UNIVERSIDADE ESTADUAL DE CAMPINAS

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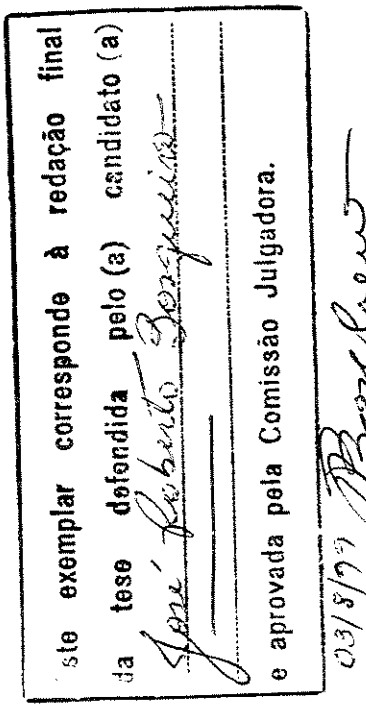
PARTICIPAÇÃO DE ESTOQUES INTRACELULARES DE Ca^{2+} NO MECANISMO DE SECREÇÃO DE INSULINA: EFEITO DO ANESTÉSICO LOCAL TETRACAÍNA



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RESUMO

Foi realizado estudo do efeito da tetracaína sobre o efluxo de ^{45}Ca, a concentração de Ca^{2+} citoplasmático, $[Ca^{2+}]_i$, e a secreção de insulina em ilhotas pancreáticas e células B isoladas. Na ausência de Ca^{2+} externo, tetracaína (0,1-2,0 mM) aumentou, de maneira dose-dependente, o efluxo de ^{45}Ca de ilhotas isoladas. Tetracaína não afetou o aumento do efluxo de ^{45}Ca causado por 50 mM de K^+ ou pela associação de carbacol (Cch – 0,2 mM) e 50 mM de K^+. Tetracaína aumentou permanentemente a $[Ca^{2+}]_i$ em células B isoladas em meio livre de Ca^{2+} e acrescido de 2,8 mM de glicose e 25 μM D-600 (metoxiveparamil). Este efeito também foi observado na presença de 10 mM de cafeína ou 1 μM de tapsigargina. Em presença de 16,7 mM de glicose, tetracaína aumentou de maneira transitória a secreção de insulina das ilhotas perfundidas, tanto na ausência quanto na presença de Ca^{2+} externo. Estes dados indicam que tetracaína mobiliza Ca^{2+} de um estoque insensível à tapsigargina e estimula a secreção de insulina na ausência de Ca^{2+} extracelular. O aumento do efluxo de ^{45}Ca causado pelas altas concentrações de K^+ e pelo Cch indica que tetracaína não interfere nos estoques sensíveis a cátions ou ao IP_3.

ABSTRACT

The effect of tetracaine on ^{45}Ca efflux, cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ and insulin secretion in isolated pancreatic islets and B-cells was studied. In the absence of external Ca^{2+}, tetracaine (0.1-2.0 mM) dose-dependently increased the ^{45}Ca efflux from isolated islets. Tetracaine did not affect the increase in ^{45}Ca efflux caused by 50 mM K^+ or by the association of carbachol (Cch - 0.2 mM) and 50 mM K^+. Tetracaine permanently increased the $[Ca^{2+}]_i$ in isolated B-cells in Ca^{2+}-free medium enriched with 2.8 mM glucose and 25 μM D-600 (methoxiverapamil). This effect was also observed in the presence of 10 mM caffeine or 1 μM thapsigargin. In the presence of 16.7 mM glucose, tetracaine transiently increased the insulin secretion from islets perifused in the absence and presence of external Ca^{2+}. These data indicate that tetracaine mobilises Ca^{2+} from a thapsigargin-insensitive store and stimulates insulin secretion in the absence of extracellular Ca^{2+}. The increase in ^{45}Ca efflux caused by high concentrations of K^+ and by Cch indicates that tetracaine did not interfere with a cation or IP_3-sensitive Ca^{2+} pool in B-cells.

INTRODUÇÃO

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1. Mecanismo de secreção de insulina

A secreção de insulina pelas células B pancreáticas apresenta um controle multifatorial, bem como uma regulação momento-a-momento, que visa o ajuste das concentrações plasmáticas de glicose dentro de estreitos limites de normalidade. Este controle da secreção tem a participação de nutrientes, hormônios e neurotransmissores. Dos nutrientes capazes de estimular a secreção de insulina, sem dúvida o mais importante é a glicose (Malaisse e cols., 1979). De acordo com as inúmeras evidências experimentais obtidas até hoje, pode-se formar um quadro complexo que explica, ao menos em parte, o mecanismo de secreção de insulina, uma vez que tal mecanismo apresenta-se como uma integração entre eventos metabólicos e iônicos.

O primeiro passo para a secreção de insulina estimulado pela glicose é o transporte da hexose para o interior da célula B pancreática, que ocorre, nos roedores, por um carregador específico, o GLUT2, membro de uma família de

transportadores com pelo menos 5 subtipos (Johnson & Newgard, 1996). A seguir, a glicose é fosforilada à glicose-6-fosfato por duas enzimas: uma glicoquinase (hexoquinase IV) de baixa afinidade (K_m alto) e uma hexoquinase de alta afinidade (K_m baixo) (Meglasson & Matschinsky, 1984). Em células B normais, o aumento nas concentrações de glicose leva à uma estimulação preferencial de eventos oxidativos mitocondriais, acarretando aumento das concentrações citoplasmáticas de ATP (Sener & Malaisse, 1987).

A descoberta da presença de canais para K^+ sensíveis ao ATP (K_{ATP}) foi fundamental na determinação do agente ou mecanismo acoplador entre os eventos metabólicos e secretórios nas células B pancreáticas (Ashcroft e cols., 1984; Cook & Hales, 1984). Assim, a elevação da concentração intracelular de ATP causa o fechamento destes canais. Na verdade, postula-se que o fator determinante para o fechamento de tais canais seja a elevação da relação de concentrações ATP/ADP, uma vez que, segundo Kakey e cols. (1986), o ADP provoca abertura dos canais K_{ATP}. De fato, o controle da abertura e fechamento destes canais é bastante complexo, uma vez que experimentos *in vitro* mostraram que concentrações micromolares de ATP (10 a 20 μM) são suficientes para fechá-los. Contudo, a concentração de ATP registrada em células B, mesmo em concentrações sublimiares de glicose (por ex. 2,8 mM), é da ordem de 3 mM. Como o potencial de membrana da célula B deve-se principalmente à saída do K^+ por esses canais, seu fechamento leva à despolarização da membrana celular. Outros canais estão presentes na membrana celular, entre eles os canais para Ca^{2+} do tipo L, dependentes de

voltagem. Desta forma, a despolarização celular leva à abertura de tais canais e, por diferença de potencial eletroquímico, os íons Ca^{2+} penetram na célula B, causando aumento da concentração intracelular do íon. Esse aumento é fundamental para a extrusão dos grânulos de insulina, através de mecanismos envolvendo a calmodulina, uma proteína reguladora que se liga ao Ca^{2+} e regula a atividade de várias enzimas, incluindo adenilato ciclase, AMPc fosfodiesterase e algumas quinases protéicas, como a quinase da miosina de cadeia leve (Valverde & Malaisse, 1984, Wilson e cols., 1998).

2. Cálcio e secreção de insulina

O íon cálcio tem função essencial no controle de uma série de processos celulares, tais como secreção, contração, ativação de enzimas e regulação do ciclo celular (Berridge, 1997). Para exercer tais funções, é necessário que sua distribuição pelos compartimentos intra e extracelulares seja regulada por mecanismos rápidos e precisos, e que possam ocorrer rápidas flutuações na concentração do íon graças a processos de transporte através das membranas.

A concentração de Ca^{2+} livre no citoplasma (cerca de 10^{-7} M), é mantida aproximadamente 10.000 vezes abaixo da concentração extracelular (em torno de 10^{-3} M). Este alto gradiente eletroquímico é essencial para a função

do íon como sinalizador bioquímico no interior celular, pois permite que a abertura de canais específicos na membrana seja acompanhada de rápida entrada de Ca^{2+}, levando a mensagem ao interior da célula (Berridge, 1997; Carafoli, 1987).

A membrana plasmática possui mecanismos de entrada de Ca^{2+} na célula mediados por canais voltagem-dependentes ou regulados por segundos mensageiros (Berridge, 1997), enquanto que o efluxo ativo do íon é promovido por uma Ca^{2+}-ATPase, que hidrolisa um ATP por Ca^{2+} translocado (Carafoli, 1994; Clark & Carafoli, 1983), ou por um sistema de trocador Na^+/Ca^{2+}, que troca um Ca^{2+} interno por três Na^+ externos, utilizando energia do gradiente de potencial eletroquímico de Na^+. Na célula B pancreática, este trocador parece estar envolvido também com a entrada de Ca^{2+} na célula quando de uma despolarização, além de representar cerca de 70% da saída do Ca^{2+} após a repolarização celular (Van Eylen e cols., 1998).

Como dito anteriormente, o mecanismo de secreção de insulina estimulado pela glicose em células B pancreáticas, envolve a abertura de canais voltagem-dependentes para Ca^{2+}, com conseqüente elevação das concentrações citosólicas do íon. Sabe-se, desde o final da década de 1960, que o influxo de Ca^{2+} desempenha papel preponderante no mecanismo de secreção de insulina, visto que a retirada do íon do compartimento extracelular resulta em inibição da secreção do hormônio (Grodsky & Bennett, 1966; Milner & Hales, 1967). Evidências posteriores, indicando que a glicose induz aumento na captação de ^{45}Ca pelas ilhotas, comprovam a importância do influxo do íon para

o processo secretório estimulado pela hexose (Hellman e cols., 1971; Malaisse-Lagae & Malaisse, 1971).

Com o aumento dos níveis de glicose no meio, ocorre inicialmente uma redução, pequena e passageira dos níveis de Ca^{2+} citoplasmáticos, que se deve à ativação do transporte ativo (Ca-ATPases) para o retículo endoplasmático pois, em baixas concentrações de glicose, o reservatório de Ca^{2+} está diminuído nesse compartimento. Concomitante à redução dos níveis de Ca^{2+}, que dura de 2 a 5 minutos, ocorre também inibição da secreção basal de insulina (Gylfe, 1989). A seguir, a concentração de Ca^{2+} é aumentada em cerca de 5 a 10 vezes e a secreção tem início. Os níveis de Ca^{2+} e a secreção de insulina permanecem elevados, enquanto o nutriente estiver presente em concentrações estimulatórias no meio.

Eletrofisiologicamente, foi demonstrado que a entrada do Ca^{2+} por esta via coincide com a atividade elétrica periódica (potencias de ação em pico) observada quando de uma elevação das concentrações de glicose (Dean & Matthews, 1970; Gilon e cols., 1993; Meissner & Schmeltz, 1974; Santos e cols., 1991). De fato, observa-se que, em presença de concentrações estimulatórias de glicose, ocorrem oscilações das concentrações intracelulares de Ca^{2+}, que refletem variações cíclicas na permeabilidade da membrana plasmática ao íon, e coincidem com a secreção pulsátil de insulina, ambos relacionados ao fluxo glicolítico, de natureza oscilatória (Bergstron e cols., 1989; Hellman e cols., 1992). Este padrão oscilatório da secreção de insulina pode ser vantajoso na

medida em que impede a "down regulation" do receptor de insulina nos órgãos alvo.

Apesar da importância que os mecanismos descritos acima representam para o processo secretório, a fisiologia da célula B pancreática com respeito à possível participação de outros mecanismos, bem como da interação entre eles permanece obscura. Sabe-se que a glicose e outros nutrientes secretagogos aumentam a produção de AMP cíclico (AMPc) nas células B (Malaisse & Malaisse-Lagae, 1984; Prentki & Matschinsky, 1987), o que potencializa os efeitos de outros iniciadores da secreção de insulina (Hellman e cols., 1992), num mecanismo aparentemente dependente do Ca^{2+}, visto que o aumento dos níveis de AMPc estimulado pela glicose é comprometido pela retirada do Ca^{2+} do meio extracelular (Valverde e cols., 1983). Este aumento dos níveis de AMPc deve-se à ativação da adenilato ciclase via calmodulina (Valverde e cols., 1979). Evidências experimentais sugerem que o AMPc, por meios dependentes da proteína quinase A (PKA) age sensibilizando a maquinaria secretória ao Ca^{2+} (Prentki & Matschinsky, 1987), provocando mobilização de estoques intracelulares do íon (Graspengiesser e cols., 1989; Sussman e cols., 1987) e favorecendo o influxo do íon nas células B pancreáticas (Eddlestone e cols., 1985; Henquin & Meissner, 1984).

A estimulação pela glicose também induz hidrólise do ácido araquidônico a partir de fosfolípideos de membrana na ilhota. Apesar dos mecanismos não serem conhecidos, dados apontam para uma participação dos estoques intracelulares de Ca^{2+} no processo (Nowatzke e cols., 1998). Nas

células B pancreáticas, demonstrou-se recentemente que os maiores estoques de Ca^{2+} intracelulares situam-se em regiões sensíveis à IP_3 e tapsigargina, uma droga que bloqueia seletivamente o transporte ativo para o interior do retículo endoplasmático através de uma Ca^{2+}-ATPase. Este estudo confirmou a existência de dois reservatórios de Ca^{2+} , proposta por Nilsson e cols. (1987), dependentes da Ca^{2+}-ATPase, sendo que somente um deles é sensível ao IP_3 (Tengholm e cols., 1998). Em células βTC3, obtidas de insulinoma de camundongos transgênicos e secretoras de insulina, as alterações de voltagem e concentração de Ca^{2+} intracelulares induzidas por glicose mostraram-se importantes no controle da produção de IP_3, e, consequentemente, na liberação de Ca^{2+} a partir de organelas intracelulares (Gromada e cols., 1996).

A presença da fosfolipase C (PLC) acoplada à membrana da célula B pancreática, ativada por nutrientes (Montague e cols., 1985; Prentki & Matschinsky, 1987; Turk e cols., 1987), origina uma série de eventos, presentes em diversos tipos celulares. A hidrólise do fosfatidilinositol 4,5-bifosfato dá origem a duas moléculas: o inositol 1,4,5-trifosfato (IP_3) e o diacilglicerol (DAG) (Best & Malaisse, 1984). O IP_3 é hidrossolúvel e tem como ação a liberação de Ca^{2+} de estoques intracelulares, predominantemente do retículo endoplasmático. Já o DAG permanece ancorado à membrana e ativa a proteína quinase C (PKC), dependente do Ca^{2+}. Como conseqüência da ação da PKC, a fosforilação de proteínas poderia sensibilizar a maquinaria secretória ao Ca^{2+}.

A liberação de Ca^{2+} dos estoques intracelulares também parece ser importante no controle do influxo do íon. A pequena queda nas concentrações

do íon, subseqüente ao estímulo pela glicose, é seguida pela liberação do Ca^{2+} das suas organelas de reserva (Kikuchi e cols., 1979; Roe e cols., 1993, 1995; Rojas e cols., 1994), o que causaria a ativação de uma corrente catiônica (I_{CRAC} – calcium release activated current, ou, corrente de cálcio ativada por liberação de cálcio) para o interior da célula (Berridge, 1997; Putney Jr., 1997; Roe e cols., 1998; Worley e cols., 1994a, b). O fator acoplador difusível seria liberado juntamente com o cálcio a partir do retículo endoplasmático (CIF – calcium influx factor) (Randriamampita & Tsien, 1993).

Além desta interação entre liberação e influxo de Ca^{2+}, foi sugerido em ilhotas a existência de um mecanismo descrito primeiramente em células musculares e presente em vários tipos de células excitáveis (Coronado e cols., 1994). Assim, a entrada de Ca^{2+} pela membrana plasmática levaria à liberação de cálcio a partir de organelas intracelulares (CICR = Calcium Induced Calcium Release). Este mecanismo envolve a participação de canais operados por receptores sensíveis à rianodina (receptor de rianodina) que, por elevação da concentração de Ca^{2+}, permitem a saída do íon a partir do retículo endoplasmático (Islam e cols., 1992). A importância de tal mecanismo depende da determinação da existência em quantidade significativa dos receptores, e da caracterização de sua modulação por substâncias presentes nas células. Como em outros tipos celulares, a ação da molécula de ADP ribose cíclica tem gerado controvérsia (Islam & Berggren, 1997; Okamoto e cols., 1997). A compreensão completa dos eventos intracelulares que acoplam a membrana citoplasmática,

local de influxo do Ca^{2+}, e o retículo endoplasmático, local de liberação de Ca^{2+} via IP_3 e/ou receptor de rianodina, está longe de ser alcançada (Mackrill, 1999).

A participação destes estoques intracelulares no processo de secreção de insulina estimulada pela glicose aguarda futuras investigações, uma vez que tal mecanismo não encontra aceitação total nos meios científicos.

Finalizando esta seção, há que se registrar trabalhos publicados desde o início da década de 90, onde identificaram-se mecanismos diferentes de secreção de insulina. Um deles diz respeito a um processo independente da ativação dos K_{ATP} (Gembal e cols., 1992; Sato e cols., 1992). Nestes trabalhos, a glicose aumentou a secreção de insulina mesmo com a abertura dos K_{ATP} (utilizando-se diazoxida) e despolarização com altas concentrações de KCl. Entretanto, esta via é dependente do aumento das concentrações intracelulares de Ca^{2+} e atua para aumentar o efeito estimulatório do aumento de Ca^{2+} na secreção.

Em outro grupo de experimentos, modo diverso de ação da glicose sobre a célula B pancreática foi indicado (Komatsu e cols., 1995). Neste trabalho, a glicose estimulou a secreção de insulina na ausência de Ca^{2+} extracelular e de qualquer aumento da concentração de Ca^{2+} no citosol, mediante ativação simultânea da PKA e PKC, num processo dependente de GTP (Komatsu e cols., 1998). Dúvidas a respeito da importância fisiológica deste mecanismo também encontram suporte na literatura (Sato e cols., 1998).

3. Anestésicos locais e secreção de insulina

Existem várias informações na literatura científica a respeito da ação dos anestésicos locais sobre a secreção de insulina e outros parâmetros da fisiologia das ilhotas pancreáticas. Por exemplo, a incubação de porções de pâncreas de coelhos com lidocaína e procaína (Tjalve e cols., 1974) e de pâncreas de ratos com mepivacaína, lidocaína (Bressler & Brendel, 1971) e tetracaína (Camu, 1973), resultaram em inibição da secreção de insulina estimulada por glicose. Esta inibição também ocorreu em preparações utilizando ilhotas isoladas (Brisson e cols., 1971; Freinkel e cols., 1975). Entretanto, em preparações de ilhotas de camundongos obesos (*ob/ob*), tetracaína potencializou a secreção de insulina induzida por glicose, manose, leucina e gliburida (Norlund & Sehlin, 1983). Efeitos variados também foram descritos quando os anestésicos foram utilizados em diferentes concentrações. Assim, procaína e lidocaína, na concentração de 1mM inibiram a secreção de insulina estimulada por glicose, enquanto concentrações menores potencializaram o efeito secretório da glicose (Tjalve e cols., 1974). Por outro lado, a secreção de insulina foi inibida por baixas concentrações de lidocaína e mepivacaína e estimulada por altas concentrações dos anestésicos (Bressler & Brendel, 1971).

Mais recentemente, novos trabalhos utilizando-se tetracaína demonstraram efeitos variáveis na secreção de insulina em função das concentrações de cálcio e glicose presentes no meio extracelular (Bordin e cols.,

1997; El Motal e cols., 1987). Bolaffi e cols. (1993) registraram inibição ou estimulação da secreção de insulina por tetracaína dependendo da fase secretória em que o anestésico foi aplicado.

Outros parâmetros da fisiologia da ilhota são alterados pelos anestésicos locais, como o metabolismo dos fosfolípideos de membrana (Freinkel e cols., 1975), resultando em inibição tanto da síntese quanto do catabolismo do fosfatidilinositol.

Outro dado interessante quanto à ação da tetracaína em ilhotas pancreáticas é sua habilidade em alterar o manejo do Ca^{2+}. Assim, a captação do $^{45}Ca^{2+}$ é inibida pelo anestésico enquanto o efluxo é estimulado (Brisson e cols., 1971; El Motal e cols., 1987; Norlund & Sehlin, 1985). Experimentos recentes demonstram que a tetracaína é capaz de mobilizar Ca^{2+} de estoques intracelulares, aumentando a concentração do íon no citosol das células B pancreáticas e, conseqüentemente, estimulando a secreção de insulina (Bordin e cols., 1997; Mears e cols., 1999).

Neste trabalho procuramos investigar melhor o modo de ação do anestésico local tetracaína bem como analisar o estoque intracelular alvo de sua ação.

TRABALHO SUBMETIDO À PUBLICAÇÃO

Tetracaine stimulates insulin secretion through the mobilisation of Ca^{2+} from thapsigargin- and IP_3-insensitive Ca^{2+} reservoir in pancreatic B-cells

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ABSTRACT

The effect of tetracaine on ^{45}Ca efflux, cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ and insulin secretion in isolated pancreatic islets and B-cells was studied. In the absence of external Ca^{2+}, tetracaine (0.1-2.0 mM) dose-dependently increased the ^{45}Ca efflux from isolated islets. Tetracaine did not affect the increase in ^{45}Ca efflux caused by 50 mM K^+ or by the association of carbachol (Cch - 0.2 mM) and 50 mM K^+. Tetracaine permanently increased the $[Ca^{2+}]_i$ in isolated B-cells in Ca^{2+}-free medium enriched with 2.8 mM glucose and 25 μM D-600 (methoxiverapamil). This effect was also observed in the presence of 10 mM caffeine or 1 μM thapsigargin. In the presence of 16.7 mM glucose, tetracaine transiently increased the insulin secretion from islets perifused in the absence and presence of external Ca^{2+}. These data indicate that tetracaine mobilises Ca^{2+} from a thapsigargin-insensitive store and stimulates insulin secretion in the absence of extracellular Ca^{2+}. The increase in ^{45}Ca efflux caused by high concentrations of K^+ and by Cch indicates that tetracaine did not interfere with a cation or IP_3-sensitive Ca^{2+} pool in B-cells.

