43 e anti-β-catenina (Zymed) (diluição 1:1000 em SB com 3% de leite desnatado), respectivamente. Após sucessivas lavagens com solução basal, em ambos os casos, a membrana foi incubada com o anticorpo policlonal anti-IgG de camundongo (diluição 1:1500 em TBS com 3% de leite desnatado) seguido de exposição por 2 h à temperatura ambiente com proteína A marcada com I^{125} (diluição 1:1000 em TBS com 1% de leite desnatado) e lavagem final com SB (5 vezes por 3 min cada). O complexo anticorpo - [I^{125}]

proteína A foi detectado por auto-radiografia, após armazenamento desta membrana a –70°C por aproximadamente 5 dias dentro de cassete provido de uma tela amplificadora. No caso da quimioluminescência, a membrana foi exposta por no máximo 5 min em solução de luminol (SuperSignal Chemiluminescent Substrate - PIERCE) e, em seguida, detectado por auto-radiografia, após armazenamento desta membrana a temperatura ambiente por até 30 min dentro de cassete desprovido de tela amplificadora.

Após revelação, a densitometria das bandas foi medida utilizando-se o programa Scion Image Release Beta 4.0.2.

5.1 *Stripping*

No caso de reaproveitamento da membrana de nitrocelulose para detecção de outras proteínas nas mesmas amostras transferidas nela anteriormente, foi procedido o método de *stripping*. Para tal, a membrana foi, previamente, incubada por 15 - 30 min a 60°C com solução contendo: 50mM Tampão fosfato de sódio pH 7.6, 2% SDS e 0.1M 2-mercaptoetanol. Após sucessivas lavagens com solução basal, a membrana foi incubada com anticorpo primário desejado, como descrito anteriormente (seção 5).

6. *RT-PCR*

6.1 Preparação do Material a ser Utilizado para Extração de RNA

6.1.1 Utilização de Luvas

Durante todas as etapas de desenvolvimento do protocolo para extração de RNA a utilização de luvas estéreis foi imprescindível.

6.1.2 Preparação da Água

Foi utilizada água mili-Q autoclavada e a ela, foi adicionado 0.1% dedietilpirocarbonato (DEPC). A água tratada foi, então, reautoclavada e deixada mantida

sob agitação para eliminação de resíduos de DEPC, por um período de 12 h à temperatura ambiente.

6.1.3 Preparação da Vidraria e Equipamentos

Todo a vidraria foi devidamente lavada, embrulhada e mantida em estufa, por 5 h a 180 °C. As ponteiras e tubos eppedorfs utilizados eram específicos para o uso de RT-PCR, sendo eles livres de RNAse e DNAse, cubas eletroforéticas e outros utensílios foram devidamente lavados com água DEPC e esterilizados em estufa posteriormente, tratados com Peróxido de Hidrogênio a 3% (diluído em água DEPC) por 15 min.

6.2 Isolamento de RNA de Ilhotas de Langerhans

A um grupo de aproximadamente 1000 ilhotas acondicionado em um eppendorf foi adicionado 1 mL do reagente Trizol (Gibco BRL) e, posteriormente, estas amostras foram homogeneizadas em vortex, nesta solução, por aproximadamente 10 s. Em seguida, o homogeneizado foi incubado por 5 min, à temperatura ambiente, para permitir a completa dissociação dos complexos núcleo-protéicos presentes na solução. Após incubação, foi adicionado, a cada amostra 200 µL de Clorofórmio (Chemco). Após adição, os tubos eppendorfs foram devidamente tampados e as amostras vigorosamente homogenizadas durante 15 s, com as mãos. Em seguida, as amostras foram incubadas por 2 a 3 min à temperatura ambiente.

As amostras foram, então, centrifugadas a 11000 g em centrífuga refrigerada a 4°C, por 15 min. Após centrifugação, o volume, correspondente à fase aquosa presente nos tubos, foi cuidadosamente transferido para novos tubos eppendorfs estéreis.

O RNA presente nesta fase aquosa foi, então, devidamente precipitado com a adição de 500 µL de álcool isopropílico (Sigma) e homogeneizado, vagarosamente, por 10 s. As amostras foram incubadas por 10 min à temperatura ambiente e, após incubação, as amostras foram centrifugadas novamente a 11000 g em centrífuga refrigerada a 4 °C por 10 min.

Após centrifugação, todo o sobrenadante foi, então, removido e o "pellet" lavado uma vez com 1 mL de etanol 75% gelado (Sigma). Procedeu-se nova centrifugação a 7500 g por 5 min a 4 °C em centrífuga refrigerada e o "pellet" foi novamente lavado com 1 mL de etanol absoluto gelado (Sigma). Para que fosse promovida uma completa lavagem, as amostras foram agitadas em "vortex" até que o "pellet" desprendesse totalmente do tubo eppendorf. Em seguida, as amostras foram centrifugadas por 5 min a 4 °C a 7500 g em centrífuga refrigerada, o sobrenadante foi desprezado e o RNA precipitado, contido no "pellet", foi mantido em estufa a 37 °C por aproximadamente 10 min, para que se procedesse a total evaporação do álcool absoluto presente no RNA. O RNA presente em cada amostra foi ressuspendido em 10μL de água DEPC.

6.3 Visualização do RNA Extraído

Para analisar a integridade do RNA extraído, foi feito um gel de agarose a 1.2%, (Sigma) diluído em tampão MOPS 1X (composição: 20 mM MOPS; 0.5 mM de acetato de sódio e 1.0 mM EDTA) e 5% de Formaldeído (Sigma) (Figura 1).

Amostras contendo 2μg de RNA, previamente dosadas pelo Genequant pela razão da leitura de absorbância 260/280 nm, foram analisadas neste gel, para certificação da dosagem, através da visualização das bandas das subunidades 28 S e 18 S do RNA total extraído. Para isso, as amostras foram incubadas por 15 min a 56 ° C, em banho seco, com 10 μL de tampão de amostra contendo os seguintes componentes: tampão MOPS 1X, 17.5% solução de formaldeído absoluto, 0.4 μg/μL de brometo de etídio e 4 μL de tampão de corrida (50% glicerol, 2.5 mM EDTA e 0.25% de azul de bromofenol).

Após incubação, as amostras foram mantidas em banho de gelo por 2 min e posteriormente aplicadas em gel de agarose. A corrida eletroforética procedeu-se a 80 V por 1h e como tampão de corrida foi utilizado o tampão MOPS 1X.

Após a corrida, o gel contendo o RNA foi visualizado em transiluminador com luz ultravioleta e fotografado quando desejado, utilizando-se máquina fotográfica Polaroid OneStep 600 (Figura 1).

