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Decreased Insulin Content and Secretion in RIN 1046-38 Cells Overexpressing α_2-Adrenergic Receptors

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Several G_i-protein-coupled receptors normally expressed in islet β-cells inhibit insulin secretion on binding of their respective agonists. To study the effect of supraphysiologic expression of such a receptor in insulin-secreting β-cells, we stably transfected cDNA encoding the mouse α_{2a}-adrenergic receptor into RIN 1046-38 cells. Four different cell lines were selected, each overexpressing the α_{2a}-adrenergic receptor to varying degrees. Cell lines showing the highest level of receptor expression showed significantly reduced insulin content, and reduced basal and stimulated insulin secretion. Pertussis toxin (PTX) treatment of cells was able to reverse partially the reduced insulin secretory response. Our results suggest that overexpression of a G_i-protein-coupled receptor in β-cells causes tonic inhibition of both insulin synthesis and secretion. Abnormalities in expression or function of such receptors could be a contributory factor in the impaired insulin secretion present in type II diabetes.

Key Words: RIN cells; α_2-adrenergic receptor; G-protein.

Introduction

Regulation of insulin secretion from islet β-cells is complex, and involves both stimulatory and inhibitory pathways *(1–4)*. Although the latter have not been as extensively studied as the former, a variety of in vitro and in vivo studies have defined an important role for certain G-protein-coupled receptors (GPCR), including those for galanin, somatostatin, and α_2-adrenergic agonists in the physiologic inhibition of insulin secretion. By coupling to pertussis toxin-sensitive G_i- and G_o-proteins, such receptors act at multiple points, including stimulation of K^+ channels and inhibition of adenylyl cyclase, Ca^{2+} channels, and possibly a late exocytotic step to reduce glucose-stimulated insulin secretion.

GPCR may be activated not only by their physiologic agonists, but by mutations that lead to agonist-independent activation *(5)*. Such mutations are thought to stabilize a conformation of the receptor that favors G-protein activation. Recently, naturally occurring activating mutations (somatic or germline) in a number of GPCR have been identified as the cause of several diseases *(6)*. Even certain wild-type GPCR, if expressed at sufficiently high levels, will cause agonist-independent activation. Overexpression of the β_2-adrenergic receptor in the hearts of transgenic mice *(7)* and of the TSH receptor in transfected cells *(8)* leads to substantial stimulation of the corresponding pathways.

The β_2-adrenergic receptor and TSH receptor are both coupled to G_s, the G-protein responsible for stimulating cAMP formation. We sought to determine if overexpression of a G_i-coupled receptor, such as the α_2-adrenergic receptor, would also lead to inappropriate activation. α_2-Adrenergic receptors are normally expressed in islet β-cells *(9)* and their activation may result in hyperglycemia owing to inhibition of insulin secretion *(10)*. By stably transfecting this receptor into RIN 1046-38 cells, a glucose-responsive β-cell line *(11)*, we asked what the consequences of such overexpression and possible receptor activation would be for regulation of insulin secretion.

Results

In order to study the consequences of overexpressing a G_i-coupled receptor in insulin-secreting β-cells, we stably transfected the mouse α_{2a}-adrenergic receptor into the rat β-cell line RIN 1046-38. We used an epitope-tagged version of the receptor (adra2a-Tag; Fig. 1) cloned in either pREP4 or pCDNA3 mammalian expression vectors. Clones selected for either G418 (adra2aTag-pCDNA3) or hygromycin (adra2aTag-pREP4) resistance were screened for adra2a-Tag expression by RT-PCR (Fig. 2A), and four clones were selected for further study. All four clones, 130 and 150 transfected with adra2aTag-pREP4 and 1118 and 1126 transfected with adra2aTag-pCDNA3, gave a 515-bp band in the RT-PCR reaction that was specific for adra2a-Tag and not present in vector-transfected cells (line pCDNA3). Adra2a-Tag mRNA expression was also detected by Northern blot of total RNA in clones 130, 150, and 1126, but not in clone 1118 (Fig. 2B).

Cell-surface expression of receptor protein was examined by indirect immunofluorescence and binding assays.