Key words: Tetracaine, B-cells, insulin secretion, Ca^{2+}.

INTRODUCTION

Calcium ions play a pivotal role in cellular processes, including muscle contraction, neurotransmitter release, enzyme activation, regulation of the cell cycle and, insulin secretion (Prentki and Matschinsky 1987; Berridge 1997). In the latter process, an increase in $[Ca^{2+}]_i$ is generally considered the major triggering event that induces insulin secretion stimulated by glucose (Wollheim and Pozzan 1984). Quantitatively, the most important source of Ca^{2+} for insulin secretion is the extracellular compartment. Cytosolic Ca^{2+} levels may be increased by mechanisms involving glucose metabolism, followed by an increase in the intracellular ATP/ADP ratio, closure of K^+_{ATP}-channels with consequent depolarisation of the cell membrane and the opening of voltage-dependent calcium channels (Hellman et al.1992).

In certain situations, insulin secretion can occur in the absence of extracellular Ca^{2+}, perhaps through the mobilisation of internal Ca^{2+} and consequent increase in the cytoplasmic levels of Ca^{2+} (Aizawa et al. 1994; Mears et al. 1999). Insulin secretion may also occur in the absence of an apparent increase in cytosolic Ca^{2+} concentrations through the activation of PKC or PKA involving GTP (Boschero et al. 1995; Komatsu et al. 1995, 1997a, 1997b, 1998a). Free fatty acids such as palmitate and myristate also stimulate insulin release in the absence of extracellular Ca^{2+} (Komatsu et al. 1998b).

We have reported that tetracaine can stimulate insulin secretion even in the absence of extracellular Ca^{2+}. Tetracaine can also alter the Ca^{2+} handling in isolated islets, leading to an augmentation of the efflux of the cation in the presence as well in the absence of external Ca^{2+} and at high (22 mM) or low (2.8 mM) glucose (Bordin et al. 1997).

Although the physiological effects of local anaesthetics are well known, their mechanism of action is not yet fully understood. In this work, we have characterised further the effects of tetracaine on insulin secretion and Ca^{2+} mobilisation in isolated islets and B-cells. We demonstrated here that tetracaine stimulates insulin secretion through the mobilisation of Ca^{2+} from thapsigargin- and IP_3-insensitive Ca^{2+} stock in pancreatic B-cells.

MATERIAL AND METHODS

Islet and B-cell isolation

All experiments were performed with islets isolated from the pancreas of fed adult female Wistar rats using the collagenase technique Lacy and Kostianovsky 1967). Isolated B-cells were obtained by dispersing isolated islets in Ca^{2+}-free saline in the presence of 0.5 mM EGTA after a preincubation of 30 min in the same medium. The cells were then incubated for 2 to 4 days in RPMI-1640 medium supplemented with 10% foetal calf serum, 10 mM glucose, 100 IU penicillin/mL, and 100 µg streptomycin/mL in an atmosphere of 5% CO_2. The medium was replaced every 48 h.

Medium

The media used for incubating, washing or perifusing the islets and B-cells consisted of a Krebs-Ringer bicarbonate-buffered solution (pH 7.4, 37°C) having the following composition (in mM): NaCl 115, KCl 5, $CaCl_2$ 2.6, $MgCl_2$ 1, $NaHCO_3$ 24 and glucose 2.8, supplemented with 0.3% albumin (w/v) and aerated with a mixture of 95%O_2/5%CO_2. In some cases, nominally Ca^{2+} free media was supplemented with 0.5 mM EGTA [ethylene glycol-*bis*-(β-aminoethyl ether)- *N,N'*-tetraacetic acid]. The media also contained glucose, tetracaine, and thapsigargin when necessary.

Tetracaine, EGTA, carbachol and D-600 were from Sigma Chem. Co., St. Louis, USA; RPMI, penicillin and streptomycin from Gibco, BRL; Fura 2 from Molecular Probes, Eugene, USA; radioactive (^{45}Ca) from NEN Life Sciences, Boston, USA.

Dynamic insulin secretion

Insulin secretion in dynamic conditions was determined as described elsewhere (Helchuelz and Malaisse 1980). Briefly, groups of 20 islets were transferred to chambers and perifused with the desired solutions after an adaptation period of 30 min. The effluent was collected every 2 min and the insulin content measured by radioimmunoassay (Scott et al. 1981) using rat insulin as a standard.

^{45}Ca efflux measurements

The method for measurement of ^{45}Ca efflux from perifused islets has been described (Helchuelz and Malaisse 1980). Briefly, groups of 100-120 islets were labelled with $^{45}CaCl_2$ (20 μCi/mL) for 90 min and then washed four times with radioisotope-free medium and transferred to a chamber in which they were perifused for 80 min with the desired solution (see Results). ^{45}Ca efflux was considered to be the fractional outflow rate (*i.e.* as a percentage of the islet content/min).

$[Ca^{2+}]_i$ measurements

Isolated B-cells cultured in RPMI-1640 medium for 2-4 days on round glass coverslips were incubated with fura-2/AM (2μM) for 60 min at 37° C in Krebs-Ringer bicarbonate buffered solution. The coverslips were transferred to a chamber mounted on an inverted fluorescence microscope (Diaphot TDM, Nikon, Tokyo, Japan) for epifluorescence. Fura-2 fluorescence of single cells was measured by dual-excitation fluorimetry using a camera-based image analysis system (Magical of Applied Imaging Ltd, U.K.). The excitation and emissions wavelengths used were respectively 340/380 and 510 nm, and a pair of ratioable images (at the excitations of 340 and 380 nm, 30 msec interval) were taken every 2.5 s. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) was calculated from the ratios of the 340 and 380 nm signals using the equation:

$$[Ca^{2+}]i = Kd \times \frac{(R - Rmin)}{(Rmax - R)} \times \frac{Sf_2}{Sb_2}$$

were Kd is the dissociation constant for the Fura 2- Ca^{2+} complex (224 nM at 37°C), R is the ratio of fluorescence at 340 over 380 nm, Sf_2 is the fluorescence of free dye measured at 380 nm and Sb_2 is the fluorescence of bound dye measured at 380 nm. Rmax, Rmin and Sf_2/Sb_2 were determined in separate experiments by recording the fluorescence of fura 2 (free form) in the absence of extracellular Ca^{2+} or in the presence of saturating Ca^{2+} concentration (Van Eylen et al. 1998).

Data analysis

Except for the $[Ca^{2+}]_i$ measurements, the results are expressed as the mean ± S.E. of *n* experiments. The statistical significance of the differences between data was assessed by using Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Dynamic insulin secretion measurements showed that glucose (16.7 mM) increased insulin secretion only in the presence of external Ca^{2+} (Fig.1, filled circles). In contrast, tetracaine (2 mM) induced a transient monophasic insulin secretion in the absence or presence of external Ca^{2+}. Maximal release was achieved 18 min after the addition of tetracaine to the perifusion medium in the absence or presence of external Ca^{2+} . The calculated area under the curves was

64.33 ± 5.31 (n = 4) in the presence and 95.13 ± 1.54 (n = 4) in the absence of extracellular Ca^{2+} ($p < 0.05$ compared to the respective control values before the addition of tetracaine).

Tetracaine (2 mM) significantly increased the $[Ca^{2+}]_i$ in isolated B-cells perifused with Ca^{2+}-free medium containing 25 µM D-600. This effect was sustained and partially reversible (Fig. 2A). Tetracaine (2 mM) also significantly increased the $[Ca^{2+}]_i$ in Ca^{2+}-free medium containing either 10 mM caffeine (Fig. 2B) or 1 µM thapsigargin (Fig. 2C).

In another series of experiments, we used ^{45}Ca as a tracer to analyse the velocity of tetracaine action and also the Ca^{2+} pool affected by this anaesthetic. Figure 3 (A, B) shows that tetracaine (2 mM) increased ^{45}Ca efflux from perifused islets in the absence or presence of external Ca^{2+}. Tetracaine was present from the onset of the perifusion and the maximal effluxes were observed after 16 min and 18 min of perifusion in the absence and presence of external Ca^{2+}, respectively. The integrated area under the curve in the presence of external Ca^{2+} was significantly greater than that obtained in the absence of the cation. Figure 4 (A-D) shows the effect of increasing concentrations of tetracaine on the ^{45}Ca efflux from perifused islets. Concentrations of tetracaine as low as 0.1-0.2 mM increased the ^{45}Ca efflux rate from perifused islets.

Increasing the K^+ concentration from 5 mM to 50 mM in the presence of tetracaine (2 mM) in Ca^{2+}-free medium, produced a significant and transient increase in the ^{45}Ca efflux from perifused islets. The addition of 0.2 mM Cch in the

presence of 50 mM K^+, provoked an additional increase in the ^{45}Ca efflux rate (Fig. 5).

DISCUSSION

Insulin secretion induced by nutrients depends on a rise in $[Ca^{2+}]_i$. Most of this Ca^{2+} comes from the extracellular medium and enters B-cells through L-type channels following membrane depolarisation (Prentki and Matschinsky 1987). A small fraction of the Ca^{2+} involved in the insulin secretion mechanism come from IP_3-sensitive stores (Liu et al. 1998). Although the main insulinotropic effect of IP_3 is related to cholinergic activation (Boschero et al. 1995; Bordin et al. 1995), glucose may also have a stimulatory effect on IP_3 generation in pancreatic B-cells (Rasmussen et al. 1995). IP_3-dependent Ca^{2+} mobilisation accounts for about 30% to 50% of the total Ca^{2+} sequestered into internal stores (Nilsson et al. 1987; Tengholm et al. 1998), indicating that B-cells possess both IP_3-sensitive and IP_3-insensitive Ca^{2+} pools. Interestingly, a Ca^{2+}-independent pathway for insulin release has been reported when protein kinase C and protein kinase A, which modulate B-cell function, are stimulated (Komatsu et al. 1995, 1997a, 1997b, 1998a). This mechanism seems to play a minor role in the physiological regulation of insulin secretion (Sato et al. 1998).

In the present work, we used the local anaesthetic tetracaine to further explore the participation of intracellular Ca^{2+} in the mechanism of insulin secretion. The findings regarding the effect of tetracaine on insulin secretion are

controversial (Freinkel et al. 1975; El Motal et al. 1987; Norlund and Sehlin 1983; Bolaffi et al. 1993), partly because of the different methodologies used in various studies. Thus, insulin secretion was inhibited in islets incubated for long periods of time (Freinkel et al. 1975) or during the stimulation of secretion in dynamic studies (El Motal et al. 1987; Norlund and Sehlin 1983). In static incubations (60 min) and in the presence of 22 mM glucose, tetracaine inhibits insulin secretion in the presence of external Ca^{2+} but stimulates secretion in the absence of this cation (Bordin et al. 1997). In dynamic studies, in which the insulin content in the perifusate was measured every 2 min, tetracaine transiently enhanced the insulin secretion induced by 16.7 mM glucose in the absence and presence of physiological $[Ca^{2+}]_o$, thus confirming earlier studies (El Motal et al. 1987; Norlund and Sehlin 1983). Since the total insulin secretion induced by tetracaine in the absence of $[Ca^{2+}]_o$ was significantly higher than that observed in the presence of the cation ($p < 0.05$), some desensitisation of the secretory process provoked by the higher concentration of $[Ca^{2+}]_i$ may occur, as already proposed for parathyroid (Nygren et al. 1987) and B-cells (Hellman et al. 1994).

Tetracaine (0.1-2.0 mM) dose-dependently increased the ^{45}Ca efflux from prelabelled islets, even in Ca^{2+}-free medium, indicating that the anaesthetic mobilises Ca^{2+} from internal stores. This effect was observed at concentrations of tetracaine as low as 0.1-0.2 mM (Figs. 3, 4). The ^{45}Ca efflux results were confirmed by the analysis of cytoplasmic Ca^{2+} concentrations using the specific Ca^{2+} tracer Fura-2. Tetracaine (2 mM) significantly and permanently increased the $[Ca^{2+}]_i$ in isolated B-cells, even in Ca^{2+}-free medium containing D-600, an L-type

Ca^{2+} channel blocker. This effect was rapidly reverted when tetracaine was withdrawn from the perifusion medium, showing that at the concentration used the anaesthetic was not harmful to B-cells. Recent observation that tetracaine blocks L type Ca^{2+} channels in pancreatic B-cell (Mears et al. 1999) lend support that augmentation of ^{45}Ca efflux and $[Ca^{2+}]_i$ are consequence of mobilization of internal Ca^{2+} pool.

To determine the Ca^{2+} pool mobilised by tetracaine we examined the effect of tetracaine on cytoplasmic Ca^{2+} concentrations in the presence of drugs that deplete the endoplasmic reticulum (ER) Ca^{2+} store. ER is functionally the most important Ca^{2+} store in B-cells (Nilsson et al. 1987). Thapsigargin removes more than 90% of the Ca^{2+} that can be mobilised by ionophore A23187 (Tengholm et al. 1998). Fig. 2 (B,C) shows that tetracaine increased the $[Ca^{2+}]_i$ in the presence of 10 mM caffeine or 1 μM thapsigargin, but did not block the increase in ^{45}Ca efflux by agents such as Cch that generate IP_3 (Fig. 5). We can conclude that tetracaine mobilises Ca^{2+} from a thapsigargin- and IP_3-insensitive compartment. Since tetracaine competes with Ca^{2+} and other cations for different sites on biological membranes (Low et al. 1979) it is possible that the anaesthetic increases $[Ca^{2+}]_i$ by mobilising Ca^{2+} from internal membranes. Tetracaine did not block the increase of ^{45}Ca efflux provoked by 50 mM K^+. This effect was observed in the absence of external Ca^{2+} (Fig. 5), and suggests the existence of a tetracaine-insensitive Ca^{2+} pool in islet cells.

In conclusion, tetracaine induced insulin secretion in the absence of external Ca^{2+} by mobilising sufficient Ca^{2+} from thapsigargin- IP_3-, and, possibly,

cation-insensitive stores. The possibility that tetracaine increases $[Ca^{2+}]_i$ by competing with Ca^{2+} by specific sites at internal membranes, including insulin-containing granules, deserves further investigation.

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REFERENCES

Aizawa, T., Sato, Y., Ishihara, F., Taguchi, N., Mitsuhisa, K., Suzuki, N., Hashizume, K. and Yamada, Y. 1994. ATP-sensitive K^+ channel-independent glucose action in rat pancreatic β-cell. Am J. Physiol. **266**: C622-C627.

Berridge, M.J. 1997. Elementary and global aspects of calcium signalling. J. Physiol. **499**: 291-306.

Bolaffi, J.L., Rodd, G.G., Ma, Y.H. and Grodsky, G.M. 1993. Opposite, phase-dependent effects of 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester or tetracaine on islets function during three phases of glucose-stimulated insulin secretion. Endocrinology **132**: 2325-2331.

Bordin, S., Boschero, A.C., Carneiro, E.M. and I. Atwater, I. 1995. Ionic mechanisms involved in the regulation of insulin secretion by muscarinic agonists. J. Membr. Biol. **148**: 177-184.

Bordin, S., Carneiro E.M., Bosqueiro J.R. and Boschero, A.C. 1997. Tetracaine stimulates extracellular Ca^{2+}-independent insulin release. Eur. J. Pharmacol. **327**: 257-262.

Boschero, A.C., Szpak-Glasman, M., Carneiro, E.M., Bordin, S., Paul, I., Rojas, E. and Atwater, I. 1995. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M_3 muscarinic receptors. Am. J. Physiol. **268**: E336-E342.

El Motal, S.M.A.A., Pian-Smith, M.C.M. and Sharp, G.W.G. 1987. Effects of tetracaine on insulin release and calcium handling by rat pancreatic islets. Am. J. Physiol. **252**: E727-E733.

Freinkel, N., El Younsi, C. and Dawson, R.M.C.1975. Inter-relations between the phospholipids of rat pancreatic islets during glucose stimulation and their response to medium inositol and tetracaine. Eur. J. Biochem. **59**: 245-252.

Hellman, B., Gylfe, E., Bergsten, P., Grapengiesser, E., Lund, P.E., Berts, A., Dryselius, S., Tengholm, A., Liu, Y.J., Eberhardson, M. and Chow, R.W. 1994. The role of Ca^{2+} in the release of pancreatic islet hormones. Diabete Metab. **20**: 123-131.

Hellman, B., Gylfe, E., Grapengiesser, E., Lund, P.E. and Marcström, A. 1992. Cytoplasmic calcium and insulin secretion. *In*: Nutrient Regulation of Insulin Secretion. *Edited by* P. R. Flatt. Portland Press, London, UK. pp. 213-245.

Herchuelz, A. and Malaisse, W.J. 1980. Regulation of calcium fluxes in pancreatic islets: two calcium movements' dissociated response to glucose. Am. J. Physiol. **238**: E87-E95.

Komatsu, M., Schermerhorn, T., Aizawa, T. and Sharp, G.W.G. 1995. Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in the absence of any increase in intracellular Ca^{2+} in rat pancreatic islets. Proc. Natl. Acad. Sci. USA **92**: 10728-10732.

Komatsu, M., Schermerhorn, T., Noda, M., Straub, S. G., Aizawa, T. and Sharp, G.W.G. 1997a. Augmentation of insulin release by glucose in the absence of extracellular Ca^{2+}. New insights into stimulus-secretion coupling. Diabetes **46**: 1928-1938.

Komatsu, M., Sharp, G. W. G., Aizawa, T. and Hashizume, K. 1997b. Glucose stimulation of insulin release without an increase in cytosolic free Ca^{2+} concentration: a possible involvement of GTP. Jpn. J. Physiol. **47**: S22-S24.

Komatsu, M., Noda, M. and Sharp, G.W.G. 1998a. Nutrient augmentation of Ca^{2+}-dependent and Ca^{2+}-independent pathways in stimulus-coupling to insulin secretion can be distinguished by their guanosine triphosphate requirements:studies on rat pancreatic islets. Endocrinology **139**: 1172-1183.

Komatsu, M. and Sharp, G.W.G. 1998b. Palmitate and myristate selectively mimic the effect of glucose in augmenting insulin release in the absence of extracellular Ca^{2+}. Diabetes **47**: 352-357.

Lacy, P. E. and Kostianovsky, M. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes **16**: 35-39.

Liu, Y.J., Tengholm, A., Grapengiesser, E., Hellman, B. and Gylfe, E.1998. Origin of slow and fast oscillations of Ca^{2+} in mouse pancreatic islets. J. Physiol. **508**: 471-481.

Low, P.S., Lloyd, D.H., Stein, T.M. and Roger III, J.A. 1979. Calcium displacement by local anaesthetics. Dependence on pH and anaesthetic charge. J. Biol. Chem. **254**: 4119-4125.

Mears, D., Leighton, X., Atwater, I. and Rojas, E. 1999. Tetracaine stimulates insulin secretion from the pancreatic β-cell by release of intracellular calcium. Cell Calcium 1999, **25**: 59-68.

Nilsson, T., Arkhammar, P., Hallberg, A., Hellman, B. and Berggren, P.-O. 1987. Characterisation of the inositol 1,4,5-triphosphate-induced Ca^{2+} release in pancreatic B-cells. Biochem. J. **248**: 329-336.

Norlund, L. and Sehlin, J. 1983. Effect of tetracaine and lidocaine on insulin release in isolated mouse pancreatic islets. Biochim. Biophys. Acta **763**: 197-204.

Nygren, P., Larsson, R., Lindh, E., Ljunghall, S., Ratad, J., Akerström, G. and Gylfe, E. 1987. Bimodal regulation of secretion by cytosolic Ca^{2+} as evidenced by the parathyroid. FEBS Lett. **213**: 195-198.

Prentki, M. and Matschinsky, F.M. 1987. Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Physiol. Rev. **67**: 1185-1248.

Rasmussen, H., Isales, C.M., Calle, R., Throckmorton, D., Anderson, M., Gasalla-Herraiz, J. and McCarthy, R. 1995. Diacylglycerol production, Ca^{2+} influx, and protein kinase C activation in sustained cellular responses. Endocrine Rev. **16**: 649-681.

Sato, Y., Nenquin, M. and Henquin, J.C. 1998. Relative contribution of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms to the regulation of insulin secretion by glucose. FEBS Lett. **421**: 115-119.

Scott, A.M., Atwater I. and Rojas, E. 1981. A method for simultaneous measurement of insulin release and B-cell membrane potential in single mouse islets of Langerhans. Diabetologia **21**: 470-475.

Tengholm, A., Hagman, C., Gylfe, E. and Hellman, B. 1998. In situ characterisation of nonmitochondrial Ca^{2+} stores in individual pancreatic β-cells. Diabetes **47**: 1224-1230.

Van Eylen, F., Lebeau, C., Albuquerque-Silva, J. and Herchuelz, A. 1998. Contribution of Na/Ca exchange to Ca^{2+} outflow and entry in the rat pancreatic beta-cell. Studies with antisense oligonucleotides. Diabetes **47**: 1873-1880.

Wollheim, C. B. and Pozzan, T. 1984. Correlation between cytosolic free Ca^{2+} and insulin release in an insulin-secreting cell line. J. Biol. Chem. **259**: 2262-2267.

FIGURE LEGENDS

Fig. 1. Effect of tetracaine on insulin secretion.

Groups of 25 islets were transferred to a chamber and perifused with Krebs-bicarbonate solution containing 2.8 mM glucose for the initial 40 min, which was replaced with a solution containing 16.7 mM glucose for the remainder of the experiment. Tetracaine (2 mM) was added to the perifusing solution as indicated. Insulin secretion was evaluated in the absence (open cycles) or presence (closed circles) of extracellular Ca^{2+}. Each point represents the mean ± SE of 4 experiments.

Fig. 2. Effect of tetracaine on $[Ca^{2+}]_i$.

Isolated B-cells cultured for 2-4 days were labelled with fura2/AM for 1 h after which the cells were washed extensively. Cells were perifused with medium containing 2.8 mM glucose and D600 (25 µM) in DMSO. Tetracaine (2 mM) alone (panel A), in the presence of 10 mM caffeine (panel B), or in the presence of 1 µM thapsigargin (panel C) was added as indicated. The graphics represents the means of 3 experiments (total of 20-24 cells).