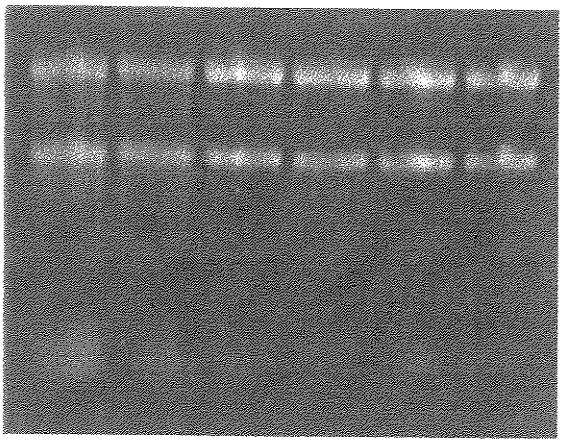

Figura 1: Demonstração da integridade do RNA extraído das ilhotas pancreáticas dos diferentes grupos experimentais: N1d, ilhotas de ratos recém-nascidos cultivadas por 1d; N3d, ilhotas de ratos recém-nascidos cultivadas por 3d e N7d, ilhotas cultivadas por 7d. A integridade do RNA é comprovada pela visualização das bandas das subunidades 18 S e 28 S.

6.4 Processamento das Amostras para RT-PCR

Inicialmente, uma amostra de 2 μg de cada RNA foi submetida a reação de transcrição reversa, em um volume final de reação de 20 μL, com a utilização de oligonucleotídeos. Para isso, foi adicionado, a cada amostra, tampão de enzima, DTT (100mM) e a enzima SuperScript II (200 U). Esta solução foi incubada por 50 min a 42 °C para a reação. A partir dos cDNAs obtidos, foram realizadas as curvas de ciclos para cada primer específico (Tabela I), com o mínimo de 5 pontos para cada curva. Após obtenção das curvas, os experimentos foram realizados com o número de ciclos equivalente a dois pontos antes da saturação da curva (platô). O ponto de saturação é obtido quando a diferença entre os pontos é inferior a 20%.

Tabela I: Lista de oligonucleotídeos usados nestes experimentos.

Gene	Sense Primer (5'-3')	Antisense Primer (3'-5')	cDNA bp
Cx43	5' CCG ACG ACA ACC AGA ATG CC 3'	3' GA ACC CTA TCG ACC CGC TTG 5'	322
Cx36	5' AGT GGT GGG AGC AAG CGA GAA G 3'	3'CCG AAG TCA CAG GGT CCC AAC A 5'	282
Insulina	5'TTG CAG TAG TTC TCC AGT T 3'	3'TGT CCC GGT ACA AC TTG TTA5'	379
Beta-Actina	5'ACC ACA AGC TGA GAG GGA AAT CG 3'	3'CGT TTA CGA AGA TCC GCC TGA C 5'	533

6.5 Análise por PCR

A reação de PCR foi realizada em um volume final de 25 µL de reação, utilizando-se 1 µL de cDNA como *template*. As reações de PCR continham: 2.5 µL de 10X PCR buffer (GIBCO Life Technologies), 0.75 µL de 50 mM $MgCl_2$ (GIBCO Life Technologies), 0.5 µL de 10 mM dNTP Mix (GIBCO Life Technologies), 0.5 µL de 100 pM de cada primer e 0.2 µL de Taq DNA Polymerase (GIBCO Life Technologies). Foi preparado um Pré-Mix com as soluções descritas anteriormente, num volume referente ao número de amostras a serem submetidas à reação de PCR, para diminuir o erro de pipetagem. O ciclo ótimo de amplificação foi determinado para cada conexina, utilizando-se uma curva, como descrita anteriormente, com as amostras do grupo N1d.

A reação teve seu início, submetendo as amostras, durante 5 min a 94 °C, para o despareamento das duplas fitas de cDNA. A amplificação de Cx43 foi obtida, utilizando-se 25 ciclos, cada um deles compostos de 30 s a 94 °C, 30 s a 57 °C, e 45 s a 72 °C, utilizando-se um termociclador Perkin Elmer 9600 (Manufacturer). Após o último ciclo, foi realizada a etapa de elongamento do fragmento obtido, por 4 min, a 72 °C. A amplificação de Cx36 foi obtida com 35 ciclos, contendo os mesmos passos descritos anteriomente, e a

amplificação da insulina foi obtida com 23 ciclos. Em todos os casos, foi realizado um controle negativo da reação submetendo uma amostra com água para amplificação. A expressão de Beta-actina foi utilizada como controle interno em todas as reações de PCR.

7. Imunocitoquímica Indireta

7.1 Preparação de Lâminas

Para execução da técnica de imunocitoquímica, lâminas de vidro foram, primeiramente, lavadas com solução sulfocrômica contendo: 1L ácido sulfúrico, 50g de $K_2Cr_2O_7$ e 50 mL de água destilada por durante 30 min. Em seguida, as lâminas foram enxaguadas em água corrente por 10 min, mergulhadas em água destilada por 15 min, e secas em estufa, a 37 °C.

Depois de secas, as lâminas foram mergulhadas em solução de Poli - L - lisina 1% (Sigma) por aproximadamente 10 min, secas em estufa a 37 °C e mantidas a 4 °C, estando assim prontas para o uso.

As ilhotas isoladas como descrito na seção 2.1.2, foram primeiramente fixadas por 30 min com solução de 2% paraformaldeído (Sigma) à temperatura ambiente. Em seguida, foram feitas duas sucessivas lavagens do material com solução tampão fosfato de sódio 0.5 M (PBS, pH 7.4). As ilhotas foram, então, submetidas ao processo de inclusão em solução de gelatina (Sigma) a 5% e 10%, ambas por 2h a 37°C em banho-maria. Em seguida, o material foi mantido em solução de gelatina a 25%, por 12 h a 37 °C em banho-maria.

As ilhotas foram, então, transferidas para um mini-cilíndro de plástico devidamente vedado em uma das extremidades com parafina e mantidas no gelo para possibilitar o endurecimento da gelatina. Após endurecimento, o material foi desenformado, coberto com meio de inclusão para criostato (*Tissue Te*, Sigma) e congelado em solução de n-hexano (Sigma) previamente resfriada em nitrogênio líquido.

As ilhotas foram, em seguida, cortadas em criostato e transferidas para lâminas devidamente identificadas. O material foi mantido à temperatura ambiente por 1 h para possibilitar a aderência do material às lâminas.