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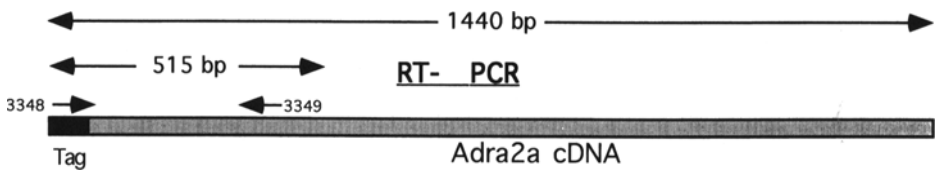

Fig. 1. Adra2a-Tag construct. A 1440-bp cDNA containing the HA epitope tag (in black) and adra2a coding region (in gray) was ligated into pCDNA3 and pREP4 mammalian expression vectors. Primers 3348 and 3349 were used in RT-PCR to detect mRNA from this construct in transfected RIN cells. The primers amplify a 515-bp fragment corresponding to the HA epitope and nucleotides 1–485 of the adra2a coding sequence. Primers 9272 and 9273 were used to amplify a 204-bp fragment (corresponding to the HA epitope and nucleotides 1–179 of the adra2a coding sequence) for use as a probe in Northern blot of RIN cell RNA.

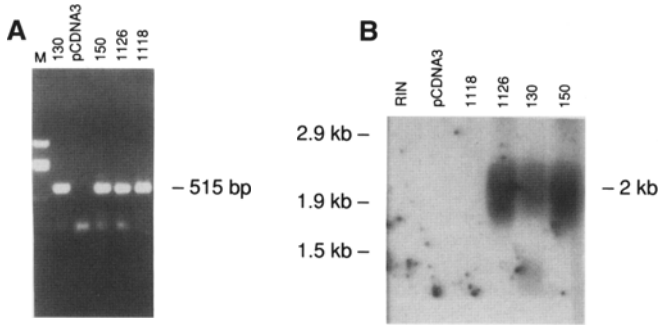

Fig. 2. RT-PCR and Northern blot of RIN 1046-38 cells expressing the adra2a-Tag receptor. **(A)** RT-PCR of vector-transfected clone (pCDNA3) and clones stably transfected with pREP4-adra2a (130 and 150) and pCDNA3-adra2a (1126 and 1118). A PCR fragment of 515 bp was specific for adra2a-Tag and detected only in clones 130, 150, 1126, and 1118, but not in vector-transfected clones. **(B)** Northern blot of the same clones. A PCR fragment of approx 200 bp was used as an adra2a-Tag probe as described in Material and Methods. Northern blot was unable to detect expression in clone 1118 even with longer times of exposure.

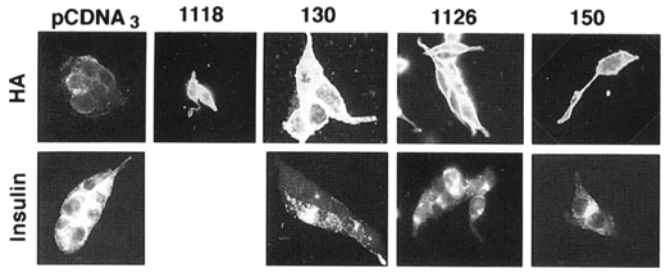

Fig. 3. Indirect immunofluorescence to detect epitope-tagged adra2a receptors (top row: "HA"). First and second antibodies (mouse anti-HA and goat antimouse FITC-conjugated) were used in nonpermeabilized cells in order to detect the presence of the adra2a-Tag receptor in RIN 1046-38 cells. Vector-transfected cells are labeled pCDNA3. Indirect immunofluorescence to detect insulin content is shown in the bottom row. In this case, cells were permeabilized with saponin, and incubated with antiporcine insulin and FITC-conjugated secondary antibodies as previously described.

Using the monoclonal antibody (MAb) 12CA5 directed against the HA epitope tag in nonpermeabilized cells, we were able to detect substantial immunoreactivity in the four clones overexpressing adra2a-Tag as compared with background staining in cells transfected with pCDNA3 alone (Fig. 3). Moreover, the binding of the selective α_2-antagonist RX 821002 was found to be markedly increased in both cells (not shown) and membranes (Table 1) of clones expressing the adra2a-Tag construct. Clones 1118, 130, 1126, and 150 showed three to four times more binding sites for RX 821002 than pCDNA3 transfected cells (Table 1).