Fig. 3. Effect of tetracaine on the ^{45}Ca fractional outflow rate.

Groups of 100 islets were prelabelled with $^{45}CaCl_2$ for 90 min and then washed four times with non-radioactive medium, transferred to a chamber and perifused for 60 min with medium containing 2.8 mM glucose in the absence (panel A) or

presence (panel B) of 2.56 mM Ca^{2+}. The effluent was collected from the beginning of the perifusion in the absence (open circles) or presence (closed circles) of 2 mM tetracaine added from the onset of the experiment. Each point is the mean ± SE of 4 experiments.

Fig. 4. Effect of increasing concentrations of tetracaine on the ^{45}Ca fractional outflow rate.

Groups of 100 islets were prelabelled with $^{45}CaCl_2$ for 90 min and then washed four times with non-radioactive medium, transferred to a chamber and perifused for 20 min to permit the necessary equilibration for a more stable basal FOR. Then, the islets were perifused for 90 min with a Ca^{2+}-free medium containing 2.8 mM glucose. Tetracaine was added as indicated and maintained until the end of the experiment. Each point is the mean ± SE of 4 experiments.

Fig. 5. Effect of K^+ and carbachol (Cch) on the ^{45}Ca fractional outflow rate.

Groups of 100 islets were prelabelled with $^{45}CaCl_2$ for 90 min and then washed four times with non-radioactive medium, transferred to a chamber and perifused for 20 min to permit the necessary equilibration for a more stable basal FOR. Then, the islets were perifused with a solution containing 2.8 mM glucose and 2 mM tetracaine in the absence of external Ca^{2+}. The increase in K^+ concentration from 5 mM to 50 mM and the inclusion of Cch (0.2 mM) in the perifusion medium are indicated. Each point is the mean ± SE of 4 experiments.

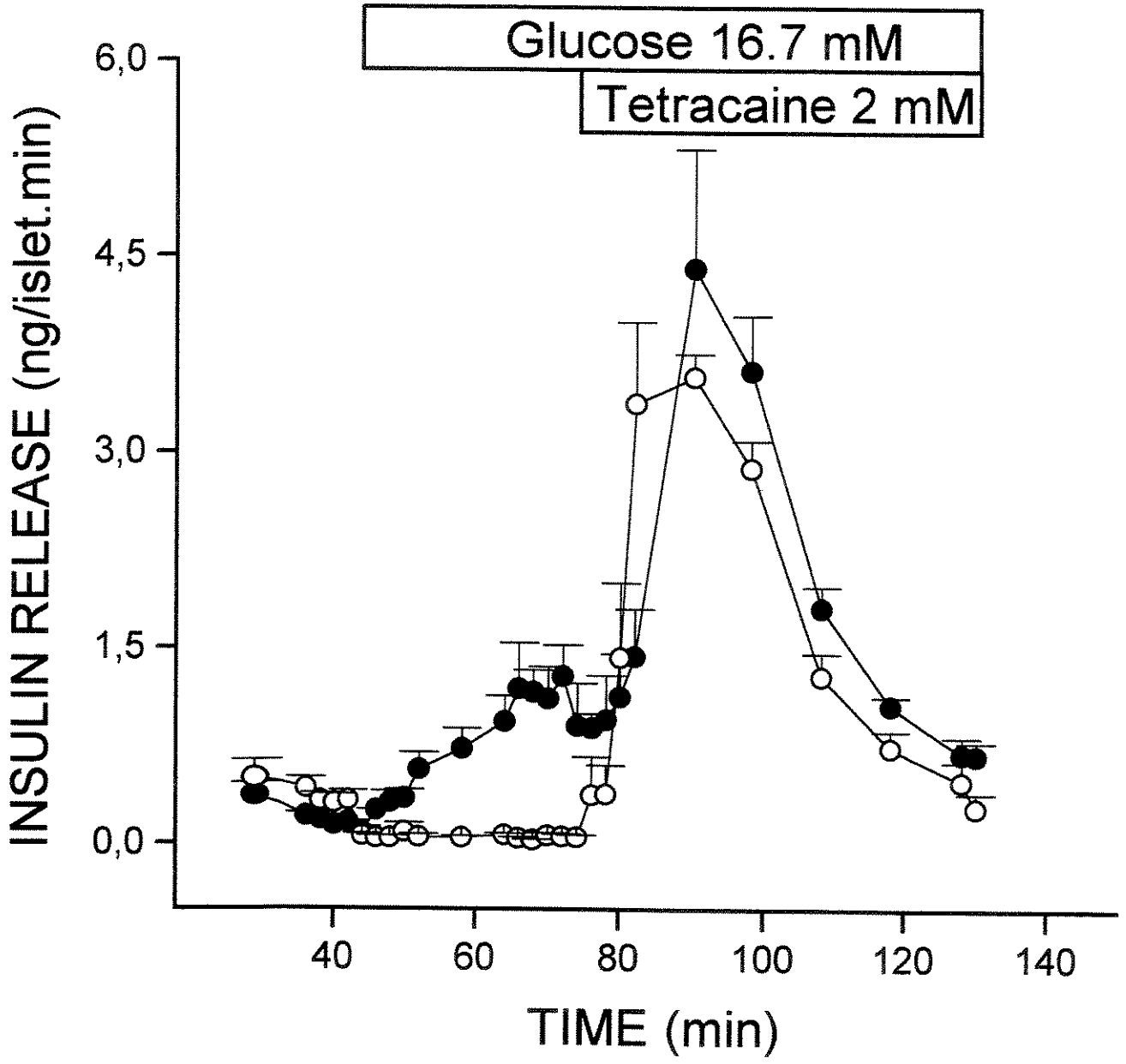

Figure 1. J. R. Bosqueiro, E. M. Carneiro, S. Bordin and A.C. Boschero

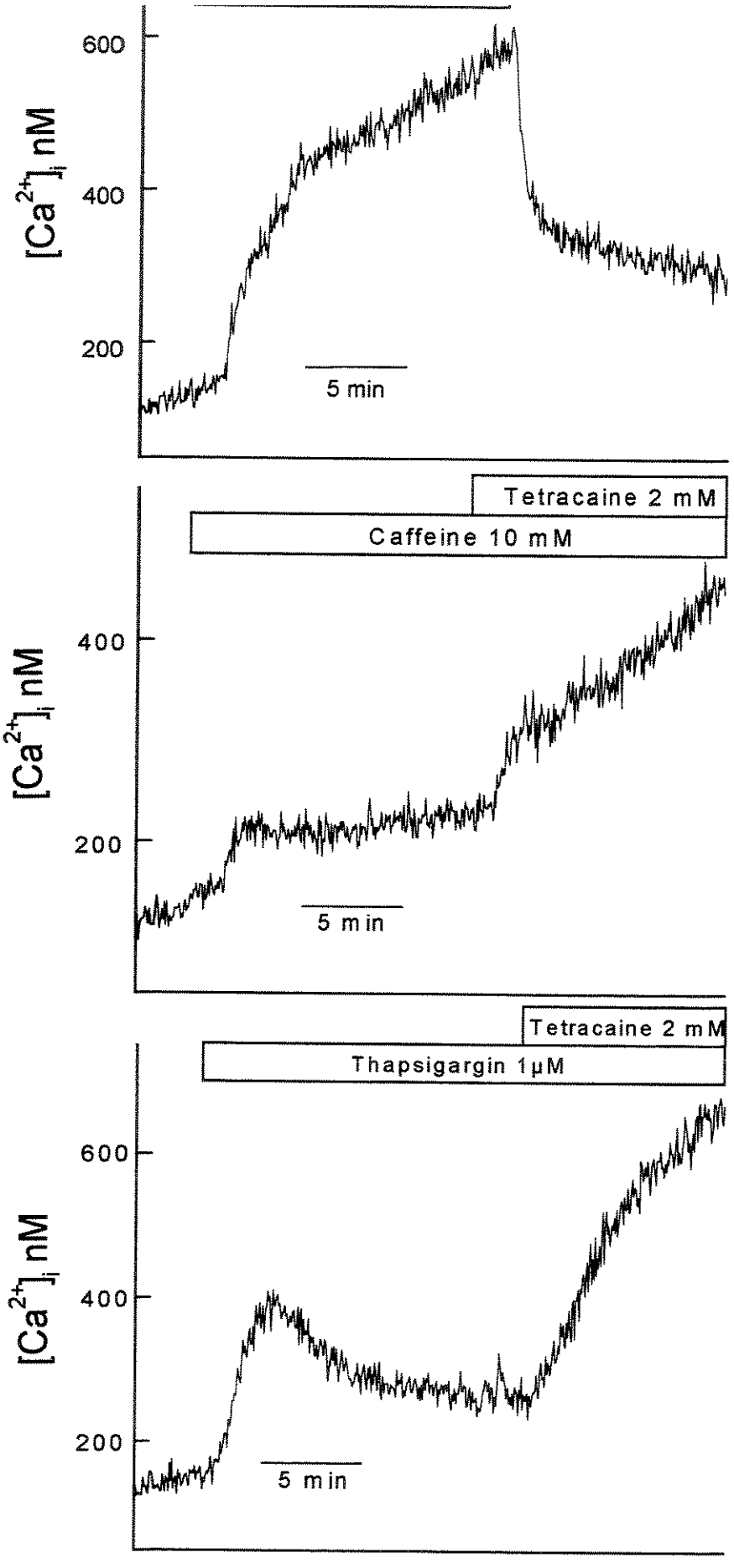

Figure 2. J. R. Bosqueiro, E. M. Carneiro, S. Bordin and A.C. Boschero

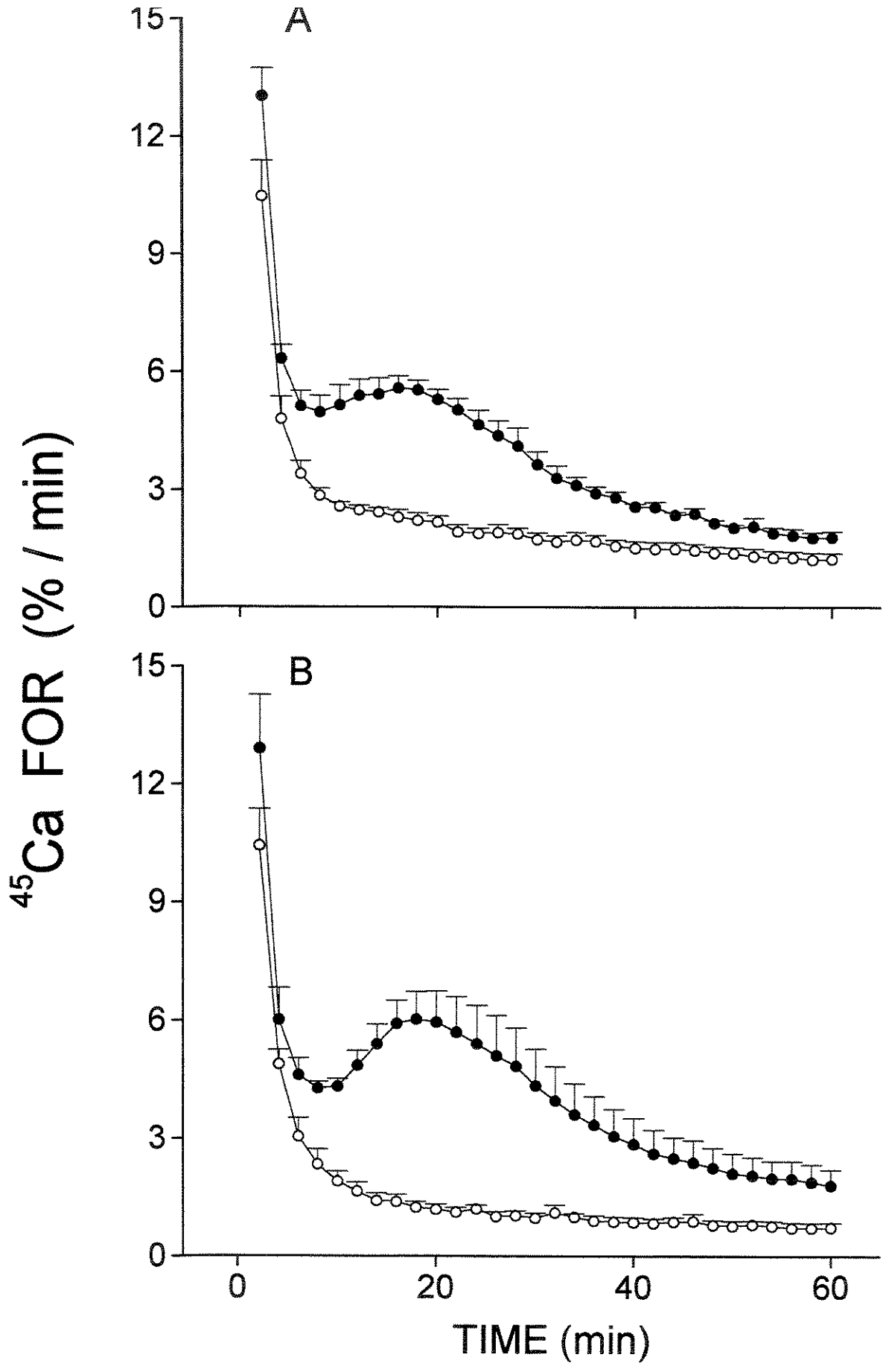

Figure 3. J. R. Bosqueiro, E. M. Carneiro, S. Bordin and A.C. Boschero

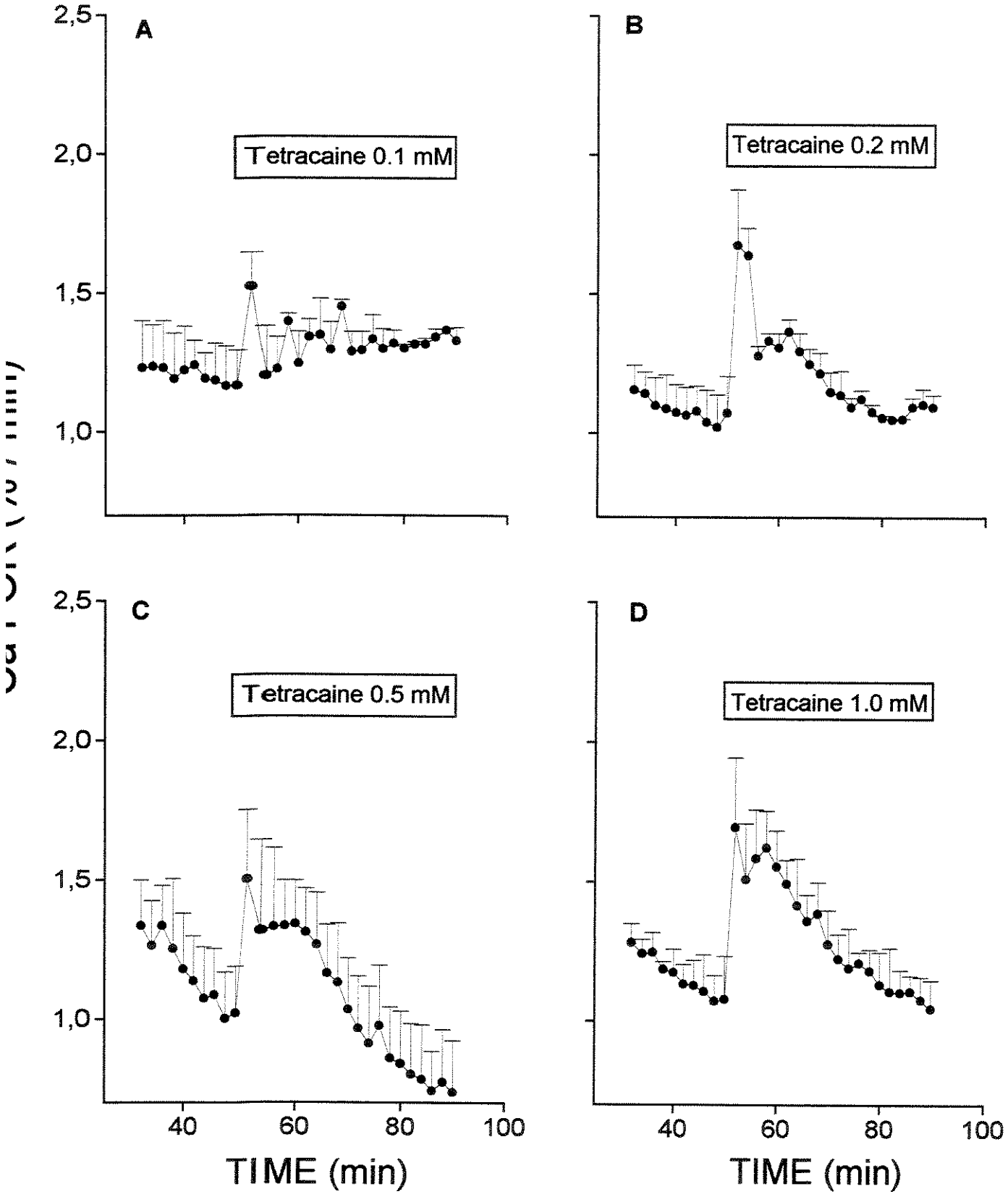

Figure 4. J. R. Bosqueiro, E. M. Carneiro, S. Bordin and A.C. Boschero

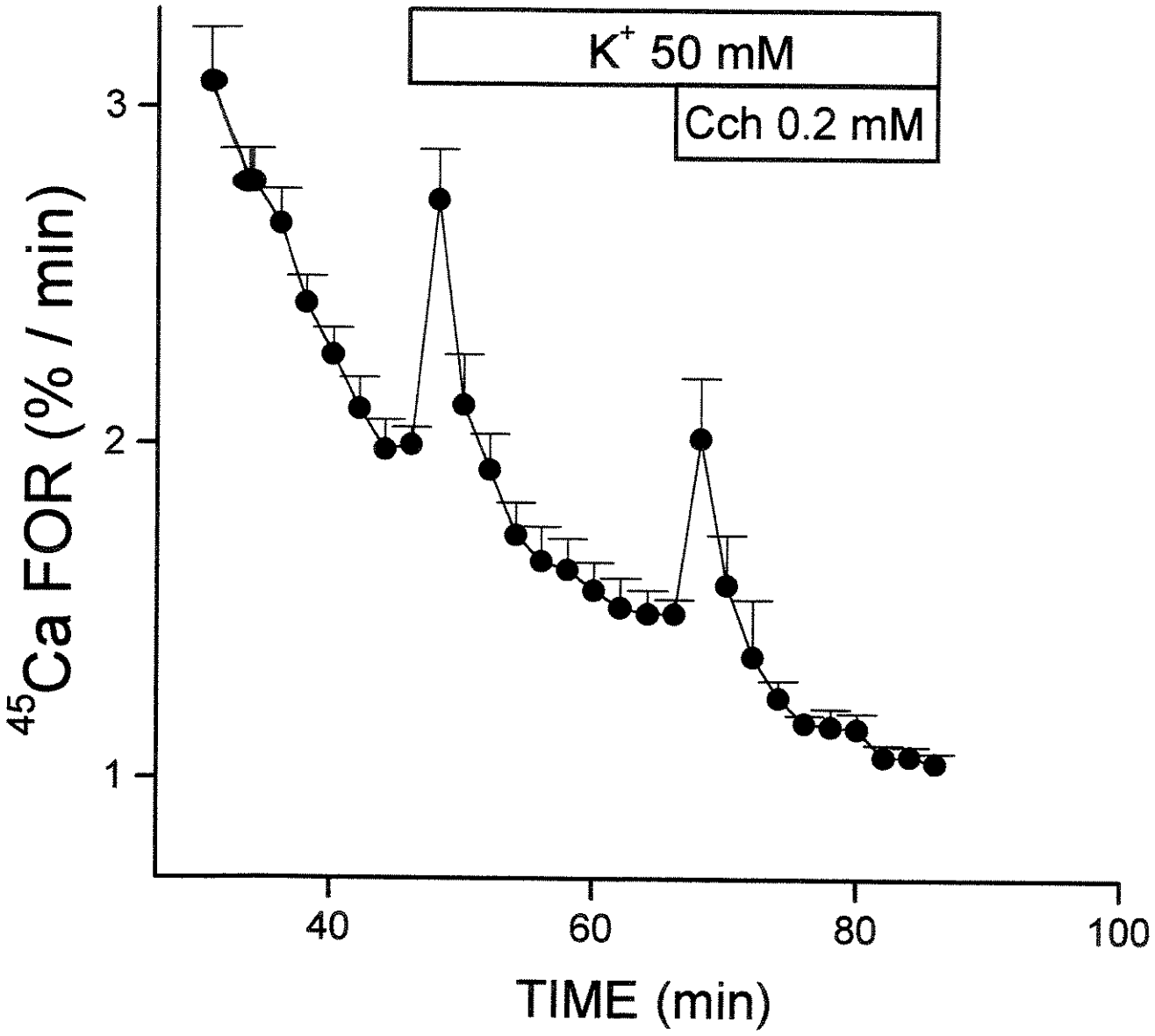

Figure 5. J. R. Bosqueiro, E. M. Carneiro, S. Bordin and A.C. Boschero

CONCLUSÕES

CONCLUSÕES

Nossos resultados indicam que:

- o anestésico local tetracaína estimula a secreção de insulina mesmo na ausência de cálcio extracelular;

- o cálcio responsável pela ativação da secreção, induzida por tetracaína, é oriundo de estoques intracelulares não mobilizados (insensíveis a) pelo IP_3, tapsigargina e cátions. É provável que o aumento das concentrações citoplasmáticas de cálcio seja consequência do deslocamento do cátion de sítios de membrana, provocado pelo anestésico.

BIBLIOGRAFIA

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Ashcroft, F.M.; Harrison, D.E.; Ashcroft, S.J.H. Glucose induces closure of single potassium channels in isolated rat β-cells. **Nature (London)**, **312**: 446-6, 1984.

Bergstron, R.W.; Fujimoto, W.Y.; Teller, D.C.; Haen, C. Oscillatory insulin secretion in perifused isolated rat islets. **Am. J. Physiol.**, **257**: E479-85, 1989.