Os cortes foram, primeiramente hidratados, 2 vezes, com solução tampão Tris- HCl 50mM (TBS) (pH 7.4) e, em seguida, permeabilizados com Triton X 100 0.1% em TBS por 10 min à temperatura ambiente. Foram procedidas 3 sucessivas lavagens do material com solução de TBS.

Para detecção da proteína, os cortes de ilhotas foram bloqueados por 1 h com solução TBS contendo 0.01% de Tween 20 (Sigma) (TTBS) e 5% de albumina bovina sérica (BSA) (Sigma). Após bloqueio, os cortes foram incubados a 4 °C, por 12 h, com anticorpos monoclonais específicos como descritos na Tabela II.

Procedeu-se, então, 3 sucessivas lavagens dos cortes com solução de TTBS e, em seguida, as ilhotas foram incubadas com anticorpos secundários, anti-IgG conjugado com FITC (Sigma) (diluição 1:75 em TBS/BSA 1%) por 2h à temperatura ambiente.

Os cortes foram, então, novamente lavados com solução TTBS por 3 vezes e as lâminas montadas em *Vectashield* (Vector) A imunofluorescência foi detectada por microscopia confocal de varredura a laser (Bio-Rad MRC 1024) do Instituto de Biologia/Faculdade de Engenharia de Alimentos UNICAMP).

Tabela II: Anticorpos utilizados na reação de imunocitoquímica.

Anticorpo	**Fabricante**	**Tipo**	**Hospedeiro**	**Imunógeno**
Anti-Cx43	Zymed	Monoclonal	Mouse	Rat
Anti-Beta-catenina	Zymed	Monoclonal	Mouse	Chicken

8. Análise Estatística

A análise estatística de múltiplas comparações foi determinada utilizando-se ANOVA (análise de variância) seguido do teste de Bonferroni. O limite de significância foi estabelecido para $p<0.05$. Para estas análises, foi utilizado o programa computacional "Prism 2.01 for Windows"

APÊNDICE B

PUBLICAÇÕES RELEVANTES

Collares-Buzato CB, Leite AR, Boschero AC. Modulation of gap and adherens junctional proteins in cultured neonatal pancreatic islets. 2001 *Pancreas 23:177-185.*

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APÊNDICE C

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Modulation of Gap and Adherens Junctional Proteins in Cultured Neonatal Pancreatic Islets

Carla B. Collares-Buzato, *Adriana R. Leite, and *Antonio Carlos Boschero

*Departments of Histology and Embryology and *Physiology and Biophysics, University of Campinas (UNICAMP), Campinas, Brazil*

Summary: Fetal and neonatal pancreatic islets have lower insulin secretory responses compared with adult islets. In culture conditions and after treatment with mammosomatotropic hormones, neonatal islets undergo maturation of the secretory machinery that might involve regulation of cell–cell contacts within the islet. This study is an investigation of the effect of prolonged culturing and in vitro treatment with prolactin on the expression of the gap junction–associated connexin 43 and the adherens junction–associated β-catenin in cultured neonatal rat islets. Pancreatic islets from neonatal Wistar rats were cultured for 24 hours or 7 days, and the treated group was exposed to 2 μg/mL prolactin daily for 7 days. Connexin 43 and β-catenin were barely detected at the cell–cell contacts in 24-hour-cultured islets, as revealed by immunocytochemical analysis. Nevertheless, both junctional proteins were well expressed at the junctional region in islet cells cultured for 7 days and showed even greater staining in islets after long-term prolactin treatment. In accordance with the morphologic data, neonatal islets cultured for 24 hours displayed a relatively low level of connexin 43, as determined by Western blot analysis. Culturing for 7 days or combined prolactin treatment induced a significant increase in connexin 43 expression; this was 40% greater in the prolactin-treated group than in the control group. Furthermore, an enhancement of the expression of β-catenin and translocation of this protein to the cell–cell contact site was also observed in neonatal islets cultured for 7 days compared with those cultured for 24 hours. In vitro prolactin treatment induced even greater expression of β-catenin in islet cells. A correlation was observed between the increased expression of these junctional proteins and an increase in insulin secretion in cultured neonatal islets. In conclusion, prolonged culturing and in vitro treatment with prolactin induce the modulation of gap and adherens junctional proteins in pancreatic islets, which may be an important event in the in vitro maturation process of neonatal islet cells. **Key Words:** Cell–cell contact—Connexin 43—β-Catenin—Islets of Langerhans—Prolactin—Islet culture.

Cell-to-cell contacts are crucial for the proper secretory response within the pancreas (1). These interactions depend on specialized membrane components known as intercellular junctions, which include the tight junction, adherens junction, desmosome, and gap junction (GJ). All these junctions have been shown to be dynamic and regulated membrane structures in several different tissues (2–4). Among the intercellular junctions, GJs have been the most studied in the pancreas. They are characterized by the presence of intercellular channels involved in cell-to-cell communication (4,5). These channels are composed of members of a family of closely related integral membrane proteins known as connexins, which are assembled in a hexagonal pattern within the membrane and delineate a central hydrophilic pore (4,6,7). Evidence has indicated the importance of the GJ-mediated intercellular communication for insulin secretion by B cells. In vivo and in vitro studies have shown that stimulated insulin secretion by glucose and other secretagogues is associated with increased GJ-mediated coupling between B cells (8,9). Experimental conditions that inhibit the insulin secretion result in reduction or blockage of coupling between B cells, whereas the pharmacologic inhibition of the GJ channels induces impairment of the secretory function of these islet cells (10,11). The observation that both synthesis and release of insulin

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Address correspondence to Dr. Carla B. Collares-Buzato, Departamento de Histologia e Embriologia, Instituto de Biologia, C.P. 6109, Universidade Estadual de Campinas 13083-970, Campinas SP, Brazil. E-mail: collares@obelix.unicamp.br

are markedly altered after dispersion of B cells in vitro and are rapidly improved after cell reaggregation further suggests that the secretory mechanisms depend on cell-to-cell contacts (1,12).

Although the importance of GJ-mediated intercellular communication in the endocrine pancreas is well established, the mechanisms underlying the regulation of these junctions in experimental models for B-cell dysfunction are still unexplored. One of these models is the fetal and neonatal rat islet that shows, compared with the adult rat islets, a reduced secretory response of insulin to glucose and other secretagogues, such as amino acids, muscarinic agonists, and phorbol ester (13,14). Prolactin and other lactogenic substances appear to play an important role in the maturation of the secretory machinery occurring in the B cells. In vitro studies have reported that treatment of neonatal rat islets with prolactin increases insulin secretion, decreases the glucose stimulation threshold, and enhances GLUT2 glucose transporter expression and intracellular glucose oxidation (15–17). It has been reported that under prolonged culture conditions, neonatal islets also show improvement of the insulin secretion response to glucose and amino acids, indicating a possible role for a culture medium–containing factor in the B-cell maturation process (14,18,19). With prolactin treatment, this hormone also increases GJ-mediated coupling between B cells, as indicated by the intercellular transference of intracellular microinjected Lucifer yellow dye between adjacent cells (17,20). This observation indicates that cell–cell contacts mediated by GJs may play an important role in hormone-induced neonatal B-cell maturation. Meanwhile, possible GJ upregulation during prolonged culture of fetal and neonatal islets has not been assessed.