Table 1
RX821002 Binding to RIN 1046-38 Cell Membranes from Vector- and Adra2a-Tag-Transfected Cells

Clone #	pmol/mg Protein
pCDNA3	0.71 ± 0.1
1118	1.9 ± 0.1
130	2.1 ± 0.1
1126	2.8 ± 0.2
150	3.0 ± 0.2

Basal cAMP levels in clones 1118, 130, 1126, and 150 were found to be slightly lower than pCDNA3, although the differences were not significant (Table 2). Forskolin (FK) was able to raise intracellular cAMP levels in all the clones, including 1126 and 150, the ones that overexpressed the highest amount of receptor. The α_2-selective agonist UK14,304 was able to inhibit this FK stimulatory response in all clones and its effect was reversed by both the α_2-selective antagonist RX821002HCl and pertussis toxin (PTX) (Table 2).

In order to study the physiological consequences of the overexpression of adra2a-Tag receptor in RIN cells, we measured insulin content by indirect immunofluorescence in permeabilized cells (Fig. 3) and by radioimmunoassay (Table 3). Clones 130, 1126, and 150 were found to have reduced insulin immunoreactivity by both immunofluorescence and radioimmunoassay. The insulin content of all three clones was 1/5th that of pCDNA3 by radioimmunoassay (Table 3). Clone 1118, however, showed no difference as compared with pCDNA3 by immunofluorescence (data not shown in Fig. 3) and radioimmunoassay (Table 3). Reduced mRNA levels for insulin in clones 130, 1126, and 150 were observed on Northern blot (data not shown).

Table 2
cAMP Response of Stably Transfected RIN 1046-38 Clones (cAMP/ATP × cAMP) × 100

				FK + UK 14,304	
	Basal	FK 50 μ*M*		+RX821002HCl	+PTX 0.1 μg/mL
pCDNA3	0.62 ± 0.11	1.63 ± 0.15[a]	1.14 ± 0.12[d]	1.78 ± 0.38	1.52 ± 0.18
1118	0.38 ± 0.05	0.91 ± 0.12[a]	0.75 ± 0.04	1.01 ± 0.09	0.96 ± 0.02
130	0.43 ± 0.03	1.62 ± 0.14[a]	1.07 ± 0.16[c]	1.22 ± 0.39	1.72 ± 0.08
1126	0.42 ± 0.04	3.32 ± 0.18[b]	1.87 ± 0.13[d]	3.38 ± 0.32	2.17 ± 0.25
150	0.41 ± 0.03	2.83 ± 0.72[a]	1.72 ± 0.35[c]	2.29 ± 0.66	2.08 ± 0.46

[a] $p < 0.05$.
[b] $p < 0.001$ as compared with basal.
[c] $p < 0.05$.
[d] $p < 0.001$ as compared with FK 50 μ*M*.

Table 3
Insulin Content of RIN 1046-38 Cell Clones Expressing Adra2a-Tag

Clone #	fg of Insulin/cell
pCDNA3	67.8 ± 5.5
1118	58.5 ± 11.5
130	12.6 ± 2.4[a]
1126	12.9 ± 1.4[a]
150	11.9 ± 1.2[a]

[a] $p < 0.01$ as compared to pCDNA3.

Finally, we studied the effect of the overexpression of adra2a-Tag receptor on insulin secretion in response to various secretagogs. Preliminary secretion studies had shown a similar insulin secretion pattern in clones 130, 1126, and 150 (data not shown); clone 1118, in contrast, showed comparable responses to that of vector-transfected cells (data not shown). Therefore, only clones pCDNA3 and 150 were studied in detail. Glucose, GIP, FK, carbachol, KCl, and glibenclamide all stimulate insulin secretion in clones pCDNA3 and 150 (Table 4). In vector-transfected cells, the stimulatory response of different stimuli as compared with basal was as follows: glucose twofold, GIP 2.4-fold, FK fourfold, carbachol threefold, KCl ninefold, and Glibenclamide fivefold. In clone 150, basal insulin secretion was reduced fivefold as compared to pCDNA3, although all secretagogs were still able to stimulate insulin secretion: glucose twofold, GIP twofold, FK fourfold, carbachol twofold, KCl eightfold, and Glibenclamide fourfold. α_2-agonist clonidine was able to suppress either basal insulin secretion or insulin secretion in response to β-cell secretagogs in both pCDNA3 and 150 clones. Clonidine inhibition was PTX-sensitive in both pCDNA3 and 150 clones (Table 4). In pCDNA3 clone, PTX increased glucose-stimulated insulin secretion in the presence of clonidine above the value without clonidine (3.38 vs 2.89, respectively). Insulin secretion in clone pCDNA3 treated with PTX and no clonidine was comparable, 3.57. In clone 150, PTX not only reversed clonidine inhibition, but increased basal and secretagog-stimulated insulin secretion above values obtained without clonidine (Table 4). PTX treatment alone increased insulin secretion of clone 150 cells to values comparable to or greater than observed with additional incubation with clonidine (basal = 0.48, glucose = 1.87, GIP = 1.81).