Berridge, M.J. Elementary and global aspects of calcium signalling. **J. Physiol.**, **499**: 291-306, 1997.

Best, L.; Malaisse, W. J. Nutrient and hormone-neurotransmitter stimuli induce hydrolysis of polyphosphoinositides in rat pancreatic islets. **Endocrinology**, **115**: 1814-20. 1984.

Bolaffi,J. L.; Rodd, G. G.; Ma, Y. H.; Grodsky, G. M. Opposite, phase-dependent effects of 3,4,5-trimetoxybenzoic acid 8-(diethylamino) octyl ester or tetracaine on islet function during three phases of glucose-stimulated insulin secretion. **Endocrinology**, **132**: 2325-31, 1993.

Bordin, S.; Carneiro, E. M.; Bosqueiro, J. R.; Boschero, A. C. Tetracaine stimulates extracellular Ca^{2+}-independent insulin release. **Eur. J. Pharmacol.**, **327**: 257-62, 1997.

Bressler, R.; Brendel, K. Effect of local anesthetics on insulin secretion by pancreas pieces. **Diabetes**, **20**: 721-8, 1971.

Brisson, G. R.; Camu, F.; Malaisse-Lagae, F.; Malaisse, W. J. Effect of local anesthetic upon calcium uptake and insulin secretion by isolated islets of Langerhans. **Life Sci.**, **10**: 445-8, 1971.

Camu, F. Effect of local anesthetics on glucose-stimulated insulin secretion from rat pancreas *in vitro*. **Arch. Int. Pharmacodyn.**, **204**: 368-72, 1973.

Carafoli, E. Intracellular calcium homeostasis. **Ann. Rev. Biochem.**, **56**: 395-433, 1987.

Carafoli, E. Biogenesis plasma membrane calcium ATPase 15 years of work on the purified enzyme. **FASEB J.**, **8**: 993-1002, 1994.

Clark, A.; Carafoli, E. The stoichiometry of the Ca^{2+} pumping ATPase of erytrocytes. **Cell Calcium**, **4**: 83-8, 1983.

Cook, D.L.; Hales, C.N. Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. **Nature (London)**, **311**: 271-3, 1984.

Coronado, R.; Morrissette, J.; Sukhareva, M.; Vaughan, A.D.M. Structure and function of ryanodine receptors. **Am. J. Physiol.**, **266**: C1485-C504, 1994.

Dean, P.M.; Matthews, E.K. Electrical activity in the pancreatic islets cells: effect of ions. **J. Physiol.**, **210**: 265-75, 1970.

Grodsky, G.M.; Bennett, L.L. Cation requirements for insulin secretion in the isolated perifused pancreas. **Diabetes**, **15**: 910-2, 1966.

Gylfe, E. Glucose induced buffering of cytoplasmic Ca^{2+} in the pancreatic B-cell. An artifact or a physiological phenomenon. **Biochem. Biophys. Res. Commun.**, **159**: 907-12, 1989.

Hellman, B.; Gylfe, E.; Grapengiesser, E.; Lund, P.-E.; Marcström. Cytoplasmic calcium and insulin secretion. *In*: **Nutrient regulation of insulin secretion**. Ed. P.R. Flatt. Portland Press. p. 213-46, 1992.

Hellman, B.; Sehlin, J.; Täljedal, I.B. Calcium uptake by pancreatic β-cells as measured with the aid of ^{45}Ca and mannitol-^{3}H. **Am. J. Physiol.**, **221**: 1795-1801, 1971.

Henquin, J.C.; Meissner, H.P. Effects of theophylline and dibutyryl cyclic adenosine monophosphate on the membrane potential of mouse pancreatic β-cells. **J. Physiol. (London), 351**: 595-612, 1984.

Islam, M.S.; Berggren, P.O. Cyclic ADP-ribose and the pancreatic beta cell: where do we stand? **Diabetologia**, **40**: 1480-4, 1997.

Islam, M.S.; Rorsman, P.; Berggren, P.O. Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells. **FEBS Lett.**, **296**: 287-91, 1992.

Johnson, J.H.; Newgard, C.B. The role of glucose transport and phosphorylation in glucose-stimulated insulin secretion. *In*: **Diabetes Mellitus**. Eds. Derek LeRoith; Simeon I. Taylor; Jerrold M. Olefsky. Lippincott-Raven, Philadelphia, New York, pp. 42-9, 1996.

Kakey, A.; Kelly, R.P.; Ashcroft, S.J.H.; Ashcroft, F.M. The ATP-sensitivity of K^+-channels in rat pancreatic B-cells is modulated by ADP. **FEBS Lett.**, **208**: 63-6, 1986.

Kikuchi, M.; Wolheim, C. B.; Siegel E. G.; Renold, A. E.; Sharp, G. W. G. Bifasic insulin release in rat islets of Langerhans and the role of intracellular Ca^{2+} stores. **Endocrinology**, **105**: 1013-19, 1979.

Komatsu, M.; Noda, M.; Sharp, G. W. G. Nutrient augmentation of Ca^{2+}-dependent and Ca^{2+}-independent pathways in stimulus-coupling to insulin secretion can be distinguished by their guanosine triphosphate requirements: studies on rat pancreatic islets. **Endocrinology**, **139**: 1172-83, 1998.

Komatsu, M.; Schermerhorn, T.; Aizawa, T.; Sharp, G. W. G. Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in the absence of any increase in extracellular Ca^{2+} in rat pancreatic islets. **Proc. Natl. Acad. Sci. USA**, **92**: 10728-32, 1995.

Mackrill, J. Protein-protein interactions in intracellular Ca^{2+}-release channel function. **Biochem. J.**, **337**: 345-61, 1999.

Malaisse, W.J.; Malaisse-Lagae, F. The role of cyclic AMP in insulin release. **Experientia**, **40**: 1068-75, 1984.

Malaisse, W.J.; Sener, A.; Herchuelz, A.; Hutton, J.C. Insulin release: the fuel hypothesis. **Metabolism**, **28**: 373-86, 1979.

Malaisse-Lagae, F.; Malaisse, W.J. Stimulus-secretion coupling of glucose-induced insulin release. III. Uptake of ^{45}calcium by isolated islets of Langerhans. **Endocrinology**, **88**: 72-80, 1971.

Mears, D.; Leighton, I.; Atwater, I.; Rojas, E. Tetracaine stimulates insulin secretion from the pancreatic β-cell by release of intracellular calcium. **Cell Calcium**, **25**: 59-68, 1999.

Meglasson, M.D.; Matschinsky, F.M. New perspectives on pancreaic islet glucokinase. **Am. J. Physiol.**, **246**: E1-E13, 1984.

Meissner, H.P.; Schmeltz, H. Membrane potential of beta-cells in pancreatic islets. **Pflugers Arch.**, **351**: 195-206, 1974.

Milner, R.D.G.; Hales, C.N. The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. **Diabetologia**, **3**: 47-9, 1967.

Montague, W.; Morgan, N. G.; Rumford, G. M.; Prince, C. A. Effect of glucose on polyphosphoinositide metabolism in isolated rats islets of Langerhans. **Biochem. J.**, **227**: 483-9, 1985.

Nilsson, T.; Arkhammar, P.; Hallberg, A.; Hellman, B.; Berggre, P.O. Characterization of the inositol 1,4,5-triphosphate-induced Ca^{2+} release in pancreatic β-cells. **Biochem. J.**, **248**: 329-36, 1987.

Norlund, L.; Sehlin, J. Effect of tetracaine and lidocaine on insulin release in isolated mouse pancreatic islets. **Biochim. Biophys. Acta**, **763**: 197-204, 1983.

Norlund, L.; Sehlin, J. Effect of tetracaine and glibenclambideon $^{45}Ca^{2+}$ handling by isolated pancreatic islets. **Br. J. Pharmacol.**, **85**: 127-32, 1985.

Nowatzke, W.; Ramanadham, S.; Ma, Z.; Hsu, F-F.; Bohrer, A.; Turk, J. Mass spectrometric evidence that agents that cause loss of Ca^{2+} from intracellular compartments induce hydrolysis of arachidonic acid from pancreatic islets

membrane phospholipids by a mechanism that does not require a rise in cytosolic Ca^{2+} concentration. **Endocrinology**, **139**: 4073-85, 1998.

Okamoto, H.; Takasawa, S.; Nata, K. The CD38-cyclic ADP-ribose signalling sistem in insulin secretion: molecular basis and clinical implications. **Diabetologia**, **40**: 1485-91, 1997.

Prentki, M.; Matschinsky, F.M. Ca^{2+}, cAMP, and phospholip-derived messengers in coupling mechanisms of insulin secretion. **Physiol. Rev.**, **67**: 1185-1248, 1987.

Randriamampita, C.; Tsien, R. Y. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. **Nature**, **364**: 809-14, 1993.

Roe, M. W.; Campbell, G.; Spencer, B.; Lancaster, M. E.; Worley, J. F.; Dukes, I. D. Glucose transiently activates cytosolic Ca^{2+} release in mouse islets by a phospholipase C-dependent mechanism. **Biophys. J.**, **68**: A 235 (Abstract), 1995.

Roe, M. W.; Lancaster, M. E.; Mertz, J.; Worley, J. F.; Dukes, I. D. Voltage-dependent intracellular calcium release from mouse islets stimulated by glucose. **J. Biol. Chem.**, **268**: 9953-6, 1993.

Roe, M. W.; Worley, J. F.; Qian, F.; Tamarina, M.; Mittal, A. A.; Dralyuk, F.; Blair, N. T.; Mertz, R. J.; Philipson, L. H.; Dukes, I. D. Characterization of a Ca^{2+} release-activated nonselective cation current regulating membrane potential and $[Ca^{2+}]_i$ oscillations in transgenically derived β-cells. **J. Biol. Chem.**, **273**: 10402-10, 1998.

Rojas, E.; Carroll, P. B.; Ricordi, C.; Boschero, A. C.; Stojilkovic, S. S.; Atwater, I. Control of cytosolic free calcium in cultured human pancreatic β-cells occurs by external calcium-dependent and independent mechanisms. **Endocrinology**, **134**: 1771-81, 1994.

Santos, R.M. Rosario, L.M.; Nadel, A.; Garcia-Sancho, J.; Soria, B.; Valdeolmillos, M. Widespread synchronous $[Ca^{++}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. **Pflugers Arch.**, **418**: 417-22, 1991.

Sato, Y.; Aizawa, T.; Komatsu, M.; Okada, N.; Yamada, T. Dual functional role of membrane depolarization/Ca^{2+} influx in rat pancreatic β-cell. **Diabetes**, **41**: 438-43, 1992.

Sato, Y.; Nenquin, M.; Henquin, J. C. Relative contribution of Ca^{2+}-dependent and Ca^{2+}-independent mechanisms to the regulation of insulin secretion by glucose. **FEBS Lett.**, **421**: 115-9,1998.

Sener, A; Malaisse, W.J. Stimulation by D-glucose of mitochondrial oxidative events in islets cells. **Biochem. J.**, **246**: 89-95, 1987.

Sussman, K.E.; Leitner, J.W.; Draznin, B. Cytosolic free-calcium concentrations in normal pancreatic islets cells. **Diabetes**, **36**: 571-7, 1987.

Tengholm, A.; Hagmen, C.; Gylfe, E.; Hellman, B. *In situ* characterization of nonmitochondrial Ca^{2+} stores in individual pancreatic β-cells. **Diabetes**, **47**: 1224-30, 1998.

Tjalve, H.; Popov, D.; Slanina, P. Effect of the local anesthetics procaine and lidocaine on insulin secretion from rabbit pancreas pieces. **Horm. Metab. Res.**, **6**: 106-10, 1974.

Turk, J.; Wolf, B. A.; McDaniel, M. L. Glucose-induced accumulation of inositol triphosphates in isolated pancreatic islets. Predominance of the 1,3,4-isomer. **Biochem J.**, **237**: 259-63, 1987.

Valverde, I.; Garcia-Morales, P.; Ghiglione, M.; Malaisse, W.J. The stimulus-secretion coupling of glucose-induced insulin release. III. Calcium dependency of the cyclic AMP response to nutrient secretagogue. **Horm. Metab. Res.**, **15**: 62-8, 1983.

Valverde, I.; Malaisse, W.J. Calmodulin and pancreatic B-cell function. **Experientia**, **40**: 1061-8, 1984.

Valverde, I.; Vandemeers, A.; Anjeneyulu, R.; Malaisse, W.J. Calmodulin activation of adenylate cyclase in pancreatic islets. **Science**, **206**: 225-7, 1979.

Van Eylen, F.; Lebeau, C.; Albuquerque-Silva, J.; Herchuelz, A. Contribution of Na/Ca exchange to Ca^{2+} outflow and entry in the rat pancreatic β-cell. Studies with antisense oligonucleotides. **Diabetes**, **47**: 1873-80, 1998.

Wilson, J.R.; Ludowyke, R.I.; Biden, T.J. Nutrient stimulation results in rapid Ca^{2+}-dependent threonine phosphorylation of myosin heavy chain in rat pancreatic islets and RINm5F cells. **J. Biol. Chem.**, **273**: 22729-37, 1998.

Worley, J. F.; McIntyre, M.; Spencer, B.; Dukes, I. D. Depletions of intracellular Ca^{2+} stores activates a maitotoxin-sensitive non-selective cationic current in β-cells. **J. Biol. Chem.**, **269**: 32055-8, 1994a.

Worley, J. F.; McIntyre, M.; Spencer, B.; Mertz, R. J.; Roe, M. W.; Dukes, I. D. Endoplasmic reticulum calcium store regulates membrane potential in mouse islets β-cells. **J. Biol. Chem.**, **269**: 14359-62, 1994b.

APÊNDICE

Canais de K^+ Sensíveis ao ATP e a Secreção de Insulina: Aspectos Fisiopatológicos da Hiperinsulinemia Familiar

revisão

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RESUMO

Glicose provoca a secreção de insulina através do aumento da relação ATP/ADP no citoplasma das células ß. Isto leva ao bloqueio de canais de K^+ sensíveis ao ATP (K_{ATP}), redução da saída deste cátion da célula, despolarização celular, ativação da permeabilidade ao Ca^{2+} sensível à voltagem, entrada e acúmulo deste cátion nas células e consequente secreção de insulina. O canal K_{ATP} parece ser composto por duas unidades distintas; uma delas, denominada Kir6.2, constitui o canal propriamente dito, por onde fluem as correntes de K^+. A outra é o receptor de sulfoniluréias (SUR1), que é provida de sítios de ligação para o referido fármaco, para ATP, MgADP e diazoxida, atuando como unidade regulatória. Neste artigo, fazemos uma breve revisão da fisiologia dos canais KATP, considerando também sua importância na fisiopatologia do processo secretório. (**Arq Bras Endocrinol Metab** 1998;42/1: 29-35).

Unitermos: Secreção de insulina; Células ß; Canais K_{ATP}; Glicose; Sulfoniluréias; Hiperinsulinemia familiar.

ABSTRACT

Glucose induces insulin secretion in pancreatic ß -cells by increasing the intracellular ATP/ADP ratio. This increase provokes the closure of the ATP-sensitive K^+ channels (K_{ATP}) and depolarises the -cell membrane, opening the voltage-sensitive Ca^{2+}-channels, and raising the intracellular Ca^{2+} concentrations to trigger exocytosis of the insulin containing granules. The K_{ATP} channel consists of a complex of an inward rectifier K^+ channel (Kir6.2) and the sulphonylurea receptor protein (SUR1). Potassium currents flow through Kir6.2, whereas SUR1 acts as a regulatory subunit. SUR1 is equipped with binding sites for ATP, MgADP, sulfonylureas and diazoxide. We have shortly reviewed here the physiology of the K_{ATP} channel also considering its possible role in the physiopathology of the insulin secretion mechanism. (**Arq Bras Endocrinol Metab** 1998;42/1: 29-35).

Keywords: Insulin secretion; ß-cell; K_{ATP} channels; Glucose; Sulphonylureas; Familial hyperinsulinemia.

A MANUTENÇÃO DE NÍVEIS adequados de nutrientes circulantes, em especial da glicose, é fundamental para a sobrevivência do homem. Em indivíduos normais, o nível de glicose é continuamente ajustado entre 4 e 5 mM, evitando-se, dessa maneira, grandes flutuações, sabidamente prejudiciais ao organismo. Essa regulação perfeita depende, durante o jejum, da ação de vários hormônios contra-regulatórios e, no estado alimentado, da insulina. Falhas que provocam redução da secreção de insulina, associadas a alterações da sensibilidade à insulina nos órgãos-alvo, podem induzir o aparecimento do diabetes mellitus (1). Por outro lado, a secreção aumen-

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tada de insulina, tal como ocorre na hiperinsulinemia familiar, pode provocar hipoglicemias indesejadas (2). Nesta revisão, focalizamos a participação dos canais K_{ATP} no mecanismo de secreção de insulina bem como sua possível função na fisiopatologia do processo secretório.

SECREÇÃO DE INSULINA ESTIMULADA POR GLICOSE

A figura 1 mostra as principais etapas da secreção de insulina induzida por glicose nas células ß pancreáticas, com destaque para a função dos canais iônicos no referido processo. Nessas células, o potencial de repouso (-60 a -70 mV) é mantido quase que exclusivamente pelo efluxo de K^+ através de milhares de canais K_{ATP}. Com a entrada de glicose nas células, e sua subsequente metabolização, ocorre aumento intracelular de ATP e redução do ADP. O aumento de ATP, a redução do ADP, ou ainda o aumento da relação ATP/ADP, induz o fechamento dos canais K_{ATP}, provocando despolarização da membrana das células ß, pelo aumento intracelular de K^+. Esta despolarização provoca abertura de canais de Ca^{2+} sensíveis à voltagem (canais L), resultando na entrada e conseqüente acúmulo deste cátion no citoplasma das células ß, finalizando com a extrusão dos

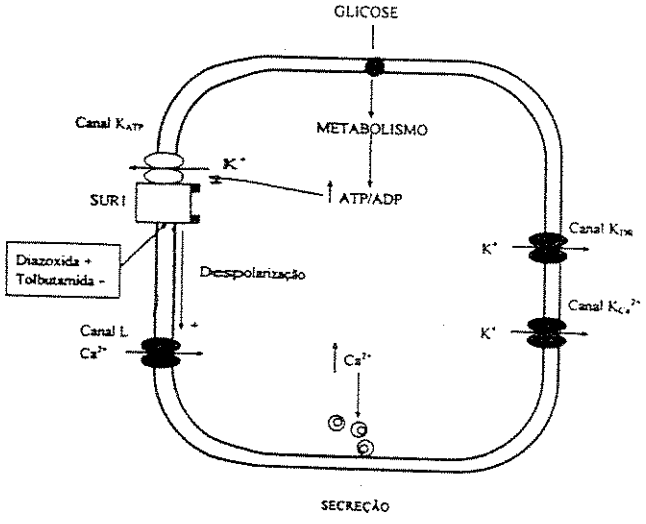

Figura 1 - Esquema simplificado de uma célula pancreática. Na membrana estão identificados os canais iônicos mais importantes para o processo de secreção de insulina. Os sinais + e - que acompanham as setas indicam que as substâncias em questão promovem abertura ou fechamento dos canais, respectivamente. Abreviações: K_{ATP}: Canal de K^+ regulado por ATP; **KDR**: Canal de K+ retificador tardio; **KCa2+**: Canal de K+ ativado por Ca2+; **Canal L**: Canal de **Ca2+** sensível à voltagem; SUR1: Receptor de sulfoniluréia (Adaptado de Boschero AC, ref. 3).

grânulos que contém insulina (secreção). Com a redução da glicose no meio extracelular, o processo se inverte. Ocorre abertura dos canais K_{ATP} e fechamento dos canais de Ca^{2+} (tipo L). Assim, parte substancial do K^+, acumulado durante o período de estímulo, sai das células, pela ativação temporária de pelo menos mais dois tipos de canais de K^+: um sensível à voltagem e outro sensível ao Ca^{2+} (3).

É muito relevante o fato de os canais K_{ATP} serem fechados por sulfoniluréias, tais como tolbutamida e glibenclamida, usadas comumente para tratamento do diabetes tipo 2 e, pelo anestésico tiopental (4-6). Por outro lado, podem ser abertos por derivados das benzotiazinas, como a diazoxida ou, ainda, por um grupo significativo de drogas vasodilatadoras (Cromocalina, SR 44866, SDZ PCO-400, Nicorandil, Pinacidil e RP 49356), o que indica que sua presença não é exclusiva das células pancreáticas (7).

Diante da importância da atuação dos canais K_{ATP} em vários processos fisiológicos, especialmente na secreção de insulina, é justificável o interesse de muitos pesquisadores por conhecer detalhes do seu funcionamento. Aliás, a menor secreção de insulina, provocada pela glicose (ou outros nutrientes), em fetos e recém-nascidos, quando comparado com aquela em adultos, é conseqüência de um número reduzido de canais de K_{ATP} ou da insensibilidade dos mesmos quando do aumento dos níveis de ATP proveniente da metabolização do nutriente (8,9). A figura 2 ilustra as diferenças de efeito da glicose (fig 2A) e da tolbutamida e diazoxida (fig 2B) sobre o efluxo do ^{86}Rb (usado como substituto do ^{42}K) entre ilhotas de ratos adultos e de recém-nascidos. O processo de maturação da resposta secretória à glicose pode ser acelerado, submetendo-se ilhotas fetais ou neonatais ao tratamento crônico com prolactina in vitro (fig 2C) (10).