The intracellular mechanisms involved in this increase of cellular communication induced by prolactin are still unknown and might involve one or several possible levels of intercellular gate control. The regulation of gap junctional communication potentially can occur at multiple levels: transcription of the connexin gene, translation of connexin transcripts, assembly of connexins into connexons and transport to the plasma membrane, assembly of connexons into GJ intercellular channels, gating of functional GJ channels, and disassembly and degradation of the connexin proteins (4,7). Cell-to-cell adhesion mediated by the cadherin-catenin complexes within the adherens junction may also affect intercellular communication by modulating some of these GJ channel formation steps (21–23). The aim of this study was to investigate the effect of prolonged culturing and in vitro treatment with prolactin on the cellular expression and localization of the GJ-associated connexin 43 and the adherens junction–associated β-catenin in neonatal rat islets. We report here that those experimental conditions induce upregulation of the expression of both junctional proteins. A correlation was observed between the increased expression of Cx43 and β-catenin and an increase in insulin secretion followed by 7-day culture or combined with chronic prolactin treatment in neonatal islets. We suggest that the regulation of gap and adherens junctional proteins by prolactin and culture medium–containing factors may be an important event for the maturation process of B cells observed in vitro.

MATERIALS AND METHODS

Materials

All chemicals, cell culture media, and supplements were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). All sterile plastics (Petri dishes and multiwell plates) were purchased from Falcon (New York, NY, U.S.A.) and Corning (Sunnyvale, CA, U.S.A.) Radiolabeled insulin and protein A were obtained from Amersham Life Sciences (Cleveland, OH, U.S.A.). Fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Sigma Chemical. Anti-Cx43 and anti–β-catenin monoclonal antibodies were supplied by Zymed Laboratories (San Francisco, CA, U.S.A.). Rat prolactin used in this study was obtained through NHPP, NIDDK, and Dr. Parlow.

Culture of islets of Langerhans

Islets from neonatal (2 to 36 hours) Wistar rats were obtained as previously described (14). They were maintained in culture within sterile Petri dishes at 37°C in a humidified 5% carbon dioxide and air atmosphere. The culture medium consisted of Roswell Park Memorial Institute (RPMI)-1640 medium containing 10 m*M* glucose and supplemented with 5% fetal calf serum (heat inactivated) and 100 IU penicillin/mL and 100 μg streptomycin/mL. The dishes were divided between three experimental groups: neonatal islets cultured for 24 hours (group N24), neonatal islets cultured for 7 days (group NC), and neonatal islets cultured for 7 days that received a daily dose (2 μg/mL) of prolactin (group NPRL). The culture medium was changed every second day for 7 days. At the end of the culture period, the islets were collected individually under a dissecting microscope using a micropipette. Some of these islets were used to determine the insulin secretion levels, and the remaining islets were fixed in aldehyde for immunocytochemical analysis or frozen to -70°C in RPMI-1640 medium containing 2 m*M* phenylmethylsulfonyl fluoride and 0.05% glycerol for immunoblotting.

Immunocytochemical analysis

The cell locations of connexin 43 and β-catenin proteins were determined using a standard indirect immunofluorescence technique (3,24). A pool of at least 1,500 islets were pelleted within an Eppendorf, fixed in 2% paraformaldehyde (in phosphate buffer saline, pH 7.4) for 30 minutes at room temperature. Then they were included in increasing concentrations of gelatin solutions (5%, 10%, and 25%) at 37°C. After incubation, the gelatin blocks were embedded in OCT compound and frozen in n-hexane with liquid nitrogen. Cryostat sections (6 μm thick) were picked up on poly-L-lysine–coated glass slides, air dried, and treated with 0.1% Triton X-100 (in phosphate-buffered saline) for 10 minutes at room temperature. Sections were washed three times with TTBS (10 m*M* Tris, 150 m*M* NaCl, pH 7.4, 0.1% Tween 20) and incubated with TTBS containing 5% bovine serum albumin for 1 hour at room temperature. For immunofluorescence labeling of connexin 43, these islet sections were first incubated with anti-connexin 43 or anti–β-catenin monoclonal antibodies (dilution 1:50 in TBS plus 1% bovine serum albumin) overnight at 4°C and incubated with the specific secondary antibody conjugated with fluorescein (fluorescein isothiocyanate–labeled anti-mouse immunoglobulin G, dilution 1:75 in TBS plus 1% bovine serum albumin) for 2 hours at room temperature. After the final wash, the sections were mounted in a commercial antifading agent (Vectashield; Vector Laboratories, Burlingame, CA, U.S.A.) and viewed by confocal laser scanning microscopy (CLSM; BioRad MRC 1024UV; Bio-Rad, Hercules, CA, U.S.A.) using an inverted fluorescence microscope.

Western blotting

A pool of at least 1,000 islets from each experimental group was homogenized by sonication in an antiprotease cocktail (10 m*M* imidazole [pH 7.4], 4 m*M* ethylenediaminetetraacetic acid, 1 m*M* ethyleneglycol-bis-[β-aminoethylether]-N,N,N',N'-tetraacetic acid, 0.5 μg/mL pepstatin A, 200 KIU/mL aprotinin, 2.5 μg/mL leupeptin, 30 μg/mL trypsin inhibitor, 200 μ*M* DL-dithiothreitol, and 200 μ*M* phenylmethylsulfonyl fluoride). After a 10-minute centrifugation of the sonicate at 3,000*g* and 4°C, the supernatant was collected and its total protein content was determined using the DC protein assay kit (Bio-Rad Laboratories). A sample of the crude membrane preparation of each experimental group containing 70 μg total protein was incubated for 60 minutes at 37°C with 20% of the total volume with ×5 concentrated Laemmli sample buffer (1 *M* sodium phosphate pH 7.8, 0.1% bromophenol blue, 50% glycerol, 10% sodium dodecyl sulfate, and 2% mercaptoethanol). These samples were fractionated by electrophoresis in an 8% polyacrylamide gel. Electrotransfer of proteins from gel to nitrocellulose (Bio-Rad) or PVDF (Millipore, Bedford, MA, U.S.A.) membranes was performed for 60 minutes at 20V constant in the absence of methanol and sodium dodecyl sulfate. After being checked for efficient transfer by Ponceau S staining, the membranes were saturated with 5% dry skimmed milk in TTBS (10 m*M* Tris, 150 m*M* NaCl, 0.5% Tween 20) overnight at 4°C. Connexin 43 was detected in the membrane after a 2-hour incubation at room temperature with a mouse monoclonal antibody against heart connexin 43 (dilution, 1:1,000 in TTBS plus 3% dry skimmed milk) or anti-β-catenin (dilution 1:1,000 in TTBS plus 3% dry skimmed milk). The membrane was incubated with rabbit anti-mouse immunoglobulin G (dilution, 1:1,500 in TTBS plus 3% dry skimmed milk) followed by a further 2-hour incubation at room temperature with I-125–labeled protein A (dilution, 1:1,000 in TTBS plus 1% dry skimmed milk). Radiolabeled protein bound to the antibodies was detected by autoradiography. Band intensities were quantified by optical densitometry (Molecular Dynamics) of the developed autoradiogram.