Discussion

We stably transfected RIN 1046-38 cells *(11)* with an epitope-tagged mouse α_{2a}-adrenergic receptor using two different mammalian expression vectors. There are at least three subtypes of α_2-adrenergic receptor *(12)*, and the subtype(s) endogenously expressed in islet β-cells is not clear *(9)*. A homolog of the human α_2-C2 receptor was identified in neonatal rat islets, but in a cultured β-cell line, homologs of the α_2-C4 and α_2-C10 receptors were detected *(13)*. Since significant differences between these subtypes in terms of G_i coupling have not been defined, we chose the mouse α_2-C10 homolog for this study.

Four different clones (two each for each of the expression vectors) were isolated that overexpressed α_2-adrenergic receptors as shown by RT-PCR and Northern analysis of the corresponding mRNA, by immunocytochemistry using an MAb specific for the epitope tag, and by direct binding studies using an α_2-adrenergic receptor-specific radioligand. We measured basal and forskolin-stimulated cAMP levels in the four clones in comparison with vector-transfected cells. If α_2-adrenergic receptor overexpression leads to tonic activation of G_i-proteins, we expected that cAMP levels would be lower and less responsive to forskolin. Although basal cAMP levels were lower in the receptor-transfected clones, the difference was not quite significant. Forskolin stimulation, moreover, was as high as or even higher for some clones than that in vector-transfected control cells. The higher values for forskolin stimulation in clones 150 and 1126 compared with clone 130 could reflect clonal variation in postreceptor components of the cAMP pathway. All of the cells were still susceptible

Table 4
Insulin Secretory Response (ng/10^6 cells/90 min)
of RIN 1046-38 Cells Transfected with Vector (pCDNA3) or Adra2a-Tag (Clone# 150)

		Clonidine 1 μ*M*	Clonidine 1 μ*M*
pCDNA3			PTX 0.1 μg/mL
Basal	1.39 ± 0.09	1.03 ± 0.13[c]	1.42 ± 0.14
Glucose 1 m*M*	2.89 ± 0.36[b]	1.04 ± 0.12[d]	3.38 ± 0.74
GIP 1 μ*M*	3.31 ± 0.71[a]	1.4 ± 0.3[c]	1.67 ± 0.15
FK 50 μ*M*	5.44 ± 0.77[b]	2.75 ± 0.62[c]	3.36 ± 0.67
Carbachol 100 μ*M*	3.81 ± 0.52[b]	2.23 ± 0.51[c]	2.87 ± 0.74
KCl 20 m*M*	12.11 ± 1.0[b]	5.63 ± 1.27[d]	13.01 ± 1.13
Glibenclamide 1 μ*M*	6.80 ± 0.69[b]	2.38 ± 0.7[c]	6.77 ± 0.81
150			
Basal	0.28 ± 0.07	0.21 ± 0.08	0.55 ± 0.02
Glucose 1 m*M*	0.41 ± 0.16[a]	0.27 ± 0.03	1.50 ± 0.86
GIP 1 μ*M*	0.57 ± 0.06[a]	0.32 ± 0.18	1.55 ± 0.65
FK 50 μ*M*	1.21 ± 0.14[a]	1.08 ± 0.69	2.03 ± 0.62
Carbachol 100 μ*M*	0.41 ± 0.07[a]	0.63 ± 0.44	1.40 ± 0.65
KCl 20 m*M*	2.09 ± 0.79[a]	1.73 ± 0.56	4.29 ± 0.18
Glibenclamide 1 μ*M*	1.03 ± 0.22[a]	0.31 ± 0.01	4.56 ± 0.02