O CANAL K_{ATP}

O canal K_{ATP} foi primeiramente identificado em miócitos (11). Sua importância como fator essencial de ligação entre o metabolismo celular e o controle do potencial de membrana está amplamente caracterizada, não só no músculo cardíaco, como também no músculo liso e esquelético, neurônios e, logicamente, em ilhotas de Langerhans. (7,11-13). Disseminados amplamente pelos vários tipos de tecidos, os canais K_{ATP} têm como característica comum a modulação por nucleotídeos e por vasta gama de fármacos, alguns já citados. Contudo, a ação desses agentes varia amplamente de tecido para tecido, apresentando inquestionável seletividade. Assim, drogas vasodilatadoras, como cromocalina, nicorandil e

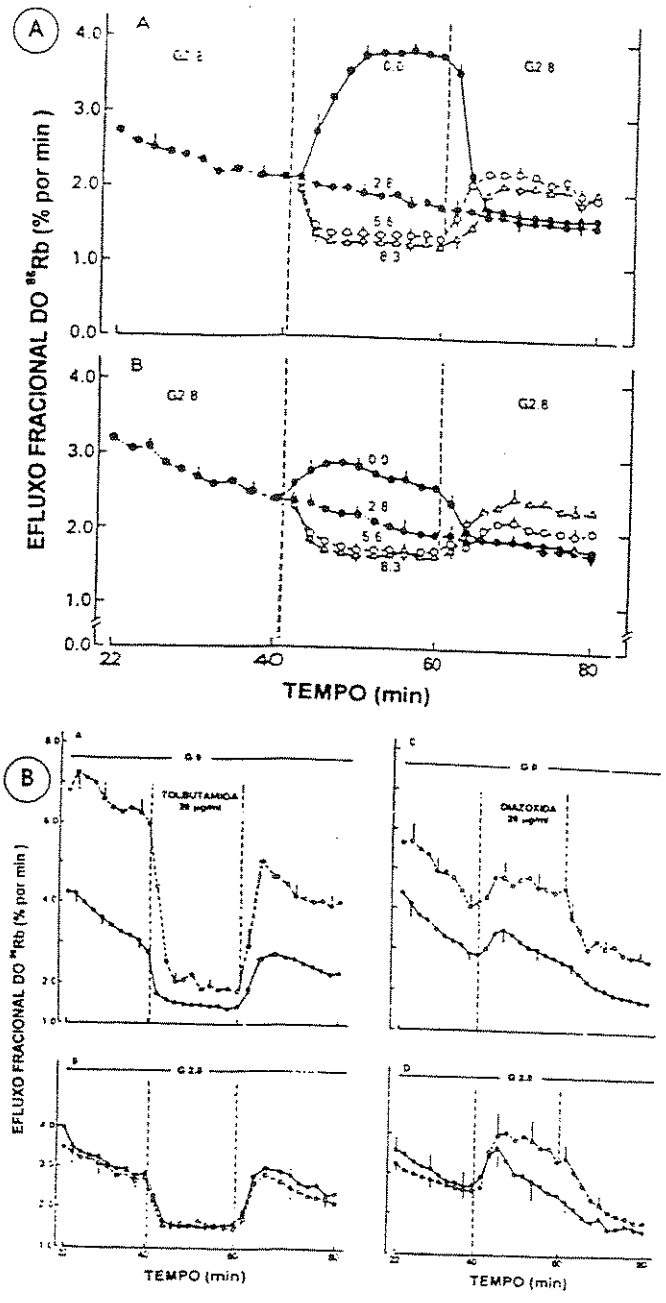

Figura 2 - Efeito da glicose sobre o efluxo do ^{86}Rb de ilhotas de ratos recém-nascidos ou adultos. Após isolamento, as ilhotas (grupos de 100 a 150) foram marcadas com o ^{86}Rb (usado como substituto de ^{42}K) por 90 min, lavadas 4 vezes com meio sem o radioisótopo e perfundidas pelo tempo indicado nas figuras. **A)** Diferença do efeito da glicose sobre o efluxo do ^{86}Rb de ilhotas de ratos recém-nascidos e adultos (Adaptado de Boschero et al., ref. 8). **B)** Diferença do efeito da tolbutamida e diazoxida sobre o efluxo do ^{86}Rb de ilhotas de ratos recém-nascidos e adultos (Adaptado de Boschero et al., ref. 9). **C)** Efeito do tratamento crônico com prolactina sobre a capacidade da glicose para reduzir o efluxo do ^{86}Rb em ilhotas de ratos recém nascidos (Adaptado de Boschero et al., ref. 10).

pinacidil, são muito potentes em alterar o funcionamento dos canais K_{ATP} no músculo liso, mas pouco efetivas no músculo cardíaco. Diazoxida, por sua vez, hiperpolariza a membrana das células ß (mantendo os canais K_{ATP} abertos por mais tempo), enquanto que nos miócitos funciona como bloqueador dos canais K_{ATP} (7).

Recentemente identificou-se uma proteína de membrana, em linhagens de células ß de hamsters e camundongos, com alta afinidade para a sulfoniluréia glibenclamida, a qual recebeu inicialmente a denominação SUR (*sulphonylurea receptor*) (14,15) e, mais recentemente SUR1 (16). Esta proteína, de peso molecular 145 kDa, apresenta 13 segmentos hidrofóbicos que atravessam a membrana plasmática, além de possuir dois sítios intracelulares com alta afinidade por nucleotídeos. Sua seqüência de aminoácidos indica que, juntamente com as proteínas da fibrose cística (CFTR) e da resistência a multidrogas (MDR), faz parte de uma superfamília de ATPases, denominadas *proteínas de tráfego*.

A membrana de oócitos transfectados com a referida proteína revelou a presença de sítios com alta afinidade pela glibenclamida. Contudo, estudos eletrofisiológicos nessas células não demonstraram correntes iônicas semelhantes àquelas registradas em outras células normalmente equipadas com canais K_{ATP}. Especulou-se, então, que o sistema completo deva ser composto pelo SUR1 e por outra proteína.

Nesse sentido e, em paralelo, uma nova proteína da superfamília de canais de K^+, a Kir6,2 (*potassium inward rectifier*), foi clonada usando-se a *biblioteca* do genoma humano e de uma linhagem de células de camundongos (17,18). A co-expressão da Kir6,2 e da SUR1, em células indiferenciadas COS-1 e COSm6, reconstituiu correntes de K^+ semelhantes àquelas registradas em canais K_{ATP} de células ß(fig. 3). Nessas células transfectadas, glibenclamida inibiu e diazoxida estimulou as correntes iônicas à semelhança do observado em células ß pancreáticas. Contudo, quando expressas individualmente, nenhuma das preparações celulares apresentou corrente elétrica, indicando claramente a necessidade da concorrência dos dois tipos de proteínas, para o funcionamento do canal. Como Kir6,2 não apresenta sítios de ligação para nucleotídeos, é muito provável que essa proteína represente o canal por onde fluem as correntes de K^+,

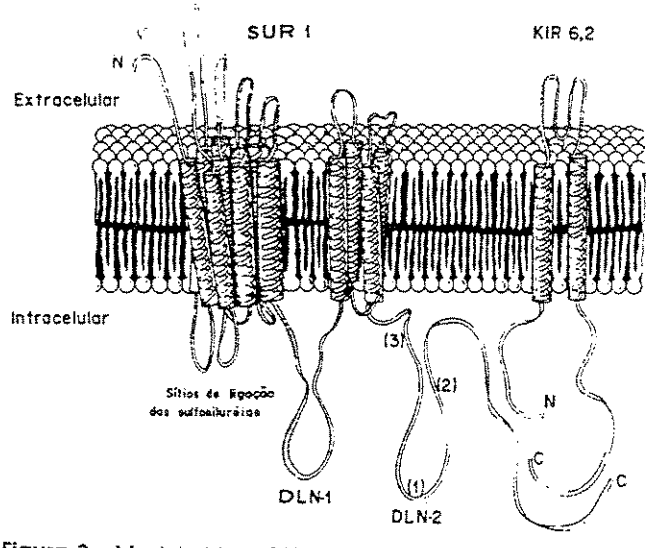

Figura 3 - Modelo hipotético do canal K_{ATP}, que mostra o canal Kir6,2 acoplado à unidade regulatório SUR1 (Adaptação baseada nas refs. 15,40,41). Notar que a unidade regulatória possui os sítios DLN-1 e DLN-2, onde se ligam ATP e MgADP, respectivamente. A região dos sítios de ligação para sulfoniluréias está indicada. Nas células pancreáticas, ATP e sulfuniluréias bloqueiam, enquanto MgADP e diazoxida abrem os canais K_{ATP}. SUR1 possui ainda 20 sítios potenciais para ação da proteína quinase C e três sítios para a proteína quinase A. No modelo, (1), (2) e (3) indicam mutações observadas em indivíduos com HF.

enquanto que o SUR1, com dois sítios internos de alta afinidade por nucleotídeos, constitui a unidade regulatória (17). No homem, essas duas proteínas estão codificadas no cromossomo 11, mais precisamente na posição 11p15.1 (19).

Digno de nota é que 90% dos sítios de ligação para as sulfoniluréias se encontrem na membrana das vesículas que contém os grânulos de insulina (20,21). Isso deixa em aberto várias questões sobre a função dos SUR1 localizados nessas vesículas. Seriam apenas receptores de reserva (*spare receptors*), ou teriam alguma função durante o processo da exocitose? Se esta última colocação for verdadeira, qual a função dos íons K^+ no processo de fusão das membranas vesiculares com a membrana plasmática? Finalmente, qual a importância dessas proteínas para o processo secretório, em indivíduos que apresentam mutações nos genes que expressam SUR1 e Kir6,2 (22)?

CONTROLE DOS CANAIS K_{ATP}

Ainda existem controvérsias sobre a maneira pela qual os nucleotídeos afetam a abertura e o fechamento dos K_{ATP}. Primeiro, porque a atividade desses canais é alterada por vários componentes intracelulares, tais como: ácidos graxos não-esterificados, fosfolípides, ésteres da Acil-CoA e nucleotídeos de piridina, cujas concentrações variam com a metabolização da glicose (23,24). Segundo, porque há evidências de que os níveis citoplasmáticos de ATP das células ß praticamente não variam, em concentrações de glicose acima de 5 mM, quando efetivamente ocorre secreção de insulina (25). Contudo, é inquestionável que a aplicação de ATP do lado interno da membrana de células ß provoca fechamento dos canais K_{ATP} (12,13) . É provável que variações locais de ATP, ou da relação ATP/ADP ocorram sem que os métodos bioquímicos atuais sejam capazes de detectá-las (26). A hipótese de que variações dos níveis de MgADP sejam mais importantes que variações do ATP, para as alterações da atividade dos canais K_{ATP}, é bastante atrativa. Isto porque as concentrações intracelulares de ATP nas células insulares estão próximas de 3 µmoles/l, enquanto as de ADP estão em torno de 30 µmoles/l, sendo, portanto, cem vezes menores que as primeiras (26). Como esses dois nucleotídeos exercem ações antagônicas sobre o comportamento dos referidos canais, é lícito esperar-se que variações nas concentrações de MgADP sejam mais facilmente detectadas (27).

Já citamos anteriormente que a unidade regulatória SUR1 possui dois sítios com alta afinidade para nucleotídeos. É interessante o fato do primeiro sítio ter maior afinidade pelo ATP e o segundo pelo MgADP.

Este último funciona em antagonismo com o primeiro sítio, uma vez que a probabilidade de abertura dos canais K_{ATP} diminui com o decréscimo da concentração intracelular de ADP (28). Finalmente, além dos nucleotídeos, discute-se ainda a possibilidade dos canais K_{ATP} serem regulados por equivalentes reduzidos, em especial pelo NAD(P)H, embora evidências concretas sobre o assunto ainda não tenham sido obtidas (26).

Quanto aos fármacos que atuam sobre os canais K_{ATP}, sabe-se que as sulfoniluréias (ex. tolbutamida e glibenclamida), ou seus análogos (como a meglitinida) induzem fechamento desses canais, e aumento da secreção de insulina. Aliás, existe estreita correlação entre a capacidade de ligação destas drogas e a respectiva potência secretória. Em experimentos com patch clamp, a ordem de potência para o fechamento dos canais é: glibenclamida > glipizida > meglitinida > tolbutamida (29). A mesma ordem de potência é obedecida quanto à capacidade destas drogas para inibirem o efluxo do ^{86}Rb de células de insulinoma (30), e quanto à capacidade de ligação em membrana de células secretoras de insulina (30,31). O local de ligação das sulfoniluréias não é o mesmo dos nucleotídeos (ATP ou MgADP) (32), sendo também diferente das substâncias que promovem a abertura dos referidos canais (ex. diazoxida) (33). Tanto as sulfoniluréias quanto a diazoxida são efetivas em alterar o comportamento dos canais K_{ATP}, quando aplicadas fora ou dentro das células. Inclusive, podem atingir seus sítios de ligação através de difusão lateral na fase lipídica da membrana (34). Isso indica que seus respectivos sítios de ligação poderiam estar localizados na parte hidrofóbica da molécula. Concluindo, os canais K_{ATP} são controlados por, pelo menos, quatro diferentes sítios de ligação, quais sejam: um sítio para o ATP (e outros nucleotídeos que fecham os canais), um para os complexos MgADP (e outros nucleotídeos que abrem os canais), outro para sulfoniluréias e, finalmente, um sítio para a diazoxida (35).

ANOMALIAS DOS CANAIS KATP

Diante da grande importância dos canais K_{ATP} para a secreção de insulina, bem como da existência de fármacos hipoglicemiantes que atuam nesses canais e ainda, da possibilidade de mutações no gene que expressa SUR1 e Kir6,2 induzirem alterações no processo secretório, vários pesquisadores têm-se dedicado ao seu estudo. Uma das anomalias, felizmente rara, relacionada com disfunção dos canais K_{ATP} é a hiperinsulinemia familiar (HF), também conhecida por nesidioblastose ou, ainda, hipoglicemia hiperinsulinêmica permanente da infância (*Online Mendelian Inheritance in Man:* 256450) (2,3,36). Em indivíduos portadores de HF, os sintomas da hipoglicemia podem ser aliviados por ingestão de carboidratos, associada ao uso de drogas inibidoras da secreção de insulina, tais como diazoxida e somatostatina. Contudo, o quadro invariavelmente evolui para a necessidade de pancreatectomia parcial ou mesmo total (para revisão ver refs. 2 e 36).

A análise de correntes iônicas em células ß de pacientes com HF identificou potenciais de ação espontâneos e dependentes de Ca^{2+}, contrariamente ao que ocorre em células ß de indivíduos normais, onde se detectaram apenas correntes de saída de K^+. Essas observações claramente indicam que, nas células de indivíduos com HF, os canais de K_{ATP} permanecem fechados por mais tempo provocando despolarização permanente das células ß. Isto, invariavelmente, leva à ativação dos canais de Ca^{2+} (tipo L), acúmulo desse cátion no citosol e conseqüente aumento da secreção de insulina (37). Análise na posição 11p15.1, em famílias portadoras da anomalia, detectou mutações localizadas próximas à seqüência que expressa o sítio de ligação de nucleotídeos na subunidade SUR1, mais precisamente na dobra de ligação do MgADP, indicadas na figura 3 através dos números entre parênteses (38). É sugestiva a observação de que, em células ß de um indivíduo portador de HF, os canais K_{ATP} responderam adequadamente ao ATP mas não ao ADP, cuja ação antagônica (função de abrir os canais) estava significativamente alterada (39). Variações no gene que expressa SUR1 e Kir6,2 em caucasianos portadores de diabetes tipo 2, também foram observadas, indicando que mutações no referido gene poderiam estar associadas não só à HF, como também ao diabetes tipo 2 (40). Essas mutações não se restringem à região que expressa SUR, pois alterações na seqüência que expressa a subunidade Kir6,2 também foram detectadas (41). Como vários resultados experimentais são ainda contraditórios, é necessário cautela quanto à associação das mutações, observadas na região 11p15.1, com as alterações das permeabilidades iônicas. Assim, não significa que as referidas mutações sejam responsáveis diretas pelas alterações dos fluxos iônicos, registradas em células ß de indivíduos com HF. Contudo, é provável que essas mutações, mesmo não sendo diretamente responsáveis pela alteração da função dos canais K_{ATP}, contribuam de alguma forma para tal anomalia.

Concluindo, evidências recentes indicam que o canal K_{ATP}, de fundamental importância para o processo de secreção de insulina, está relacionado com a fisiopatologia da hiperinsulinemia familiar e, provavemente, com o diabetes tipo 2. Assim, do ponto de vista clínico, justifica-se o estudo aprofundado do funcionameto do referido canal, especialmente com vistas

ao desenvolvimento de fármacos mais específicos para o tratamento das várias patologias com ele relacionadas.

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REFERÊNCIAS

1. DeFronzo RA. Pathogenesis of type 2 diabetes: metabolic and molecular implication for identifying diabetes genes. Diabetes Rev 1997;5:177-269.

2. Stanley CA, Baker L. Hyperinsulinemia in infants and children: diagnosis and therapy. Adv Pedriatr 1976;23:315-55.

3. Boschero AC. Acoplamento estímulo-secreção de insulina pelas células B pancreáticas. Arq Bras Endocrinol Metab 1996;40:149-55.

4. Ashcroft SJH, Ashcroft FM. The sulphonylurea receptor. Biochem Biophys Acta 1992;1175:45-9.

5. Boschero AC, Malaisse WJ. The stimulus-secretion coupling of glucose-induced insulin release. The regulation of $^{86}Rb^{+}$ efflux from perifused islets. Am J Physiol 1979;236:E139-46.

6. Gonçalves AA, Dias O, Langone F, Reis LC, Boschero AC. Thiopental inhibits K^{+} permeability of rat and mouse pancreatic B-cells. Eur J Pharmacol 1986;125:119-25.

7. Dunne MJ, Harding EA, Jaggar JH, Ayton BJ, Squires PE. Endogenous and chemical activators of ATP-regulated potassium channels in insulin secreting cells: possible mechanisms and physiological significance. In: Flatt PR, Lenzen S, eds. Frontiers of Insulin Secretion and Pancreatic B-cell Research. Smith-Gordon & Nishimura Co. 1994:153-9.

8. Boschero AC, Tombaccini D, Atwater IJ. Effects of glucose on insulin release and ^{86}Rb permeability in cultured neonatal and adult rat islets. FEBS Lett 1987;236:375-9.

9. Boschero AC, Tombaccini D, Carneiro EM, Atwater IJ. Differences in K^{+} permeability between cultured adult and neonatal rat islets of Langerhans in response to glucose, tolbutamide, diazoxide and theophylline. Pancreas 8;44-49.

10. Boschero AC, Crepaldi SC, Carneiro EM, Delattre E, Atwater IJ. Prolactin induces maturation of glucose sensing mechanisms in cultured neonatal rat islets. Endocrinology 1993;133:515-20.

11. Noma A. ATP-regulated K^{+} channels in cardiac muscle. Nature 1983;305:147-8.

12. Cook DL, Hales CN. Intracellular ATP directly blocks K^{+}-channels in pancreatic -cells. Nature 1984;312:271-3.

13. Ashcroft FM, Harrison DE, Ashcroft SJH. Glucose induces closure of single potassium channels in isolated rat pancreatic -cells. Nature 1983;312:446-8.

14. Aguilar-Bryan L, Nichols CG, Rajan AS, Parker C, Bryan J. Co-expression of sulfonylurea receptors and K_{ATP} channels in hamster insulinoma tumour (HIT) cells. J Biol Chem 1992;267:14934-40.

15. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP IV, Boyd AE III, Gonzalez G, et al. Cloning of the B cell high-affinity sulphonylurea receptor: a regulator of insulin secretion. Science 1995;268:423-6.

16. Inagaki N, Gonoi T, Clement JP, Wang C-Z, Aguilar-Bryan L, Bryan J, et al. A family of sulphonylurea receptor determines the pharmacological properties of ATP-sensitive K^{+} channels. Neuron 1996;16:1011-7.

17. Inagaki N, Gonoi T, Clement JP, Namba N, Inazawa J, Gonzalez G, et al. Reconstitution of IKATP: an inward rectifier subunit plus the sulphonylurea receptor. Science 1995;270:1166-70.

18. Sakura H, Ammala C, Smith PA, Gribble FM, Ashcroft FM. Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic B-cells, brain, heart and skeletal muscle. FEBS Lett 1995;377:338-44.

19. Glacer B, Chiu KC, Anker R, Nestorowicz A, Landau H, Ban-Bassat H, et al. Familial hyperinsulinism maps to chromosome 11p15.1 30 cM centromeric to the insulin gene. Nature Genet 1994;7:185-8.

20. Carpentier JL, Sawano F, Ravazzola M, Malaisse WJ. Internalisation of 3H-glibenclamide in pancreatic islet cells. Diabetologia 1986;29:259-66.

21. Ozanne SE, Guest PC, Hutton JC, Hales CN. Intracellular localisation and molecular heterogeneity of the sulphonylurea receptor in insulin-secreting cells. Diabetologia 1995;38:277-82.

22. Rorsman P. The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. Diabetologia 1997;40:487-95.

23. Eddlestone GT. ATP-sensitive K channel modulation by products of PLA2 action in insulin-secreting HIT cell. Am J Physiol 1995;268:C181-C190.

24. Dunne MJ, Findlay I, Peterson OH. Effects of pyridine nucleotides on the gating of ATP-sensitive potassium channels in insulin secretion cells. J Membr Biol 1988;102:205-16.

25. Ghosh A, Ronner P, Cheong E, Khalid P, Matschinsky FM. The role of ATP and free ADP in metabolic coupling during fuel-stimulated insulin release from islet beta-cells in the isolated perfused rat pancreas. J Biol Chem 1991;266:22887-92.

26. Prentki M, Tornheim K, Corkey BE. Signal transduction mechanisms in nutrient-induced insulin secretion. Diabetologia 1997;40:S32-S41.

27. Kakei M, Kelly RP, Ashcroft SJH, Ashcroft FM. ATP-sensitivity of K^+ channels in rat pancreatic beta-cells is modulated by ADP. FEBS Lett 1986;208:63-6.

28. Hopkins WF, Fatherazi S, Peter-Riesch B, Corkey BE, Cook DL. Two sites for adenosine-nucleotide regulation of ATP-sensitive potassium channels in mouse pancreatic -cells and HIT cells. J Membr Biol 1992;129:287-95.

29. Zünkler BJ, Lenzen S, Männer K, Panten U, Trube G. Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells. Naunyn-Schimiedberg's Arch Pharmacol 1988;337:225-30.

30. Schmid-Antomarchi H, De Weille J, Fosset M, Lazdunski M. The receptor for antidiabetic sulphonylureas control the activity of the ATP-modulated K^+ channel in insulin secreting cells. J Biol Chem 1987;262:15840-4.