Insulin secretion

After culture, the islets were detached gently from the plates while being viewed with a dissecting microscope. Clean islets were washed with a bicarbonate-buffered solution (pH 7.4; composition: 115 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 24 m*M* $NaHCO_3$ supplemented with 3 mg/mL bovine serum albumin and 10 m*M* glucose and equilibrated with a mixture of 95% oxygen and 5% carbon dioxide) and then transferred to 24-multiwell plates. Groups of 8 to 10 islets from the different experimental groups were placed within each well of 24-multiwell plates and incubated with 1 mL supplemented RPMI-1640 medium containing 10 m*M* glucose for 2 hours. After the incubation period, aliquots of the supernatant were taken and stored at -20°C. The insulin content of these samples was determined by radioimmunoassay and was expressed as nanograms per islet/hour (13).

Statistical analysis

All the results are expressed as means ± SEM with the number of individual experiments (n). The statistical significance of differences among more than two experimental groups was assessed by analysis of variance; for multiple comparisons between pairs of groups, the Bonferroni test was used. The significance level was set at $p < 0.05$.

RESULTS

Islets of Langerhans from different experimental groups showed distinct insulin secretory responses to 10 m*M* glucose (Table 1). Neonatal islets treated with prolactin for 7 days displayed an insulin secretion level of 1.6 ($p < 0.02$), which was 3.5 times ($p < 0.02$) greater than those untreated neonatal islets cultured for 7 days (NC group) or for only 24 hours (N24 group), respectively. Furthermore, the NC group showed significantly greater secretion compared with the N24 group ($p < 0.05$; Table 1).

Figure 1 shows the increase in connexin 43 expression observed after chronic treatment with prolactin, as revealed by Western blotting of homogenized islets samples. Prolactin treatment (2 $\mu g \cdot mL^{-1} \cdot day^{-1}$) for 7 days in vitro resulted in an approximately 40% increase in the expression of this GJ-associated protein as compared with untreated islets cultured for 7 days that were part of the NC group. Islets from this latter group showed higher connexin 43 expression compared with neonatal islets cultured for only 24 hours, which showed a relatively low amount of this protein (Fig. 1A).

This increased connexin 43 expression was correlated with an increase in GJ channel number located at the cell membrane as revealed by immunocytochemical analysis (Fig. 2A–C). Immunolabeling for connexin 43 protein showed a punctate staining pattern at the intercellular membrane region in 7-day-cultured neonatal islets (Fig. 2B,C); this connexin 43 labeling was not observed in all cells. The immunoreaction for connexin 43 was significantly stronger at regions of cell–cell contact in 7-day-cultured neonatal islets treated with prolactin (Fig. 2C) than in untreated neonatal islets (Fig. 2B). More cells seemed to be labeled in prolactin-treated islets (Fig. 2C) compared with those in untreated islets (Fig. 2B). Conversely, neonatal islets cells cultured for only 24 hours showed a very faint labeling at the cell–cell contact region (Fig. 2A).

TABLE 1. *Chronic treatment with PRL or prolonged culture* per se *induced increase in the insulin secretion by islets of Langerhans in vitro*

Groups[a]	Insulin secretion (ng/islets.h)[b]
N24	0.20 ± 0.02 (16)
NC	0.44 ± 0.05 (21)[c]
NPRL	0.70 ± 0.07 (26)[d,e]

[a] The experimental groups were as followed: N24, neonatal islets cultured for 24 hours; NC, neonatal islets cultured for 7 days; NPRL, neonatal islets cultured for 7 days and treated daily with PRL (2 μg/mL/day).

[b] Islets were stimulated for 2 hours with 10 m*M* glucose added to the culture media. Insulin released within the medium was measured by radioimmunoassay. The results were expressed as means ± SEM (number of islets).

[c] $p < 0.05$.

[d] $p < 0.01$ in relation to N24 group.

[e] $p < 0.02$ in relation to NC group (Student *t* test).