[a] $p < 0.05$.
[b] $p < 0.001$ as compared with basal.
[c] $p < 0.05$.
[d] $p < 0.001$ as compared with no clonidine.

to inhibition by a high concentration of a specific α_2-adrenergic receptor agonist, and this inhibition was reversed by PTX. Lack of a reduction in forskolin-stimulated cAMP production does not exclude activation of G_i-proteins in clones overexpressing α_2-adrenergic receptors. The insulin secretion inhibitory pathway involves multiple receptors, G-proteins, and effectors with varying degrees of specificity in their interactions (*see 4* for review). Norepinephrine, acting through α_2-adrenergic receptors, can markedly inhibit insulin secretion without any reduction in cAMP levels *(14)*, and relatively high concentrations of norepinephrine are required to see significant inhibition of forskolin-stimulated cAMP levels *(15)*. Although supersensitivity (compensatory elevation in cAMP response after chronic stimulation with norepinephrine) was not observed in RINm5F cells compared with other cell types, such as NG108-15 *(15)*, this type of adaptive change may have occurred in the RIN 1046-38 cell clone we used in our studies.

We found a reduction in insulin content and in mRNA in clones overexpressing α_2-adrenergic receptors. The clone with the lowest degree of receptor overexpression, 1118, showed the least reduction in insulin content, and was not significantly different from vector-transfected cells in studies of insulin secretion. The other three cell clones in contrast showed significantly impaired insulin secretion that could be partially reversed by PTX treatment. Note that PTX treatment did not simply reverse the modest inhibition seen with clonidine in clone 150 cells, but actually increased insulin secretion to values above those seen without clonidine, although not to values as high as those of the control clone pCDNA3. We believe that these observations reflect inappropriate activation of G_i-type proteins caused by α_2-adrenergic receptor overexpression. Chronic (48 h in a cell-culture model) α_2-adrenergic receptor activation has been shown to decrease insulin mRNA levels *(16)*, and the effects we observed presumably reflect such longer-term activation. α_2-Adrenergic receptor agonists are also known to have powerful inhibitory effects on insulin secretion at steps relatively distal in the exocytotic pathway. Such inhibition occurs without any measurable reduction in cAMP levels *(4)*, and presumably also contributed to the impaired insulin secretion we observed. The partial reversibility we noted with PTX treatment implicates G_i-type proteins. Lack of full reversal could imply involvement of other types of G-proteins, but could also be owing to the inability to replenish fully insulin content over the course of PTX treatment. Our studies were not directed at identification of the specific G-proteins and effectors activated by α_2-adrenergic receptor overexpression, but the availability of these clones should permit such studies.

Although the results were obtained in a cell-culture model, they may be relevant in vivo. Overexpression of the same receptor in islet β-cells of transgenic mice also leads to impaired insulin secretion (Rodriguez-Pena et al., unpublished observations). These results suggest that inappropriate activation of α_2-adrenergic receptors, and possibly other inhibitory receptors, such as somatostatin and galanin receptors or the G-proteins to which they are coupled, could contribute to reduced insulin secretion in pathologic states,

such as noninsulin-dependent diabetes mellitus (NIDDM). Although insulin resistance is a key feature of NIDDM, several lines of evidence (*see 17* for review) suggest that an additional defect in insulin secretion is required. In certain rare forms of NIDDM, specific defects in the normal stimulatory pathway for insulin secretion, e.g., deficient glucokinase activity, have been identified (*see 18* for review). The possibility that inappropriate activation of components of the inhibitory pathway regulating insulin secretion contribute to the development of NIDDM deserves further study.

Materials and Methods

Buffers, salts, bovine serum albumin (BSA), 1-methyl-3-isobutylxanthine (IBMX), FK, GIP, carbachol, glibenclamide, and cAMP were from Sigma Chem. Co. (St. Louis, MO). α_2-Adrenergic agonists and antagonists were obtained from RBI (Natick, MA). Tissue-culture media and reagents were obtained from either Biofluids (Rockville, MD) or Gibco (Gaithersburg, MD). Restriction enzymes and expression vectors were purchased from Promega (Madison, WI) and Invitrogen (San Diego, CA), respectively.