31. Gaines KL, Hamilton S, Boyd AE. Characterization of the sulphonylurea receptor on beta cell membranes. J Biol Chem 1988;263:2589-92.

32. Schwanstecher M, Löser S, Rietze I, Panten U. Phosphate and thiophosphate group donating adenine and guanidine nucleotides inhibit glibenclamide binding to membrane from pancreatic islets. Naunyn-Schimiedeberg's Arch Pharmacol 1991;343:83-9.

33. Schwanstecher M, Brandt C, Behrends S, Schaupp U, Panten U. Effects of MgATP on pinacidil-induced displacement of glibenclamide from the sulphonylurea receptor in a pancreatic B-cell line and rat cerebral cortex. Br J Pharmacol 1992;106:295-301.

34. Trube G, Rorsman P, Ohno-Shosaku T. Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic B-cells. Pflügers Arch 1986;407:493-9.

35. Schwanstecher C, Panten U. Interactive control by sulphonylureas and cytosolic nucleotides of the ATP-sensitive K^+ channel in pancreatic B-cells. In Flatt P, Lenzen S, eds. Frontiers of Insulin Secretion and Pancreatic B-cell Research. Smith-Gordon & Nishimura Co.1994:161-6.

36. Permutt MA, Nestorowicz A, Glaser B. Familial hyperinsulinism: an inherited disorder of spontaneous hyperglycemia in neonates. Diabetes Rev 1996;4:347-55.

37. Kane C, Sheperd RM, Squires PE, Johnson PMV, Lames RFL, Milla PJ, et al. Loss of functional K_{ATP} channel in pancreatic ß-cell causes persistent hyperinsulinemic hypoglycaemia of infancy. Nature Med 1996;2:1344-7.

38. Thomas PM, Cote GJ, Wohlk N, Haddad B, Mathew PM, Rabl W, et al. Mutations in the sulphonylurea receptor gene in familial hyperinsulinemic hypoglycemia of infancy. Science 1995;268:426-9.

39. Nichols CG, Shyng SL, Nestorowicz A, Glasser B, Clement JP, Gonzalez G, et al. Adenosine diphosphate as an intracellular regulator of insulin secretion. Science 1996;272:1785-7.

40. Inoue H, Ferrer J, Welling CM, Elbein SC, Hoffman M, Mayorga R, et al. Sequence variants in the sulphonylurea receptor (SUR) gene are associated with NIDDM in Caucasians. Diabetes 1996;45:825-31.

41. Nestorowicz A, Inagaki N, Gonoi T, Schoor KP, Wilson BA, Glaser B, et al. A nonsense mutation in the inward rectifier potassium channel gene, Kir6.2, is associated with familial hyperinsulinism. Diabetes 1997;40:1743-8.

42. Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, et al. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature 1990;346:362-5.

43. Mimura CS, Holbrook SR, Ames GF. Structural model of the nucleotide binding conserved component of periplasmic permeases. Proc Nat Acad Sci USA 1991;88:84-8.

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Tetracaine stimulates extracellular Ca^{2+}-independent insulin release

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Abstract

The effect of the local anesthetic, tetracaine, on ^{45}Ca efflux, cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ and insulin secretion in pancreatic B-cells was studied. At a physiological level of $[Ca^{2+}]_o$, tetracaine (0.1–5 mM) dose-dependently inhibited insulin secretion induced by 22 mM glucose. Paradoxically, at the same glucose concentration but in the absence of external Ca^{2+}, tetracaine dose-dependently increased insulin secretion. At a low glucose level (2.8 mM) tetracaine failed to affect secretion, either in the presence or absence of external Ca^{2+}. At high (22 mM) or low (2.8 mM) glucose, $[Ca^{2+}]_i$ was increased by tetracaine in a dose-dependent manner. Tetracaine (2 mM) also increased the ^{45}Ca efflux from isolated islets. This effect was of the same magnitude at both low and high glucose concentrations, and was independent of the presence of extracellular Ca^{2+}. Finally, tetracaine increased ^{45}Ca efflux from islets perifused in the presence of thapsigargin. In conclusion, our data indicate that tetracaine releases Ca^{2+} from a thapsigargin-insensitive store in pancreatic B-cells. Under suitable experimental conditions, insulin release can be elicited by a $[Ca^{2+}]_o$-independent pathway. The existence of a ryanodine-like Ca^{2+} channel in pancreatic B-cells is proposed.

Keywords: Pancreatic B-cell; Insulin secretion; $[Ca^{2+}]_i$; Tetracaine

1. Introduction

The nature of pancreatic B-cell glucose-sensing is based on two well-defined but not exclusive pathways that couple biochemical and electrical events to insulin release. The first pathway involves the inhibition of K^+ efflux through K_{ATP} channels, which is modulated by glucose metabolism and leads to cell membrane depolarization. The second involves a rise in intracellular Ca^{2+} concentration, as a consequence of membrane depolarization and Ca^{2+} influx through L-type Ca^{2+} channels (Prentki and Matschinsky, 1987). However, in some experimental conditions, these two events can not account for the triggering of insulin secretion. When the membrane potential of B-cells is voltage-clamped with depolarizing agents, e.g., combining glibenclamide or diazoxide with a high $[K^+]_o$ (Gembal et al., 1992; Sato et al., 1992), glucose is still able to stimulate exocytosis. A K_{ATP}-independent hypothesis has therefore been proposed to explain the increased insulin release when the membrane potential is not altered by the presence of glucose (Aizawa et al., 1994). A Ca^{2+}-independent pathway for insulin secretion has also been observed when protein kinase C and protein kinase A, which modulate B-cell function, are stimulated (Komatsu et al., 1995).

The release of Ca^{2+} from intracellular stores seems to play a minor role in B-cell stimulation. Although glucose has a stimulating effect on phosphoinositide (PI) hydrolysis (Rasmussen et al., 1995), the main insulinotropic effect of IP_3 generation is related to cholinergic activation (Wollheim and Biden, 1986; Bordin et al., 1995; Boschero et al., 1995). However, in mouse pancreatic B-cells, IP_3-dependent Ca^{2+} mobilization accounts for about 30% of the total Ca^{2+} sequestered into intracellular stores (Nilsson et al., 1987), indicating that B-cells possess both IP_3-sensitive and IP_3-insensitive intracellular Ca^{2+} pools.

In most excitable cells, the main mechanisms of Ca^{2+} signaling are mediated by the activation of a specific intracellular channel, namely the ryanodine receptor (RyR) (Clapham, 1995; Pozzan et al., 1994). A variety of pharmacological agents that interact with RyR have been used to study biochemical and functional aspects of intracellular Ca^{2+} release (Coronado et al., 1994). Indeed, modulators of RyR such as caffeine and local anesthetics are known to induce Ca^{2+} mobilization and insulin secretion.

In the present study, we used the local anesthetic tetracaine to explore the participation of intracellular Ca^{2+} release in the stimulus-secretion coupling. We observed

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that tetracaine produces a marked increase in the $[Ca^{2+}]_i$, as a result of Ca^{2+} mobilization from an IP_3-insensitive, non-mitochondrial Ca^{2+} store. Furthermore, we found that a tetracaine-sensitive Ca^{2+} pool modulates glucose-induced insulin secretion.

2. Materials and methods

2.1. Islets and B-cell isolation

Islets from fed adult female Wistar rats were isolated by collagenase digestion. To prepare B-cells, islets were dispersed into cells in Ca^{2+}-free saline in the presence of 0.5 mM EGTA followed by preincubation for 30 min in the same medium. Cell viability as assessed by Trypan blue exclusion was about 95%. The cell culture was maintained at 37°C for 2 to 4 days in RPMI-1640 medium with 2 mM glutamine, 10% fetal calf serum, 10 mM glucose, penicillin (100 IU/ml) and streptomycin (100 μg/ml) in an atmosphere of 5% CO_2. The medium was renewed every 48 h. After 2 days the cells were firmly attached to glass coverlips.

2.2. Medium

The medium used in all experiments was a Hepes-bicarbonate buffer containing (in mM): 100 NaCl, 5 KCl, 2.56 $CaCl_2$, 20 Na-Hepes (pH 7.4), 0.5% bovine albumin and different concentrations of glucose and tetracaine (see Results). Ca^{2+}-deprived medium, used in some perifusion experiments, contained 0.5 mM EGTA. The medium was equilibrated with a mixture of 95%O_2 and 5%CO_2.

2.3. Insulin secretion

For insulin secretion, groups of five islets were first preincubated at 37°C in 0.75 ml of the Hepes-buffer containing 5.6 mM glucose. This medium was then replaced with fresh buffer and the islets further incubated for 1 h under various experimental conditions. The insulin content in the supernatant of each sample and the insulin extracted from the islets at the end of the incubation period, were measured by radioimmunoassay as previously described (Scott et al., 1981) using rat insulin as a standard. Insulin release was expressed as a percentage of the total islet insulin content.

2.4. $[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurements were performed as described elsewhere (Rojas et al., 1994) using indo-1 as a cytoplasmic Ca^{2+} indicator. Briefly, isolated B-cells attached to glass coverslips were loaded with indo-1/AM by incubating them for 1–2 h at room temperature in a Na-Hepes bicarbonate medium containing 2 μM indo-1/AM and pluronic acid (0.02%). The coverslips were then transferred to a perifusion chamber and the cells were continuously perifused with identical medium free of indo-1 and pluronic acid. Different concentrations of tetracaine were applied using the same perifusion system. Changes in cytosolic Ca^{2+} were measured by a micro-fluorimetric technique using an excitation wavelength of 355 nm. The resulting fluorescence (F) at 410 and 485 nm was measured continuously. A computer program calculated the fluorescence ratio ($R = F_{410}/F_{485}$), which was converted to $[Ca^{2+}]_i$ using a calibration curve for Ca^{2+}.

2.5. ^{45}Ca measurements

^{45}Ca efflux from perifused islets was performed as previously described (Herchuelz and Malaisse, 1980). Briefly, groups of 100 islets were labeled with $^{45}CaCl_2$ (20 μCi/ml) for 90 min. The islets were then washed four times with a radioisotope-free medium and transferred to a small chamber in which they were perifused for 80 min with medium containing different concentrations of glucose, Ca^{2+}, and tetracaine. ^{45}Ca efflux was expressed as the fractional outflow rate (percentage of islet content per min).

2.6. Data analysis

The data are presented as the means ± S.E. of n experiments. The statistical significance of the differences between means was assessed by analysis of variance followed by Dunnett's test when several experimental groups were compared with the control group. When only two groups were involved, Students' t-test was used. Differences were considered significant at $P < 0.05$.

3. Results

In the presence of physiological $[Ca^{2+}]_o$ and 22 mM glucose, insulin release was dose dependently reduced by tetracaine (0.1–5 mM; solid bars in Fig. 1A). The secretory response was completely blocked at 5 mM of the anesthetic ($P < 0.01$) whereas 50% inhibition was observed at 0.5 mM tetracaine ($P < 0.01$), as previously observed (Freinkel et al., 1975). In contrast, at the same glucose concentration but in the absence of extracellular Ca^{2+}, tetracaine evoked a dose-dependent increase in insulin release (Fig. 1B, solid bars). A two-fold increase above basal was observed at 1 mM tetracaine ($P < 0.05$). Maximal insulin secretion was achieved at 5 mM tetracaine, and was approx. 9-times greater than the basal secretion (4.44 ± 0.68% and 0.48 ± 0.03% of islet insulin content, respectively; $P < 0.01$). No alterations in the secretory response were observed when the medium contained a low glucose concentration (2.8 mM), either in the presence or in the absence of extracellular Ca^{2+} (open bars in Fig. 1).

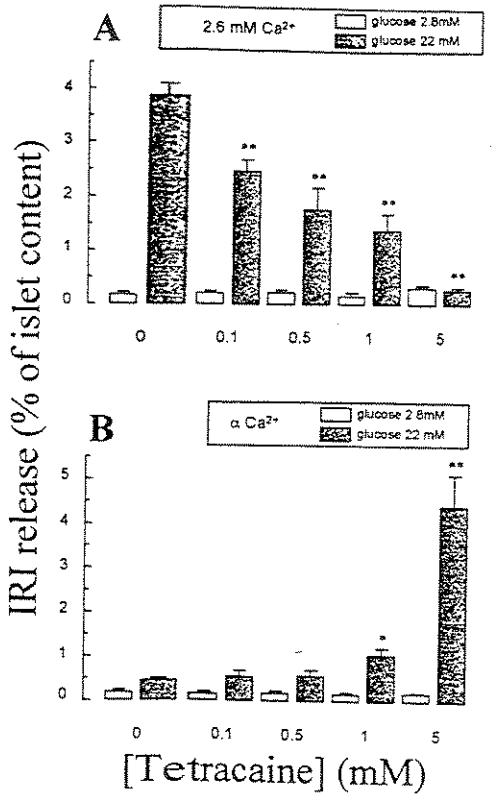

Fig. 1. Effect of tetracaine on insulin secretion. Prior to the application of tetracaine, groups of five islets each were preincubated for 45 min at 37°C in a Hepes-bicarbonate medium containing 5.6 mM glucose. This preincubation medium was then replaced with Hepes-bicarbonate containing 2.8 mM or 22 mM glucose and increasing concentrations (0–5 mM) of tetracaine. (A) Experiments carried out in the presence of 2.6 mM Ca^{2+}. (B) Experiments performed in the absence of Ca^{2+} (α Ca^{2+}). Columns represent the cumulative (1 h) insulin secretion, expressed as a percentage of the total islet content. Values are means ± S.E. of 7–19 experiments. * $P < 0.05$; ** $P < 0.01$.

Previous observations that local anesthetics alter Ca^{2+} permeability in different cell types, including B-cells (Norlund and Sehlin, 1985), provided evidence that tetracaine could affect insulin secretion by altering Ca^{2+} handling in the islet. To verify this hypothesis, we performed further experiments using two different techniques in order to measure Ca^{2+} movements under experimental conditions similar to those above. In the first, we used a microfluorimetry technique to measure changes in $[Ca^{2+}]_i$ induced by tetracaine in isolated B-cells. Fig. 2 shows the net increase in $[Ca^{2+}]_i$ relative to the baseline values in the absence of tetracaine. The values attained before the addition of the anesthetic were 98.5 ± 6.1 nM and 137.8 ± 19 nM for low (Fig. 2A) and high (Fig. 2B) glucose concentrations, respectively. At both low and high glucose levels, tetracaine elicited rapid elevations in $[Ca^{2+}]_i$ which were clearly not dependent on the glucose concentration. The dose-dependent rises in $[Ca^{2+}]_i$ were rapidly reverted when tetracaine was removed from the medium. The net increases in $[Ca^{2+}]_i$ at both glucose concentrations were 30, 90, 180 and 880 nM, respectively, for 0.1, 0.5, 1 and 5 mM tetracaine.

We also studied the effects of tetracaine on ^{45}Ca fluxes from perifused islets. In agreement with the $[Ca^{2+}]_i$ results, the pattern of ^{45}Ca efflux was identical at both levels of glucose (2.8 mM and 22 mM; Fig. 3A). In Ca^{2+}-deprived medium, tetracaine also increased the efflux of Ca^{2+} (Fig. 3B). Inspite of the difference in resting ^{45}Ca efflux rates, maximal efflux values in the presence of tetracaine and with either 2.8 mM or 22 mM glucose were essentially the same. Together, the $[Ca^{2+}]_i$ and ^{45}Ca measurements indicate that tetracaine mobilizes Ca^{2+} from intracelluiar stores(s) in a glucose-independent pathway.

To further understand the mechanism of action of tetracaine on Ca^{2+} release, we performed ^{45}Ca efflux measurements in the presence of thapsigargin. This drug is a specific blocker of the Ca^{2+}-ATPase pump and is generally used to promote depletion of the IP_3-sensitive Ca^{2+} store in the endoplasmic reticulum (ER) (Thastrup et al., 1990). Fig. 4 shows that thapsigargin did not alter the

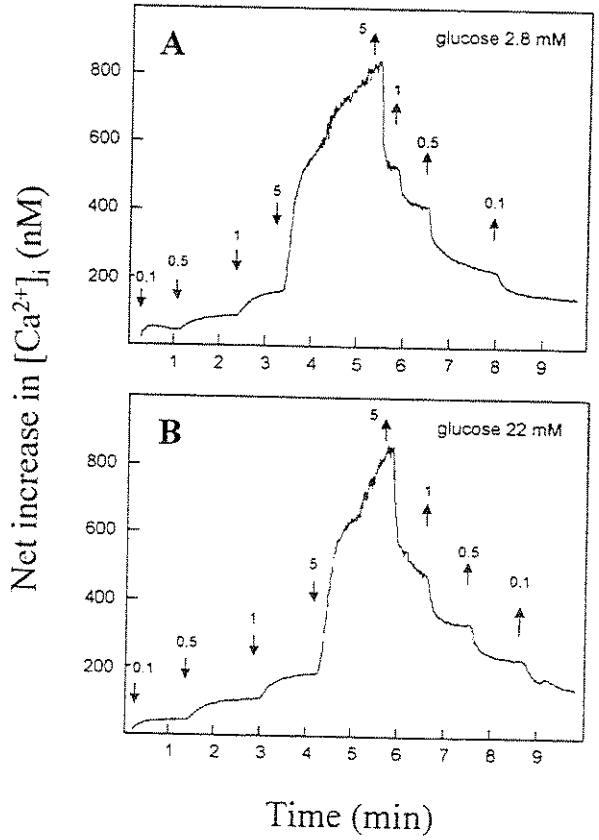

Fig. 2. Effect of tetracaine on $[Ca^{2+}]_i$. Isolated B-cells, cultured for 2–4 days, were prelabeled with the intracellular Ca^{2+} marker indo-1/AM for 1 h after which the cells were extensively washed and treated with increasing concentrations (0–5 mM) of tetracaine. The experiments were performed with medium containing 2.6 mM Ca^{2+} and 2.8 mM glucose (A) or 22 mM glucose (B). The arrows indicate either the addition (↓) or removal (↑) of tetracaine in order to achieve the concentration indicated above the arrows. The traces are representative of at least three experiments.

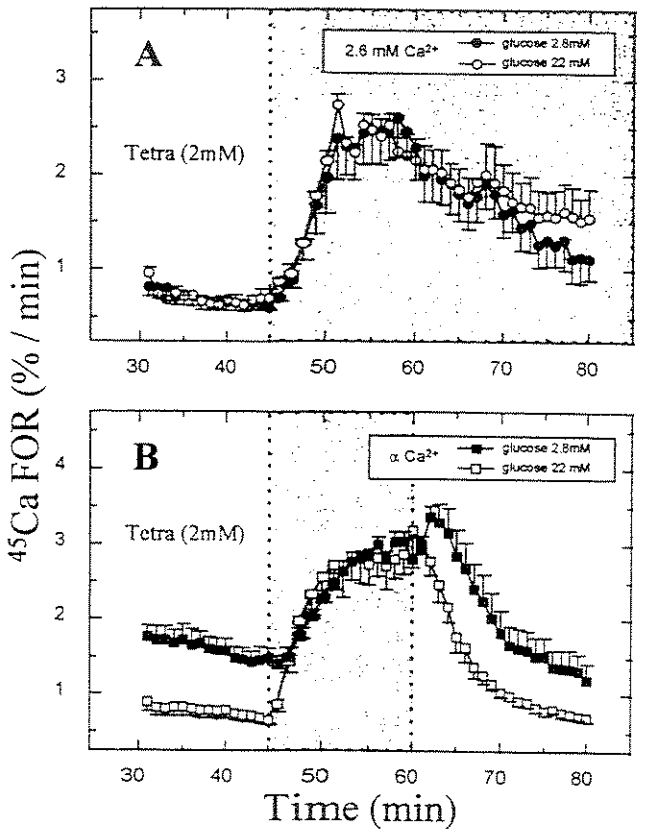

Fig. 3. Effects of tetracaine on the ^{45}Ca fractional outflow rate. Groups of 100 islets each were prelabeled with $^{45}CaCl_2$ for 90 min. The islets were then washed four times with non-radioactive medium, placed in a small chamber and perifused for 80 min in the presence of 2.6 mM Ca^{2+} (A) or in the absence of the cation (B). Glucose 2.8 or 22 mM was present throughout the perifusion period. Tetracaine (2 mM) was present during the period indicated by the shadowed areas. Each point is the mean ± SE of four experiments.

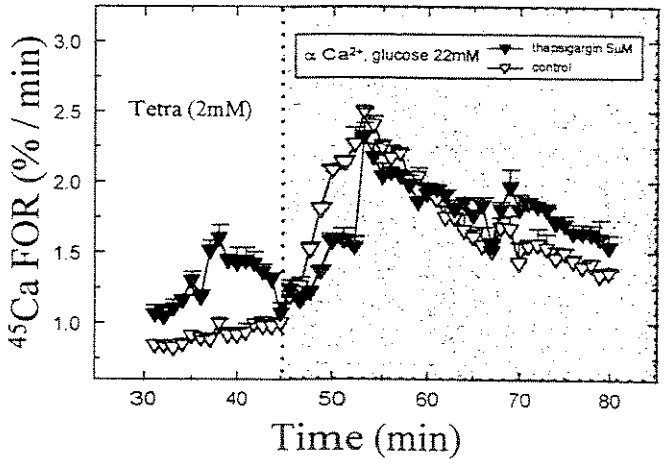

Fig. 4. Effect of thapsigargin (5 μM) on tetracaine-induced increase in ^{45}Ca efflux. Groups of 100 islets, prelabeled with $^{45}CaCl_2$, were perifused for 80 min with a Ca^{2+}-deprived medium containing 22 mM glucose. Tetracaine (2 mM) was introduced at the 45th min and maintained until the end of perifusion period (shaded area), either in the presence (open triangles) or the absence (solid triangles) of thapsigargin (5 μM). The latter was introduced at min 30th and maintained until the end of the experiments. Each point is the mean ± S.E. of four experiments.

amount of ^{45}Ca released by tetracaine. The initial increase in ^{45}Ca efflux (30th to 45th min) from thapsigargin-treated islets reflects the Ca^{2+} leak from ER (solid triangles in Fig. 4). When tetracaine (2 mM) was added to the solution (45th min), the ^{45}Ca efflux increased significantly attaining essentially the same level, irrespective of whether the thapsigargin-sensitive Ca^{2+} reservoir had previously been depleted or not. The small differences in the effluxes with or without thapsigargin after the 45th min indicate different time responses. Integrating the data to eliminate the differences in time delays showed that the areas under the curves were identical and corresponded to 25% of the initial ^{45}Ca content. These results rule out the possibility of tetracaine acting by the same mechanism as thapsigargin.