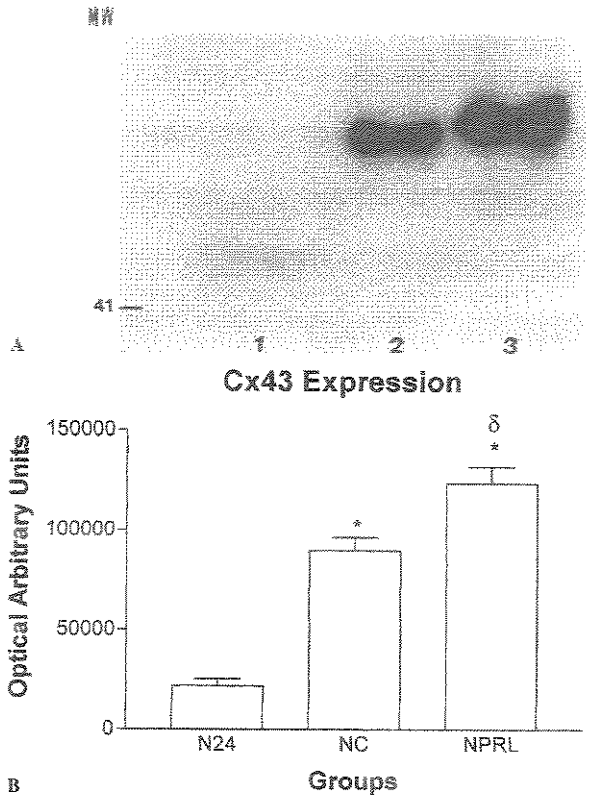

FIG. 1. Connexion 43 expression was increased in neonatal pancreatic islets induced by in vitro treatment with prolactin or prolonged culture alone. The level of connexin 43 was measured by Western blotting in islet sonicate. Compared with culturing for 24 hours (N24, lane 1), culture of islets for 7 days alone (NC, lane 2) or 7-day treatment with prolactin (2 $\mu g \cdot mL^{-1} \cdot day^{-1}$; NPRL, lane 3) induced (A) a substantial increase in the expression of connexin 43. **B:** Expression of connexin 43 after prolactin treatment was 40% greater than the 7-day cultured control as measured by densitometry. Values of densitometry for each experimental group are shown in panel b and expressed as means ± standard errors. The result shown in panel a is representative of five independent experiments. N24, neonatal islets cultured for 24 hours; NC, neonatal islets cultured for 7 days; NPRL, neonatal islets cultured for 7 days and treated daily with 2 $\mu g \cdot mL^{-1} \cdot day^{-1}$ prolactin. $^*p < 0.05$ in relation to N24 group; $\delta p < 0.05$ in relation to NC group (Bonferroni test).

Besides the connexin 43, prolonged culture or 7-day treatment with prolactin also induced changes in expression of another junctional protein, β-catenin, which is associated with the adherens junctions in several epithelial cells (Fig. 3). As revealed by Western blotting, we observed relatively high expression of β-catenin in the NC and NPRL groups. Prolonged culture or prolactin treatment induced, respectively, a 4-fold ($p < 0.05$) and 6.5-fold ($p < 0.05$) increase in β-catenin expression in

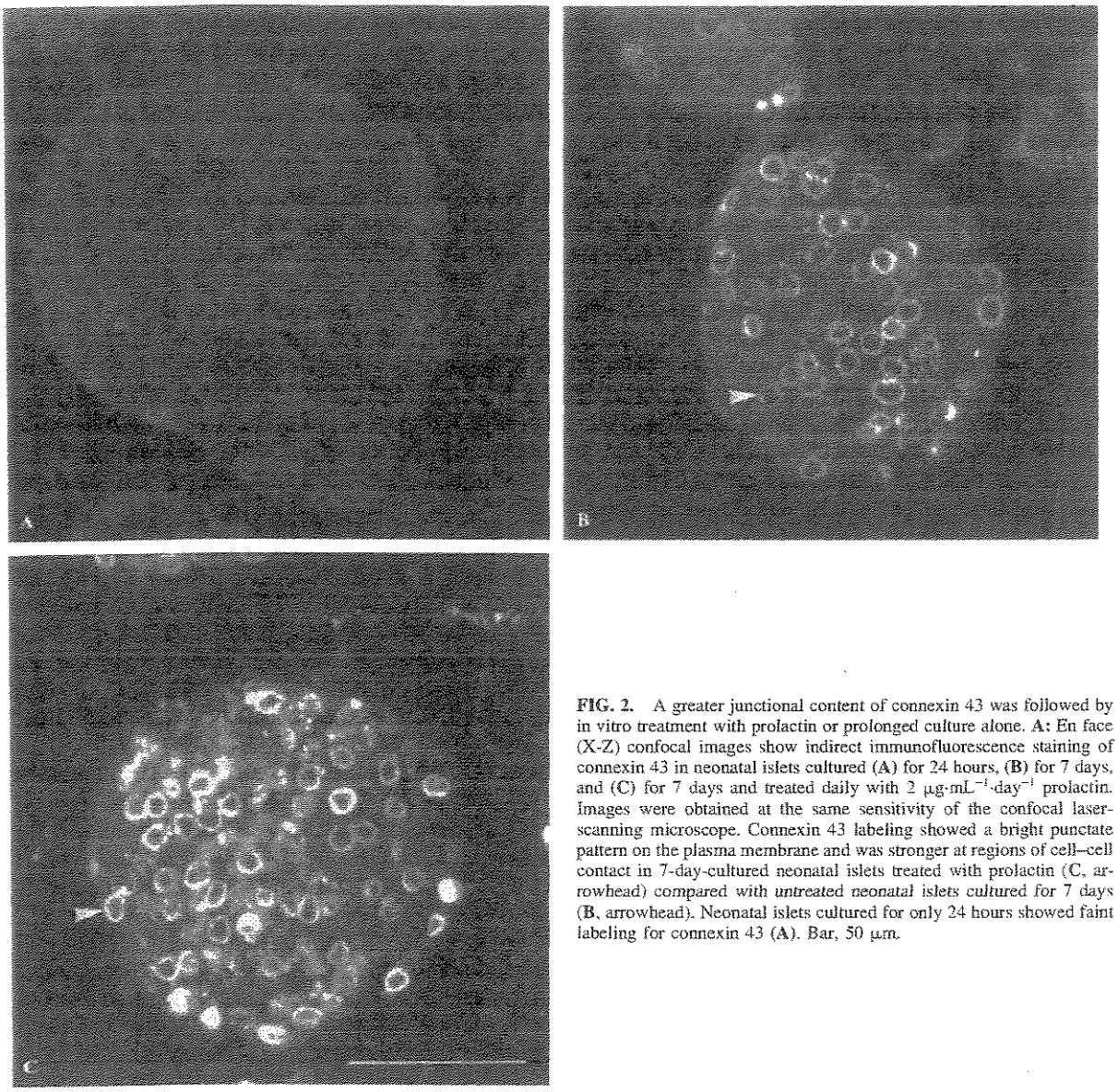

FIG. 2. A greater junctional content of connexin 43 was followed by in vitro treatment with prolactin or prolonged culture alone. **A:** En face (X-Z) confocal images show indirect immunofluorescence staining of connexin 43 in neonatal islets cultured **(A)** for 24 hours, **(B)** for 7 days, and (C) for 7 days and treated daily with 2 $\mu g \cdot mL^{-1} \cdot day^{-1}$ prolactin. Images were obtained at the same sensitivity of the confocal laser-scanning microscope. Connexin 43 labeling showed a bright punctate pattern on the plasma membrane and was stronger at regions of cell–cell contact in 7-day-cultured neonatal islets treated with prolactin (C, arrowhead) compared with untreated neonatal islets cultured for 7 days (**B**, arrowhead). Neonatal islets cultured for only 24 hours showed faint labeling for connexin 43 (**A**). Bar, 50 μm.

neonatal islets compared with those cultured for only 24 hours.

In agreement with the immunoblotting data, 7-day culturing followed by prolactin treatment increases the junctional expression of β-catenin in islet cells (Fig. 4B,C). In both groups, a bright labeling at the perijunctional region of all islet cells was observed (Fig. 4B,C). In contrast, islets cultured for only 24 hours showed very few labeled cells, located mainly at the islet periphery, probably representing non–B cells (Fig. 4A), after immunostaining for β-catenin. Labeling in these cells was apparent throughout the cell cytoplasm (Fig. 4A).