Plasmids and Vectors

A cDNA encoding a mouse homolog (Adra2a) of the human α_2-C10 adrenergic receptor with a nonapeptide epitope tag (HA) derived from the influenza virus hemagglutinin protein at the amino-terminus (Fig. 1) was obtained from Link and Kobilka (Stanford) in pBC12BI *(12)*. An *NcoI*/*SalI* restriction fragment containing the epitope-tagged receptor cDNA was gel-purified and ligated into both pCDNA3 and pREP4 vectors, yielding plasmids pCDNA3-adra2a and pREP4-adra2a, respectively, for stable transfection into RIN cells.

Cell Culture and Stable Transfection

The RIN 1046-38 cell line was kindly provided by S. Clark (Betagene, Dallas). Cells were cultured in Dulbecco's Modified Eagle's Medium DMEM/F-12 containing 10% fetal calf serum, 2 m*M* glutamine, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 17.5 m*M* glucose at 37° (95% O_2/5% CO_2) in a humidified incubator. Stable transfection was carried out by lipofection at 37° for 18 h. RIN cells (passage 20–25) were grown in 100-mm plates until they reached ~50% confluence. Five micrograms of plasmid DNA (pCDNA3, pREP4, pCDNA3-adra2a, or pREP4-adra2a) were mixed with 40 μg lipofectamine (Gibco BRL) and added to cells in serum- and antibiotic-free media. Selection with 400 μg/mL G418 (for pCDNA3) or 500 μg/mL hygromycin (for pREP4) was started on the third day after transfection, and clones were hand-picked 2 wk after the selection was started.

RNA Preparation, RT-PCR, and Northern Blot

Total RNA was extracted from RIN 1046-38 cells and clones using TRIZOL reagent (Gibco BRL). Reverse-transcription reaction was carried out with 2 μg total RNA, 300 ng random primers, 40 U Ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) in a final volume of 50 μL, with buffer and incubations according to the instructions of Stratagene (La Jolla, CA). Separate reactions tubes without reverse transcriptase were used as controls. PCR was performed in a 9600 Perkin-Elmer/Cetus (Norwalk, CT) thermal cycler using 5 μL of cDNA template, 0.5 μ*M* of each primer, 3348 (forward: 5' TACCCATACGACGTC CCAGACTACG 3') and 3349 (reverse: 5' GCCGAGATG ACCCACACGGTGACAA 3') and 2.5 U of Taq polymerase in 50 μL final volume with standard buffer conditions according to the manufacturer. The thermal conditions were as follows: denaturation at 94°C for 5 min, then 30 cycles of (94°C, 15 s; 60°C, 1 min), and a final extension at 72°C for 5 min. Primers 3348 and 3349 were designed to amplify specifically adra2a-Tag mRNA (Fig. 1). Northern Blot was performed with 5 μg of total RNA; PCR fragments containing cDNA sequences of mouse adra2a and insulin were used as probes. Primers 9272 (forward: 5' GTGAAC ACCGCGATAATAACC 3') and 9273 (reverse: 5' ATA CGACGTCCCAGACTACG 3') were used to amplify a 204-bp fragment within the 5'-region of adra2a-Tag (Fig. 1). Primers 303 (forward: 5' CATCAG CAAGCAGGTTAT TGT 3') and 304 (reverse: 5' CTCCAC TTCACGGCG GGACAT 3') were used to amplify a 204-bp fragment in both rat insulin I and II mRNAs.

Indirect Immunofluorescence Assays

Approximately 2×10^4 cells were seeded into individual wells in a 6-well Costar multiwell plate containing a 18-mm sterilized glass cover slip. After 48–72 h, cells were fixed with 2% formaldehyde in PBS for 15 min at room temperature. Saponin 0.1% was added in subsequent steps whenever permeabilization was required. After two rinses with PBS, nonspecific binding was blocked with 10% FCS in DMEM for 1 h at 37°C. Cells were then incubated with the primary antibodies: mouse 12CA5 (10 μg/mL) (Boehringer, Indianapolis, IN) directed against the HA epitope tag or guinea pig polyclonal antiporcine insulin (DAKO, Carpinteria, CA) (1:100) in DMEM/10% FCS for 2 h at 37°C. The cover slips were rinsed again in the same buffer and then incubated in a 1:100 dilution of FITC-conjugated goat antimouse or anti-guinea pig IgG (Sigma and Kierkegard & Perry, Gaithersburg, MD, respectively) for 1 h at 37°C. Cover slips were then mounted on microscope glass slides in a drop of glycerol/PBS (1:1, v/v) for viewing in a fluorescence microscope.