4. Discussion

Early studies reported opposite effects of tetracaine on glucose-induced insulin secretion. Freinkel et al. (1975) demonstrated that glucose-induced insulin secretion was inhibited when isolated rat islets were incubated with tetracaine. On the other hand, dynamic insulin secretion experiments using rat (El Motal et al., 1987) or mouse (Norlund and Sehlin, 1983) islets showed that tetracaine potentiated the secretory response induced by glucose. In our hands, tetracaine inhibited the secretory response at a physiological $[Ca^{2+}]_o$, and stimulated insulin release when islets were incubated in a Ca^{2+}-deprived medium. One possible explanation for these apparently paradoxical effects could be that B-cells regulate $[Ca^{2+}]_i$ within a very narrow range (Rojas et al., 1994). Since tetracaine elicited a very high increase in the $[Ca^{2+}]_i$, a rise in this cation above a critical level could result in a decrease in the effect of the sugar by collapsing the secretory machinery. In parathyroid cells (Nygren et al., 1987) and pancreatic B-cells (Hellman et al., 1994), desensitization of the secretory mechanism by continuous exposure of the cells to high Ca^{2+} has been observed and is accompanied by a reduction in protein phosphorylation (Jones et al., 1992). However, we can not discard the possibility that, in addition to desensitization by exposure to high Ca^{2+}, the inhibition of insulin secretion observed in the presence of high glucose and a physiological $[Ca^{2+}]_o$ could be associated to an intra-islet regulatory mechanism involving the participation of glucagon and somatostatin (for a review, see Marks and Morgan, 1994). This could partially explain the discrepancy between our results (obtained using static incubations) and those based on dynamic analysis (El Motal et al., 1987; Norlund and Sehlin, 1983). In the present study, we have demonstrated that by stimulating intracellular Ca^{2+} release with tetracaine, it is possible to induce insulin secretion in a $[Ca^{2+}]_o$-independent manner. As reported for the K_{ATP}-independent pathway (Gembal et al., 1993), the stimulatory effect of tetracaine involves a glucose-dependent mechanism. Considering that the effect of the anesthetic in inducing insulin release was only

observed in the presence of stimulatory glucose concentrations we speculate that a tetracaine-sensitive Ca^{2+} store can play a role in the mechanism of insulin secretion under physiological conditions.

Local anesthetics may interact with multiple cellular sites, including membrane receptors and ionic channels (reviewed by Butterworth and Strichartz, 1990). Our study has shown that tetracaine evoked the release of Ca^{2+} from an IP_3-insensitive intracellular pool. This conclusion is based on the observation that tetracaine increases $[Ca^{2+}]_i$ regardless of the presence or absence of extracellular Ca^{2+}, and that thapsigargin does not affect the mechanism of intracellular Ca^{2+} release stimulated by tetracaine. We also observed that tetracaine-induced Ca^{2+} release was not altered by addition of the metabolic poison sodium azide (data not shown), indicating that the $[Ca^{2+}]_i$ increase is not due to mitochondrial Ca^{2+} leakage.

In most excitable cells, two well-defined mechanisms for internal Ca^{2+} release are present. The first of these is represented by the IP_3 pathway and the second by the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. CICR is a well-known process, first described in skeletal muscle, whereby an increase in $[Ca^{2+}]_i$ causes further release of Ca^{2+} by acting on a specific receptor-operated channel known as the ryanodine receptor (RyR). Many drugs, including ryanodine, caffeine, sulphydryl reagents, and local anesthetics have been used to study the activity of RyR (Coronado et al., 1994). Although RyR has not yet been identified in B-cells, several lines of evidence suggest that ryanodine-like channels are linked to islet function. The modulation of an IP_3-insensitive intracellular Ca^{2+} store by thimerosal (Islam et al., 1992), as well as by the combination of ryanodine and caffeine (Chen et al., 1996) is strong evidence for the existence of RyR in insulin-secreting cells. The demonstration of a voltage-sensitive mechanism for Ca^{2+} extrusion (Roe et al., 1994) also suggests the activation of ryanodine-like channels. In addition, the controversial endogenous RyR agonist cADP-ribose was reported to cause Ca^{2+} release and to stimulate insulin secretion from pancreatic B-cells (Takasawa et al., 1993).

Perhaps the most intriguing observation made in the present study is that tetracaine elicited a dose-dependent increase in $[Ca^{2+}]_i$, in all of experimental conditions used. This seems to contradict recent observation showing that the local anesthetic procaine inhibits caffeine-induced Ca^{2+} release (Chen et al., 1996). However, local anesthetics are known to have complex effects on cellular function which depend on experimental conditions such as pH, ATP and Mg^{2+} concentrations as well as the cell type. It is noteworthy that tetracaine, but not procaine, evoked a dose-dependent Ca^{2+} release in rat myotubes (Jaimovich and Rojas, 1994). In this context, our data clearly demonstrate the presence of a tetracaine-sensitive mechanism for intracellular Ca^{2+} release in B-cells which may involve a channel functionally related to RyR.

In summary, we have demonstrated that insulin secretion can be elicited by a $[Ca^{2+}]_o$-independent pathway which triggers Ca^{2+} release from a specific intracellular Ca^{2+} store. Our results also suggest the participation of a ryanodine-like Ca^{2+} channel in intracellular Ca^{2+} handling during glucose-induced B-cell stimulation.

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References

Aizawa, T., Sato, Y., Ishihara, F., Tagushi, N., Komastu, M., Suzuki, N., Hashizume, K., Yamada, T., 1994. ATP-sensitive K^+ channel-independent glucose action in rat pancreatic β-cell. Am. J. Physiol. 266, C622.

Bordin, S., Boschero, A.C., Carneiro, E.M., Atwater, I., 1995. Ionic mechanisms involved in the regulation of insulin secretion by muscarinic agonists. J. Membr. Biol. 148, 177.

Boschero, A.C., Szpak-Glasman, M., Carneiro, E.M., Bordin, S., Paul, I., Rojas, E., Atwater, I., 1995. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M_3 muscarinic receptors. Am. J. Physiol. 268, E336.

Butterworth, J.F. IV, Strichartz, G.R., 1990. Molecular mechanisms of local anesthesia: a review. Anesthesiology 72, 711.

Chen, T.H., Lee, B., Yang, C., Hsu, W.H., 1996. Effects of caffeine on intracellular calcium release and calcium influx in a clonal β-cell line RINm5F. Life Sci. 58, 983.

Clapham, D.E., 1995. Calcium signaling. Cell 80, 259.

Coronado, R., Morrissette, J., Sukhareva, M., Vaughan, D.M., 1994. Structure and function of ryanodine receptors. Am. J. Physiol. 266, C1485.

El Motal, S.M.A.A., Pian-Smith, M.C.M., Sharp, G.W.G., 1987. Effects of tetracaine on insulin release and calcium handling by rat pancreatic islets. Am. J. Physiol. 252, E727.

Freinkel, N., El Younsi, C., Dawson, R.M.C., 1975. Inter-relations between the phospholipids of rat pancreatic islets during glucose stimulation and their response to medium inositol and tetracaine. Eur. J. Biochem. 59, 245.

Gembal, M., Gilon, P., Henquin, J.C., 1992. Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B cells. J. Clin. Invest. 89, 1288.

Gembal, M., Detimary, P., Gilon, P., Gao, Z.Y., Henquin, J.C., 1993. Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K^+ channels in mouse B cells. J. Clin. Invest. 91, 871.

Hellman, B., Gylfe, E., Bergsten, P., Grapengiesser, E., Lund, P.E., Berts, A., Dryselius, S., Tengholm, A., Liu, Y.J., Eberhardson, M., Chow, R.H., 1994. The role of Ca^{2+} in the release of pancreatic islet hormones. Diabet. Metabol. 20, 123.

Herchuelz, A., Malaisse, W.J., 1980. Regulation of calcium fluxes in pancreatic islets: two calcium movements' dissociated response to glucose. Am. J. Physiol. 238, E87.

Islam, M.S., Rorsman, P., Berggren, P.O., 1992. Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells. FEBS Lett. 296, 287.

Jaimovich, E., Rojas, E., 1994. Intracellular Ca^{2+} transients induced by high external K^+ and tetracaine in cultured rat myotubes. Cell Calcium 15, 356.

Jones, P.M., Persaud, S.J., Howell, S.L., 1992. Ca^{2+}-induced insulin secretion from electrically permeabilized islets. Loss of Ca^{2+}-induced secretory response is accompanied by loss of Ca^{2+}-induced protein phosphorylation. Biochem. J. 285, 973.

Komatsu, M., Schermerhorn, T., Aizawa, T., Sharp, G.W.G., 1995. Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in the absence of any increase in intracellular Ca^{2+} in rat pancreatic islets. Proc. Natl. Acad. Sci. USA 92, 10728.

Marks, V., Morgan, L.M., 1994. Intra-islet interactions and the enteroinsular axis in insulin secretion, in: Frontiers of Insulin Secretion and Pancreatic B-cell research, eds. P. Flatt and S. Lenzen. Smith-Gordon, Kent, p. 319.

Nilsson, T., Arkhammar, P., Hallberg, A., Hellman, B., Berggren, P.-O., 1987. Characterization of the inositol 1,4,5-triphosphate-induced Ca^{2+} release in pancreatic B-cells. Biochem. J. 248, 329.

Norlund, L., Sehlin, J., 1983. Effect of tetracaine and lidocaine on insulin release in isolated mouse pancreatic islets. Biochim. Biophys. Acta 763, 197.

Norlund, L., Sehlin, J., 1985. Effect of tetracaine and glibenclamide on $^{45}Ca^{2+}$ handling by isolated pancreatic islets. Br. J. Pharmacol. 85, 127.

Nygren, P., Larsson, R., Lindh, E., Ljunghall, S., Ratad, J., Akerström, G., Gylfe, E., 1987. Bimodal regulation of secretion by cytosolic Ca^{2+} as evidenced by the parathyroid. FEBS Lett. 213, 195.

Pozzan, T., Rizzuto, R., Volpe, P., Meldolesi, J., 1994. Molecular and cellular physiology of intracellular calcium stores. Physiol. Rev. 74, 595.

Prentki, M., Matschinsky, F.M., 1987. Ca^{2+}, cAMP and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Physiol. Rev. 67, 1185.

Rasmussen, H., Isales, C.M., Calle, R., Throckmorton, D., Anderson, M., Gasalla-Herraiz, J., McCarthy, R., 1995. Diacylglycerol production, Ca^{2+} influx, and protein kinase C activation in sustained cellular responses. Endocrine Rev. 16, 649.

Roe, M.W., Mertz, R.J., Lancaster, M.E., Worley, J.F. III, Dukes, I.D., 1994. Thapsigargin inhibits the glucose-induced decrease of intracellular Ca^{2+} in mouse islets of Langerhans. Am. J. Physiol. 266, E852.

Rojas, E., Carroll, P.B., Ricordi, C., Boschero, A.C., Stojilkovic, S.S., Atwater, I., 1994. Control of cytosolic free calcium in cultured human pancreatic β-cells occurs by external calcium-dependent and independent mechanisms. Endocrinology 134, 1771.

Sato, Y., Aizawa, T., Komatsu, M., Okada, N., Yamada, T., 1992. Dual functional role of membrane depolarization/Ca^{2+} influx in rat pancreatic B-cell. Diabetes 41, 438.

Scott, A.M., Atwater, I., Rojas, E., 1981. A method for the simultaneous measurement of insulin release and β-cell membrane potential in single mouse islets of Langerhans. Diabetologia 21, 470.

Takasawa, S., Nata, K., Yonekura, H., Okamoto, H., 1993. Cyclic ADP-ribose in insulin secretion from pancreatic β-cells. Science 259, 370.

Thastrup, O., Cullen, P.J., Drobak, P.K., Hanley, M.R., Dawson, A.P., 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA 87, 2466.

Wollheim, C.B., Biden, T.J., 1986. Second messenger function of inositol 1,4,5-triphosphate. Early changes in inositol, phosphates, cytosolic Ca^{2+}, and insulin release in carbamylcholine-stimulated RINm5F cells. J. Biol. Chem. 261, 8314.

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Synergistic effect of glucose and prolactin on GLUT2 expression in cultured neonatal rat islets

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Abstract

We studied the synergistic effect of glucose and prolactin (PRL) on insulin secretion and GLUT2 expression in cultured neonatal rat islets. After 7 days in culture, basal insulin secretion (2.8 mM glucose) was similar in control and PRL-treated islets (1.84 ± 0.06% and 2.08 ± 0.07% of the islet insulin content, respectively). At 5.6 and 22 mM glucose, insulin secretion was significantly higher in PRL-treated than in control islets, achieving 1.38 ± 0.15% and 3.09 ± 0.21% of the islet insulin content in control and 2.43 ± 0.16% and 4.31 ± 0.24% of the islet insulin content in PRL-treated islets, respectively. The expression of the glucose transporter GLUT2 in B-cell membranes was dose-dependently increased by exposure of the islet to increasing glucose concentrations. This effect was potentiated in islets cultured for 7 days in the presence of 2 µg/ml PRL. At 5.6 and 10 mM glucose, the increase in GLUT2 expression in PRL-treated islets was 75% and 150% higher than that registered in the respective control. The data presented here indicate that insulin secretion, induced by different concentrations of glucose, correlates well with the expression of the B-cell-specific glucose transporter GLUT2 in pancreatic islets.

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Key words
- Pancreatic islets
- Glucose transporter
- Prolactin
- Glucose

The first step in glucose-induced insulin secretion is the entry of the sugar into the B-cells, which is mediated by the glucose transporter GLUT2, located on the B-cell plasma membrane. Alteration in the expression of GLUT2 has been implicated in the reduction of the secretory response to glucose (1,2). Since growth and differentiation of the endocrine pancreas are controlled by glucose and the somatolactogenic hormones, growth hormone (GH) and prolactin (PRL) (3), we determined the effect of glucose on insulin secretion and GLUT2 expression in neonatal rat islets cultured in the presence or absence of PRL.

We have used islets from neonatal rats (2 to 48 h old) obtained by collagenase digestion and cultured for 7 days, as described previously (4). After 7 days, the culture medium was discarded and the islets were incubated for 90 min at 37°C in a bicarbonate-buffered solution containing different concentrations of glucose. The supernatant was withdrawn for insulin measurements and the

insulin present in the islets was extracted with 1 ml acid-ethanol. Insulin was measured by standard radioimmunoassay (5), and is reported as percent of the total islet content. For Western blot analysis, groups of approximately 1000 islets (N = 4) were homogenized in Tris-HCl buffer containing 1 mM EDTA, 1-2 µg/ml antipain, 1 µg/ml pepstatin, 1 mM benzamidine, 1-2 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, and 0.02% Tween 20. The protein content of the homogenates was measured by the BioRad protein assay (BioRad Lab., Melville, NY). Aliquots containing 50 µg of protein were submitted to electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked with 4% defatted dry milk, and 0.2% Tween 20 in Tris-buffered saline, pH 7.4, for 2 h at 20°C. These membranes were then incubated with a polyclonal rabbit antiserum against GLUT2 (1:1000) (East Acres, Southbridge, MA), and washed with Tris-buffered saline and the antibody-antigen complex was detected by incubation with a rabbit Ig ^{125}I-labeled whole antibody from donkey (Amersham, UK).

After 7 days in culture, basal insulin secretion (2.8 mM glucose) was similar in control and PRL-treated islets, achieving 1.84 ± 0.06% (N = 21) and 2.08 ± 0.07% (N = 21) of the islet insulin content, respectively, in 90 min. At threshold glucose concentrations (5.6 mM), insulin secretion was 1.38 ± 0.15% (N = 12) and 2.43 ± 0.16% (N = 12) of the islet insulin content ($P<0.05$) in 90 min for control and PRL-treated islets, respectively. In the presence of 22 mM glucose, insulin secretion was 3.09 ± 0.21% and 4.31 ± 0.24% of islet content for control and PRL-treated islets, respectively ($P<0.05$). The presence of GLUT2 in the B-cell plasma membrane of neonatal cultured islets (Figure 1) was demonstrated by immunohistochemistry (6). Interestingly, a positive reaction to the GLUT2 antibody was also detected as bright dots inside the B-cell cytoplasm, suggesting the presence of glucose transporters in membranes of internal organelles. At present we have no explanation for this finding. Immu-

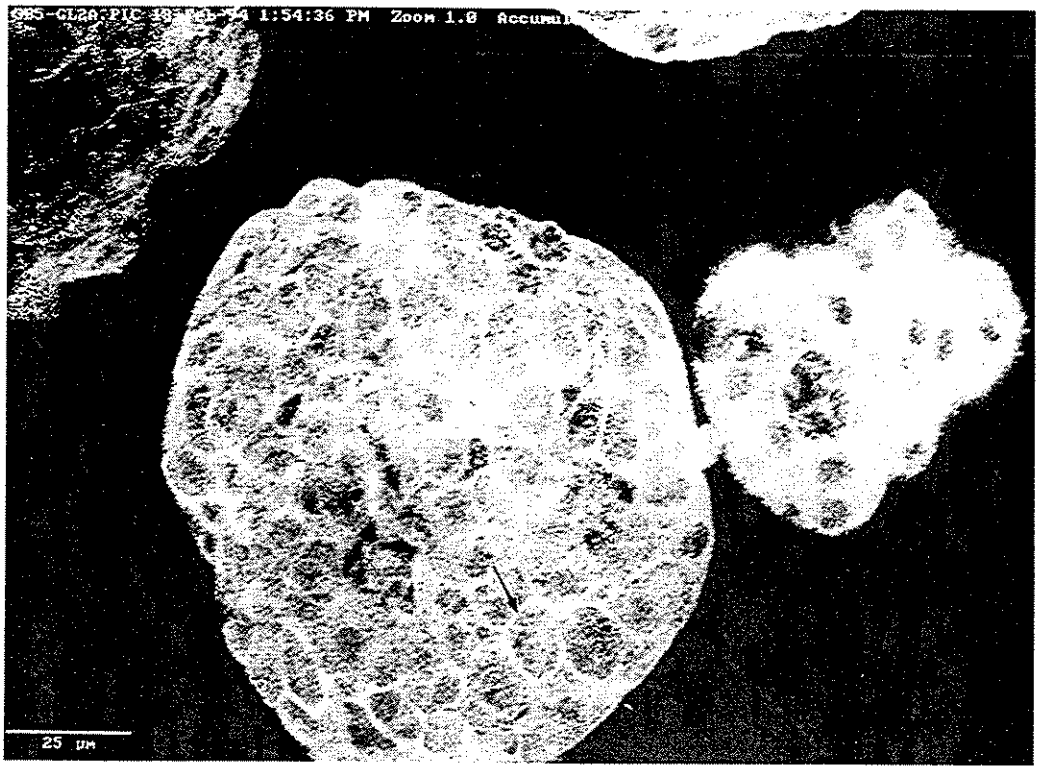

Figure 1 - Immunolocalization of the glucose transporter GLUT2 in the plasma membrane of B-cells from cultured neonatal rat islets. The presence of GLUT2 was revealed in sections (10 µm) of rat islets by indirect immunofluorescence using a polyclonal rabbit GLUT2 antiserum (dilution 1:1000) and a fluorescein isothiocyanate (FITC)-conjugated specific second antibody (dilution 1:100). Fluorescence was detected by confocal laser scanning microscopy. Note the bright reaction at the cell membrane level following GLUT2 immunolocalization (arrow).

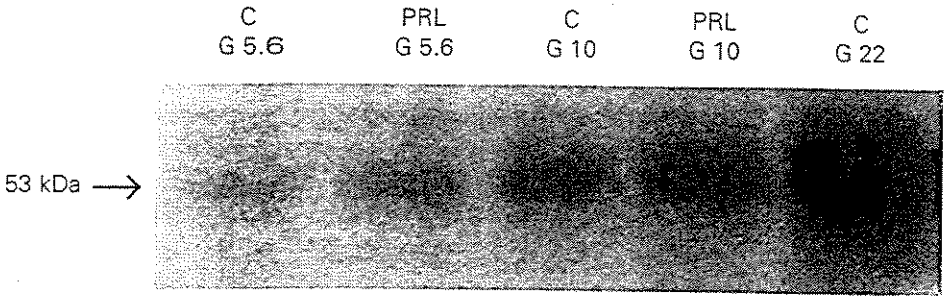

Figure 2 - Effect of glucose and prolactin (PRL) treatment on GLUT2 protein content measured by immunoblotting. Neonatal rat islets were cultured for 7 days in medium containing or not 2 µg/ml PRL and glucose at different concentrations. Neonatal islet protein (50 µg/lane) was analyzed by immunoblotting with antiserum to GLUT2. This is a representative blot of two.

blot analysis of neonatal islets with an tibody specific for the liver B-cell glucose nsporter GLUT2 is shown in Figure 2. e antibody labeled a 53-kDa band corre-onding to GLUT2 in both control and L-treated islets. PRL treatment increased GLUT2 content of the islets by approxi-tely 75% and 150% in the presence of 5.6 d 10 mM glucose, respectively (measure-nts made by densitometry and by radioac-ity analysis). A high concentration of glu-se (22 mM) alone also increased the UT2 content of the islets by about 8 times compared to control values (5.6 mM glu-se).

These data confirm prior observations t PRL treatment increases GLUT2 expression in pancreatic B-cells (7,8). Expression of GLUT2 was further enhanced with the combination of glucose and PRL. The synergistic effect of glucose and PRL treatment on GLUT2 expression correlates well with the increased insulin secretion provoked by glucose in PRL-treated islets compared to control islets (9). These observations indicate that GLUT2 may play an important role in the process of maturation of the glucose-sensing mechanism in neonatal islets.

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ferences

Johnson JH, Ogawa A, Chen L, Orci L, Newgard CB, Alam T & Unger RH (1990). Underexpression of B-cell high Km glucose transporters in non-insulin-dependent diabetes. *Science*, 250: 546-549.