DISCUSSION

Gap junctions are specialized membrane components that mediate the direct exchange of ions and small molecules between adjacent cells. In exocrine and endocrine glands, GJs can function primarily as channels for Ca^{2+}or secondary messengers (i.e., cyclic adenosine

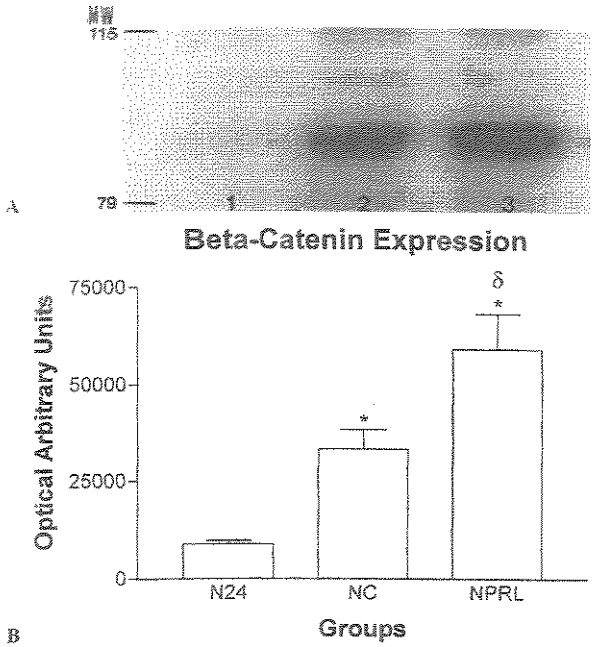

FIG. 3. Increased β-catenin expression in neonatal pancreatic islets was induced by in vitro treatment with prolactin or prolonged culture alone. The level of β-catenin was detected by Western blotting in islet sonicate. The NC (lane 2) and NPRL (lane 3) groups show significantly greater expression of this junctional protein than does the N24 group (lane 1) (A and B). Values of densitometry for each experimental group are shown in panel B and expressed as means ± standard error. The result shown in A is representative of four independent experiments. N24, neonatal islets cultured for 24 hours; NC, neonatal islets cultured for 7 days; NPRL, neonatal islets cultured for 7 days and treated daily with 2 $\mu g \cdot mL^{-1} \cdot day^{-1}$ prolactin. $^*p < 0.05$ in relation to N24; $\delta p < 0.05$ in relation to NC group (Bonferroni test).

monophosphate and IP_3) (4,7). The intercellular exchange of these elements allows the secretory gland response to be amplified because it ensures a rapid recruitment of secreting cells located distantly from the site of signaling. The importance of GJ-mediated cell coupling has been particularly well demonstrated in the endocrine pancreas; increased intercellular communication is directly correlated to an increase in the secretory insulin response in B cells (8,9). The means of upregulating GJ function can include an increase in the number of GJ channels, resulting in an overexpression of the connexin type found in the islets of Langerhans, connexin 43, or an enhancement of GJ channel permeability (4,7). Furthermore, another level of regulation for gap junctional intercellular communication may be cell–cell adhesion, which is mediated by another type of intercellular junction, the adherens junction in several tissue types. Strong evidence now exists that GJs depend functionally on the adherens junction. Impairment of intercellular adhesion with antibodies against adherens junction–associated cadherins results in GJ disassembly or intercellular uncoupling (25–27). Conversely, the transfection of a junctional communication–deficient cell line with DNA encoding cadherins increases cell coupling (23).

In this study, we found that 7-day culture alone or combined with long-term treatment with prolactin enhances the expression of connexin 43 in pancreatic islets from neonatal rats. Prolactin treatment in vitro resulted in a further increase in the junctional content of this GJ protein compared with prolonged culturing of islets in the absence of this hormone. In parallel, both experimental conditions led to an increase in the glucose-evoked insulin secretion by neonatal islets compared with 24-hour culturing. The increase in connexin 43 expression, as revealed by Western blotting, was confirmed by immunocytochemical analysis. Neonatal islets cultured for 7 days and treated with prolactin show greater levels of connexin 43 staining at the cell–cell junctional region compared with untreated neonatal islets cultured for only 24 hours. This observation indicates that the increase in cellular connexin 43 expression was accompanied by an increase in the number of connexin-formed channels at the cellular membrane site.

Increased connexin 43 expression after treatment with prolactin is in accordance with a previous observation of increased cell coupling in neonatal islets with this hormone, as shown by intercellular transference of microinjected Lucifer yellow to neighboring cells (17,20). Furthermore, the current data suggest that this prolactin-induced increase in intercellular communication may be the result of an increased number of functional GJ channels at the membrane rather than upregulation of the gating function of these channels. Prolactin and other mammosomatotropic hormones have important upregulatory effects on pancreatic islet B-cell function, including the enhancement of intercellular GJ coupling, that seem to be involved in islet adaptation to pregnancy and maturation of a B-cell glucose-sensing mechanism during the perinatal period (14,16,28,29). The mechanism of prolactin actions depends on the trigger of several intracellular signaling pathways (e.g., JAK/STAT and MAP kinases pathways (29,30)) leading to activation of nuclear promoter elements on prolactin-responsive genes. Whether these pathways also mediate the prolactin effect on GJ function requires further investigation. Concerning the stimulatory effect of prolonged islet culture (7 days) on connexin 43 expression and insulin secretion, it is plausible to suggest that lactogenic factors, hormones present within the serum, or even other

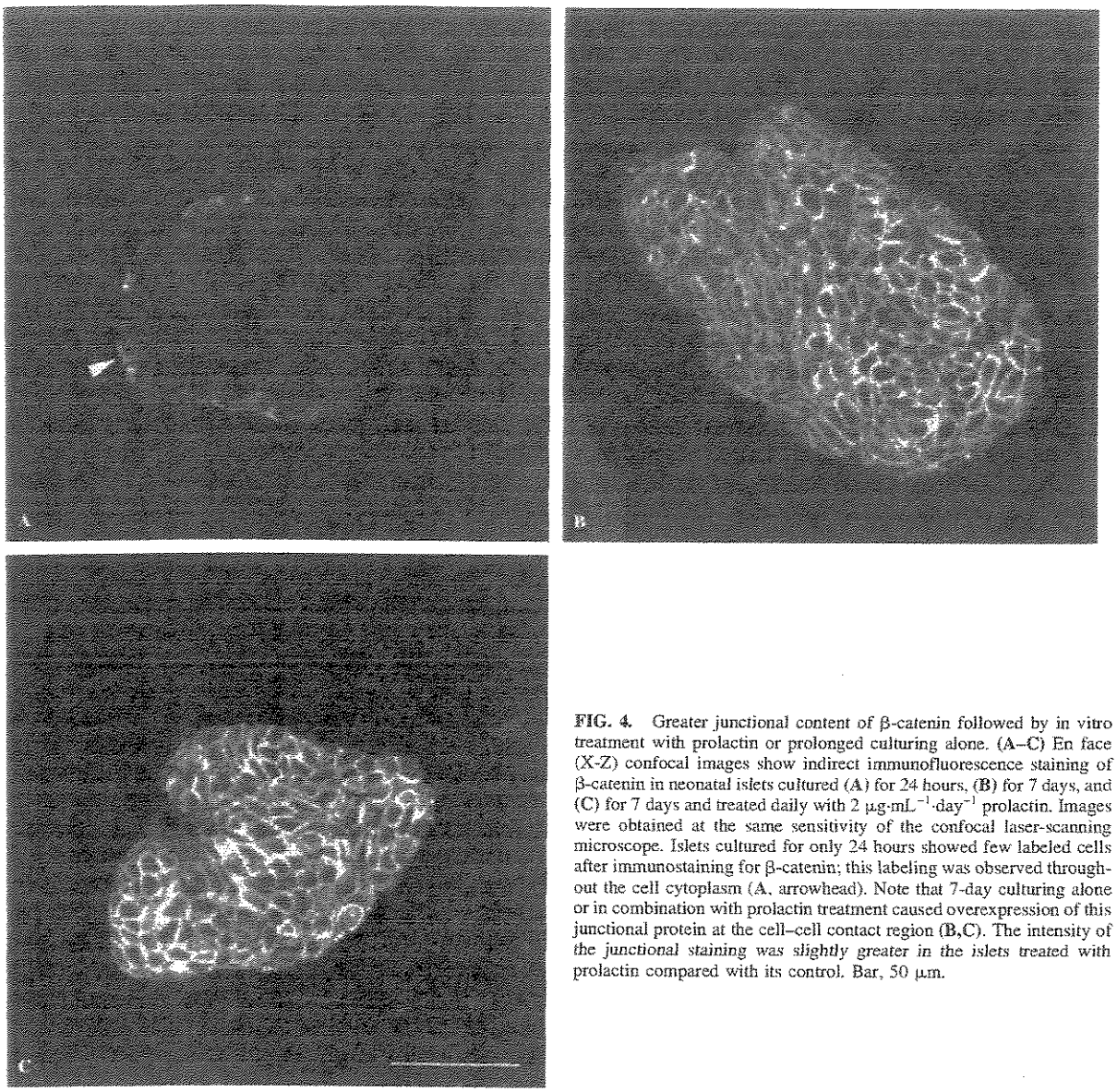

FIG. 4. Greater junctional content of β-catenin followed by in vitro treatment with prolactin or prolonged culturing alone. (**A–C**) En face (X-Z) confocal images show indirect immunofluorescence staining of β-catenin in neonatal islets cultured (**A**) for 24 hours, (**B**) for 7 days, and (**C**) for 7 days and treated daily with 2 $\mu g \cdot mL^{-1} \cdot day^{-1}$ prolactin. Images were obtained at the same sensitivity of the confocal laser-scanning microscope. Islets cultured for only 24 hours showed few labeled cells after immunostaining for β-catenin; this labeling was observed throughout the cell cytoplasm (A, arrowhead). Note that 7-day culturing alone or in combination with prolactin treatment caused overexpression of this junctional protein at the cell–cell contact region (**B,C**). The intensity of the junctional staining was slightly greater in the islets treated with prolactin compared with its control. Bar, 50 μm.

culture medium components may be accounted for these effects. The presence of serum and certain nutrients at relatively high concentrations (such as glucose and metabolizable amino acids) within the medium are essential for survival, growth, and differentiation of islet B cells in vitro (31,32). In accordance with our data, previous investigators have shown a partial maturation of the insulin response to glucose in neonatal islets during a 7-day culture using a serum-containing media compositionally similar to that used here (14,18,19). Although the cell–cell coupling has not been directly assessed in this 7-day cultured islets, we can hypothesize based on the connexin 43 expression data that this maturation of the stimulus–secretion coupling process observed after culturing may also involve an increase in GJ-mediated intercellular communication.

In addition, we found that a 7-day culture accompanied or not by prolactin treatment also induced an increase in the expression of β-catenin, a protein associated with another intercellular junction, the adherens junction. This protein plays a crucial role in cell adhesion in several epithelial cell types by modulating the linkage

of cadherins to α-catenin, which in turn interacts with the actin cytoskeleton (33–35). β-catenin is not only an essential structural and regulatory component of adhesion complexes but is also part of the Wnt/Wingless signaling pathway implicated in cell fate decision and pattern formation during development (36–38). It has been reported that the cytoplasmic β-catenin pool binds to certain transcriptional factors (e.g., TCF/LEF-1) within the nucleus and specifies gene expression (36,38). The current study is a first description of the expression and regulation of this protein in islet cells. β-catenin is expressed in low levels in poorly insulin-secreting neonatal islets cultured for 24 hours that show mainly a cytoplasmic localization for this junctional protein in cells located at the islet periphery, probably non-B cells. Prolactin treatment or culturing that seemed to improve the glucose-induced insulin secretory function induced upregulation of this protein and its translocation to the cell–cell contact region. Furthermore, there seems to be a positive correlation between upregulation on connexin 43 and β-catenin expression in islet cells with these experimental conditions. Interestingly, recent research has shown a direct association between β-catenin–associated Wnt signaling and connexin 43 expression and function in cardiomyocytes (21). Although the functional relation between adherens junction and GJ is documented extensively for several cell types (21,23,25–27), it is a matter of further investigation whether a similar relation exists in the islet cells, specifically in the case of our experimental model.

In conclusion, we found an increase in the expression of the GJ-associated connexin 43 induced by prolonged culturing per se and in vitro treatment with prolactin in neonatal rat pancreatic islets. A direct correlation was observed between the increased expression of connexin 43 and an increase in insulin secretion followed by 7-day culture or combined with chronic prolactin treatment in neonatal islets. In addition, we documented the expression of the adherens junction–associated β-catenin by islet cells and its upregulation after culture alone and prolactin treatment. The observed regulation of the gap and adherens junctions by prolactin and culture medium–containing factors may be involved in the maturation process of B cells in islets of Langerhans observed in vitro.

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