Unger RH (1991). Diabetic hyperglycemia: link to impaired glucose transporter in pancreatic B cells. *Science*, 251: 1200-1205.

Nielsen JH, Billestrup N, Moldrup A, Allevato G, Petersen ED, Amstrup J & Hansen JA (1993). Growth of the endocrine pancreas: the role of somatolactogenic hormones and receptors. *Biochemical Society Transactions*, 21: 146-149.

Boschero AC, Crepaldi SC, Carneiro EM, Delattre E & Atwater I (1993). Prolactin induces maturation of glucose sensing mechanisms of cultured neonatal rat islets. *Endocrinology*, 133: 515-520.

5. Scott AM, Atwater I & Rojas E (1981). A method for the simultaneous measurement of insulin release and B-cell membrane potential in single mouse islets of Langerhans. *Diabetologia*, 21: 470-475.
6. Saez JC, Berthoud VM, Kadle R, Traub O, Nicholson BJ, Bennet MVL & Dermietzel R (1991). Pinealocytes in rats: connexin identification and increase in coupling caused by norepinephrine. *Brain Research*, 568: 265-275.
7. Mazancourt P, Carneiro EM, Atwater I & Boschero AC (1994). Prolactin treatment increases GLUT2 but not the G protein subunit content in cell membranes from cultured neonatal rat islets. *FEBS Letters*, 343: 137-140.
8. Weinhaus AJ, Stout LE & Sorenson RL (1996). Glucokinase, hexokinase, glucose transporter 2, and glucose metabolism in islets during pregnancy and prolactin-treated islets *in vitro*: mechanism for long term up-regulation of islets. *Endocrinology*, 137: 1640-1649.
9. Crepaldi SC, Carneiro EM & Boschero AC (1996). Long-term effect of prolactin (PRL) treatment on glucose-induced insulin secretion in cultured neonatal rat islets. *Hormone and Metabolic Research* (in press).

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Development of the insulin secretion mechanism in fetal and neonatal rat pancreatic B-cells: response to glucose, K^+, theophylline, and carbamylcholine

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Abstract

We studied the development of the insulin secretion mechanism in the pancreas of fetal (19- and 21-day-old), neonatal (3-day-old), and adult (90-day-old) rats in response to stimulation with 8.3 or 16.7 mM glucose, 30 mM K^+, 5 mM theophylline (Theo) and 200 µM carbamylcholine (Cch). No effect of glucose or high K^+ was observed on the pancreas from 19-day-old fetuses, whereas Theo and Cch significantly increased insulin secretion at this age (82 and 127% above basal levels, respectively). High K^+ also failed to alter the insulin secretion in the pancreas from 21-day-old fetuses, whereas 8.3 mM and 16.7 mM glucose significantly stimulated insulin release by 41 and 54% above basal levels, respectively. Similar results were obtained with Theo and Cch. A more marked effect of glucose on insulin secretion was observed in the pancreas of 3-day-old rats, reaching 84 and 179% above basal levels with 8.3 mM and 16.7 mM glucose, respectively. At this age, both Theo and Cch increased insulin secretion to close to two-times basal levels. In islets from adult rats, 8.3 mM and 16.7 mM glucose, Theo, and Cch increased the insulin release by 104, 193, 318 and 396% above basal levels, respectively. These data indicate that pancreatic B-cells from 19-day-old fetuses were already sensitive to stimuli that use either cAMP or IP_3 and DAG as second messengers, but insensitive to stimuli such as glucose and high K^+ that induce membrane depolarization. The greater effect of glucose on insulin secretion during the neonatal period indicates that this period is crucial for the maturation of the glucose-sensing mechanism in B-cells.

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- Carbamylcholine
- Theophylline
- Potassium
- Insulin secretion

Introduction

Maturation of the insulin release mechanism in mammals occurs during the perinatal period of life. In rodents, the poor secretory response to nutrient secretagogues during the fetal period is rapidly converted to an adult response within a few days after birth (1). Despite some early controversial data, it is generally accepted that glucose starts to stimulate insulin secretion in the late stages of the gestational period (2-4); in rats, this occurs at precisely 19.5 days of fetal life (5). The insulin response at this age is monophasic and is not blocked by Ca^{2+} antagonists (4). A clear biphasic pattern of insulin secretion in response to glucose is detected only three days after birth, although at this age the insulin secretion is still well below the adult response (5).

In adult B-cells, the molecular mechanism underlying glucose-induced insulin secretion involves different steps. First the inhibition of K^+ efflux through K^+_{ATP} channels, which is modulated by glucose metabolism and leads to membrane depolarization. This is followed by an increase in the cytosolic Ca^{2+} concentration as a result of membrane depolarization and Ca^{2+} influx through L-type Ca^{2+} channels (6,7). Since K^+_{ATP} is already present and operative in fetal B-cells (8), it has been suggested that the reduced insulin secretion in response to glucose reflects the uncoupling between glucose metabolism and membrane cell depolarization in these cells (9). This aspect is still a matter of debate (10-12). Glucose-stimulated secretion is also modulated by neurotransmitters and hormones involving the phospholipase C and adenylylcyclase pathways (6,7).

In the present study we have compared the insulin secretion of fetal, neonatal and adult rat B-cells in response to glucose, K^+, theophylline and carbamylcholine. Insulin secretion in response to stimuli that involve membrane cell depolarization (glucose and high K^+) occurred at the end of the fetal period, although the greatest increase in glucose-induced insulin secretion occurred after birth. Theophylline and carbamylcholine, which increase the cAMP and the IP_3 and DAG content of pancreatic islet cells, respectively, potentiated insulin secretion after 19 days of fetal life.

Material and Methods

Animals and pancreatic tissue

Fetal (19- and 21-day-old), neonatal (3-day-old) and adult (90-day-old) rats were used. The fetal ages were determined by counting the days after the presence of sperm was detected in vaginal smears following overnight mating. The pancreas from fetal and neonatal rats was reduced to approximately 0.5 mm^3 by slicing. Isolated islets from adult rats were obtained by collagenase digestion. The rats were killed by decapitation.

Insulin secretion

To study insulin secretion, pancreatic tissue from fetal and neonatal rats (3-4 fragments/group), and adult islets (5/group) were first incubated for 45 min at 37°C in 0.75 ml of Krebs-bicarbonate buffer of the following composition: 115 mM NaCl, 5 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, 5.6 mM glucose, supplemented with 3 mg of BSA/ml and equilibrated with a mixture of 95% O_2-5% CO_2, pH 7.4. This medium was then replaced with fresh buffer and the fragments and islets were incubated for 1 h in the presence of increasing concentrations of glucose (2.8-16.7 mM). In another series of experiments, the fragments and islets were incubated in the presence of 5.6 mM glucose with the medium also containing 30 mM K^+, 5 mM theophylline, or 200 μM carbamylcholine. The insulin content in the supernatant of each sample and the insulin extracted from the fragments and

islets at the end of the incubation period were measured by radioimmunoassay as previously described (13), using rat insulin as standard. Since the amount of endocrine pancreas varied considerably between the fragments used, insulin release was expressed as percent of the total fragment or islet insulin content released during a 1-h incubation period.

Data analysis

Data are reported as the means ± SEM of *n* experiments. The significance of the differences between means was assessed by analysis of variance followed by Dunnett's test when several experimental groups were compared with the control group. The confidence limit for significance was 5%.

Results

Figure 1 shows insulin secretion induced by different concentrations of glucose from fragments of pancreas and from isolated islets obtained at different ages. Basal secretion (in the presence of 2.8 or 5.6 mM glucose) was 0.66 ± 0.08% (N = 20), 0.82 ± 0.09% (N = 25), 0.38 ± 0.04% (N = 17) and 0.88 ± 0.21% (N = 14) of the total pancreas (or islet) content for 19- and 21-day-old fetuses, 3-day-old neonatal rats and 90-day-old adult rats, respectively. Increasing the glucose concentration to 8.3 and 16.7 mM did not alter the basal release of insulin by 19-day-old fetuses. In 21-day-old fetuses, 8.3 mM glucose increased the insulin secretion to 1.16 ± 0.09% (N = 8). At this age, insulin secretion reached 1.26 ± 0.13% (N = 13) of the total pancreas content in the presence of 16.7 mM glucose ($P<0.05$). At 3 days of age, the insulin secretion induced by 8.3 and 16.7 mM glucose was 0.70 ± 0.17% (N = 17) and 1.06 ± 0.23% (N = 6) of pancreas content, respectively ($P<0.05$). At the same glucose concentrations, the insulin secretion observed for adult islets was 1.83 ± 0.22% (N = 6) and 2.62 ± 0.15% (N = 6) of the islet content, respectively ($P<0.05$).

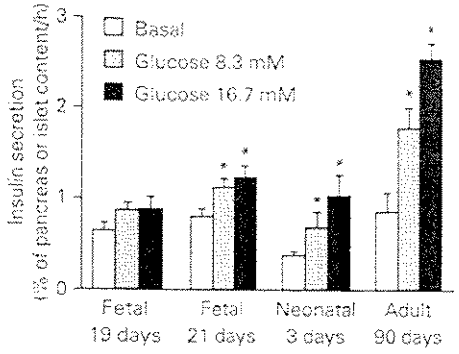

Figure 1 - Effect of 8.3 and 16.7 mM glucose on insulin secretion. Prior to exposure to different concentrations of glucose, fragments of fetal and neonatal pancreas or adult rat islets were preincubated for 45 min at 37°C in a Krebs-bicarbonate medium containing 5.6 mM glucose. This preincubation medium was then replaced with Krebs-bicarbonate containing the desired concentration of glucose (8.3 or 16.7 mM). The columns represent the cumulative (1 h) insulin secretion, expressed as percentage of the total fragment or islet content for each glucose concentration. The values are reported as the mean ± SEM of 6-25 experiments. *P<0.05 compared to basal insulin secretion.

Insulin secretion induced by K^+ (30 mM) is illustrated in Figure 2. There was a progressive increase in insulin secretion with age. However, no difference from basal secretion (5.6 mM glucose) was noticed in the fetal groups exposed to K^+. In neonatal and adult pancreas insulin secretion induced by 30 mM K^+ was 1.4- and 2.0-fold greater than basal secretion, respectively.

In the next series of experiments, we analyzed the effect of the nonspecific phosphodiesterase inhibitor theophylline, that increases the intracellular cAMP content, and carbamylcholine, an agonist that stimulates the formation of IP_3 and DAG in B-cells. Both theophylline (5 mM) and carbamylcholine (200 µM) stimulated insulin secretion at all ages. Insulin secretion in the presence of theophylline was 1.7-fold higher

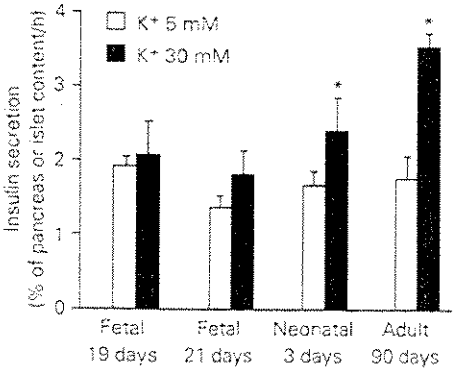

Figure 2 - Effect of 30 mM K^+ on insulin secretion. Fragments of fetal or neonatal pancreas or adult rat islets were preincubated for 45 min at 37°C in a Krebs-bicarbonate medium containing 5.6 mM glucose. This medium was then replaced with Krebs-bicarbonate containing 30 mM K^+. The osmolarity of the medium was kept constant by withdrawing an equimolar amount of NaCl. The columns represent the cumulative (1 h) insulin secretion, expressed as percentage of the total fragment or islet content, at each K^+ concentration. Values are reported as the mean ± SEM of 6-9 experiments. *P<0.05 compared to insulin secretion with 5 mM K^+.

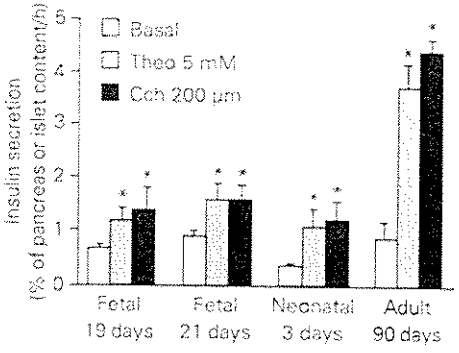

Figure 3 - Effect of 5 mM theophylline (Theo) and 200 µM carbamylcholine (Cch) on insulin secretion. Fragments of fetal and neonatal pancreas or islets from adult rats were preincubated for 45 min at 37°C in Krebs-bicarbonate medium containing 5.6 mM glucose. This medium was then replaced with Krebs-bicarbonate containing 5 mM theophylline or 200 µM carbamylcholine. The columns represent the cumulative (1 h) insulin secretion, expressed as percentage of the total fragment or islet content. The values are reported as the mean ± SEM of 6-14 experiments. *P<0.05 compared to basal insulin secretion.

in 19- and 21-day-old fetuses, 2.6-fold higher in 3-day-old rats and 4-fold higher in adult rats than each respective basal secretion (5.6 mM glucose). In the presence of carbamylcholine the increase in insulin secretion above basal values was 2- and 1.7-fold in 19- and 21-day-old fetuses, 2.9-fold in 3-day-old rats, and 5-fold in adult rats (Figure 3). As observed for supraliminal glucose concentrations and 30 mM K^+, the increase in insulin secretion in the presence of theophylline or carbamylcholine was more marked in the pancreas of 3- and 90-day-old rats.

Discussion

In the perinatal period of life, the mechanism of insulin secretion undergoes a decisive phase of functional maturation (1). During this period, insulin secretion in response to nutrient secretagogues is reduced compared to adulthood (5). In addition, the insulin response to glucose is monophasic and is similar to the pattern observed for insulin secretion during the onset of type 2 diabetes in adult humans. Thus, analysis of the glucose-sensing mechanism in fetal and neonatal islets may contribute to our understanding of the loss of glucose sensitivity associated with type 2 diabetes.

In adult islets, the reduction in K^+ permeability and the consequent membrane depolarization are important steps in the mechanism of nutrient-induced insulin secretion (6,14-16). The poor secretory response to glucose in fetal and neonatal islets is ascribed to the uncoupling of glucose stimulation and membrane depolarization which results in the opening of an insufficient number of voltage-sensitive Ca^{2+} channels and only a marginal increase in cytosolic Ca^{2+} (9). In the present experiments glucose-induced insulin secretion was observed only in the pancreas of 21-day-old fetuses, thus supporting the view that the coupling between glucose metabolism and alterations in membrane potential is not yet fully developed at earlier ages (around 19 days). The observation that high concentrations of K^+ failed to stimulate insulin secretion in the pancreas of 19-day-old fetuses also indicates that the B-cell plasma membrane is not yet mature enough to undergo sufficient depolarization to provoke the necessary accumulation of cytosolic Ca^{2+} seen in neonatal B-cells (17). Hence, there is no insulin secretion. In addition to the absence of an adequate signal derived from glucose metabolism (9,12), the poor secretory response to glucose during fetal development may also be ascribed to a reduced and/or altered behavior of K^+_{ATP} channels (18) associated with unresponsive Ca^{2+} L-type channels. The latter channels are already present in fetal B-cells, but are not yet sensitive to Ca^{2+}-antagonists (4). We measured the membrane potential of neonatal rat islet cells in the presence of depolarizing concentrations of glucose (Boschero AC, Carroll P and Atwater I, unpublished data). Only 3 of 23 cells examined depolarized in the presence of tolbutamide, while none depolarized in the presence of glucose. If one assumes that at least the tolbutamide-sensitive cells were B-cells, it becomes clear that glucose was unable to depolarize neonatal B-cells. Thus, these data, together with those discussed above, indicate that the lack of insulin secretion in response to glucose and high K^+ concentrations probably reflects the sum of different factors, including insufficient membrane depolarization, reduced entry of Ca^{2+} into B-cells, and hence reduced

accumulation of cytosolic Ca^{2+}.

Insulin secretion is modulated by second messengers, including cAMP, IP_3 and DAG. In B-cells, the intracellular levels of these second messengers can be increased by the stimulation of adenylylcyclase and phospholipase C, respectively. It is well established that both of these pathways are important modulators of insulin secretion in fetal, neonatal, and adult islets (10,19-21). In 19-day-old fetuses, theophylline stimulated insulin secretion, thus confirming that this pathway is already operative at this age and that it is fundamental for insulin secretion during fetal life. The phospholipase C-IP_3-DAG pathway was also found in 19-day-old fetuses as judged by the increase in insulin secretion induced by carbamylcholine. However, the rise in insulin secretion provoked by the muscarinic agonist was more marked in neonatal and adult pancreas. The latter observation may be partly explained by the fact that the α isoform of protein kinase C, which is important for the second phase of insulin secretion, is detected in islet B-cells only after 3 days of life (22).

In conclusion, the perinatal period of life, which extends from the 21st day of fetal life to three days after birth, is crucial for the maturation of the insulin-secreting mechanism. Initially, insulin secretion is more dependent on the presence of cAMP and can be potentiated by agents that stimulate IP_3 and DAG formation. Subsequently, the adult pattern of insulin secretion involving nutrient metabolism and alterations in K^+ and Ca^{2+} permeabilities becomes established. Finally, the mature biphasic insulin response is completed with the development of amplification systems involving the participation of several protein kinase isoforms, especially protein kinase C_α.

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References

1. Hellerstrom C & Swenne I (1991). Functional maturation and proliferation of fetal pancreatic B-cells. *Diabetes*, 40 (Suppl 2): 89-93.
2. Kervran A & Randon J (1980). Development of insulin release by fetal rat pancreas *in vitro*: effect of glucose, amino acids and theophylline. *Diabetes*, 29: 673-678.
3. Freinkel N, Lewis NJ, Johnson R, Swenne I, Bone A & Hellerstrom C (1984). Differential effects of age versus glycemic stimulation on the maturation of insulin stimulus-secretion coupling during culture of fetal rat islet. *Diabetes*, 33: 1028-1038.
4. Hole RL, Pian-Smith MCM & Sharp GWG (1988). Development of the biphasic response to glucose in fetal and neonatal rat pancreas. *American Journal of Physiology*, 254: E167-E174.
5. Bliss CR & Sharp GWG (1994). Development of glucose responsiveness from the fetal to the adult B-cell. In: Flatt P & Lenzen S (Editors), *Frontiers of Insulin Secretion and Pancreatic B-Cell Research*. Smith-Gordon, Great Britain, 69-76.
6. Boschero AC (1996). Acoplamento da estimulação-secreção de insulina pelas células B pancreáticas. *Arquivos Brasileiros de Endocrinologia e Metabologia*, 40: 149-155.
7. Prentki M & Matschinsky FM (1987). Ca^{2+}, cAMP and phospholipids-derived messengers in coupling mechanism of insulin secretion. *Physiological Reviews*, 67: 1185-1248.
8. Rorsman P, Arkhammar P, Bokvist K, Hellerstrom C, Nilsson T, Welsh M, Welsh N & Berggren P-O (1989). Failure of glucose to elicit a normal secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K^+ channels. *Proceedings of the National Academy of Sciences, USA*, 86: 4505-4509.
9. Black MA, Mealing GRA, Whitfield JF, Schwartz JL & Bégin-Heick N (1994). Insulin secretion and intracellular Ca^{2+} rises in monolayer cultures of neonatal rat B-cells. *Cellular Signaling*, 6: 897-904.
10. Ammon HPT, Glocker C, Waldner RG & Wahl MA (1989). Insulin release from pancreatic islets of fetal rats mediated by leucine b-BCH, tolbutamide, glibenclamide, arginine, potassium chloride, and theophylline does not require stimulation of Ca^{2+} net uptake. *Cell Calcium*, 10: 441-450.
11. Boschero AC, Tombaccini D & Atwater I (1988). Effects of glucose on insulin release and ^{86}Rb permeability in cultured neonatal and adult rat islets. *FEBS Letters*, 236: 375-379.
12. Boschero AC, Bordin S, Sener A & Malaisse WJ (1990). D-glucose and L-leucine metabolism in neonatal and adult cultured rat pancreatic islets. *Molecular and Cellular Endocrinology*, 73: 63-71.
13. Scott AM, Atwater I & Rojas E (1981). A method for the simultaneous measurement of insulin release and B-cell membrane potential in single mouse islets of

Langerhans. *Diabetologia*, 21: 470-475.
14. Boschero AC & Malaisse WJ (1979). Stimulus-secretion coupling of glucose-induced insulin release. XXIX. Regulation of ^{86}Rb efflux from perifused islets. *American Journal of Physiology*, 236: E139-E146.
15. Cook DL & Hales CN (1984). Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature*, 311: 271-273.
16. Ashcroft FM, Harrisom DE & Ashcroft SJH (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic B-cells. *Nature*, 312: 446-448.
17. Boschero AC, Bordin S, Plasman P & Herchuelz A (1992). Effect of glucose and K^+ depolarization on cytosolic free Ca^{2+} in cultured neonatal islets. *Hormone and Metabolic Research*, 24: 51-52.
18. Boschero AC, Tombaccini D, Carneiro EM & Atwater I (1993). Differences in K^+ permeability between cultured adult and neonatal rat islets of Langerhans in response to glucose, tolbutamide, diazoxide, and theophylline. *Pancreas*, 8: 44-49.
19. Lambert AE, Kanazawa Y, Burr IM, Orci L & Renold AE (1971). On the role of cyclic AMP in insulin release: I. Overall effects in cultured fetal rat pancreas. *Annals of the New York Academy of Sciences*, 185: 232-244.
20. Mourmeaux JL, Remacle C & Henquin JC (1989). Effects of stimulation of adenylate cyclase and protein kinase C on cultured fetal rat B-cells. *Endocrinology*, 125: 2636-2644.
21. Tuch BE, Palavidis Z & Turtle JR (1988). Activators of protein kinase C stimulate insulin secretion from the human fetal pancreas. *Pancreas*, 3: 675-680.
22. Fletcher DJ & Ways DK (1991). Age-dependent expression of protein kinase C isoforms in rat islets. *Diabetes*, 40: 1496-1503.