Membrane Preparation

Cells were grown in 162-cm flasks until confluency. Then, they were washed twice with PBS, harvested from the culture flask, and homogenized with a Polytron (30 s at maximum speed) in ice-cold 5 m*M* Tris-HCl, pH 7.4, and

2 m*M* EDTA. Membranes were pelleted at 30,000*g* for 15 min at 4° and resuspended in 2 mL of membrane buffer (75 m*M* Tris-HCl, pH 7.4 containing 2 m*M* EDTA and 12.5 m*M* $MgCl_2$). Protein content was determined according to the method of Bradford (Bio-Rad, Hercules, CA). Membranes were then frozen at –80° until use.

Radioligand Binding Assays

Binding experiments were performed in both cells (grown in 24-well plates for 48 h before the experiment) and 50 μg of membranes in 0.2 mL membrane buffer containing 0.9% NaCl (v:w), 0.1% BSA (v:w), 1 n*M* [^{3}H]RX 821002 (59 Ci/mmol; Amersham, Arlington Heights, IL) and varying concentrations of RX 821002 for 2 h at room temperature. Nonspecific binding was determined in the presence of 1 μ*M* RX 821002. Separation of bound and free radioactivity was carried out in cells, by several washes with membrane buffer/0.9% NaCl (v:w), followed by lysis with 0.5 mL of 0.1 *N* NaOH, and in membranes by vacuum filtration through GF/C filters. Bound radioactivity was then collected in tubes and counted in a β-counter. Data were analyzed using the iterative program LIGAND *(19)*.

cAMP Assays

25–50 × 10^4 Cells were loaded in 24-well plates and allowed to grow for at least 48 h in DMEM/10% FCS. Twelve hours before the assay, the cells were labeled by adding [^{3}H]adenine (23 Ci/mmol; Amersham) 2 μCi/mL in the same medium with or without 0.1 μg/mL of PTX (Gibco BRL). On the day of the assay, the medium was removed and the cells were washed with DMEM containing 10 m*M* HEPES, pH 7.4, 1 m*M* IBMX, and 50 μ*M* forskolin. Incubations with agonist (UK 14,304 1 μ*M*) and antagonist (RX 821002 10 μ*M*) were performed at 37°C for 15 min, and then, the assay medium was removed and the cAMP extracted with 1 mL of 5% TCA containing 0.1 m*M* cAMP. cAMP was separated by chromatography using a Dowex gel, followed by aluminum oxide *(20)*.

Insulin Secretion Studies

Insulin content and insulin secretion were measured in cells growing in 24-well plates. Briefly, the cells were rinsed twice at 37°C for 30 min with HEPES-balanced Krebs-Ringer bicarbonate buffer (HBKRB) containing HEPES 20-m*M*, pH 7.4, 114 m*M* NaCl, 4.7 m*M* KCl, 1.16 m*M* $MgSO_4$, 1.21 m*M* KH_2PO_4, 25.5 m*M* $NaHCO_3$, 2.5 m*M* $CaCl_2$, and BSA 0.1% (v:w) equilibrated with 95% O_2/5% CO_2. Secretagogs were added to fresh media in the absence or presence of 1 μ*M* clonidine or 1 μ*M* clonidine + 0.1 μ*M* PTX at the final concentrations shown in Table 4. Cells were incubated with agonists at 37°C for 90 min. Then, media were collected and frozen at –30°C until assayed, and cells were trypsinized and counted in a Neubauer chamber. Intracellular insulin extraction was carried out with acid-ethanol at –20°C for 12 h. Insulin was determined by radioimmunoassay using rat insulin as standard (Bios Pacific, Emeryville, CA; standard curve range from 0.05–5 ng/mL), guinea pig–antirat insulin polyclonal antibody and monoiodinated human insulin as tracer (LINCO Res Inc., St. Charles, MO). Bound insulin was pelleted by immunoprecipitation with a goat anti-guinea pig IgG and counted in a γ-counter. Interassay and intraassay coefficients of variation were <10%, and detection limit was 0.05 ng/mL.

Statistics

Results were expressed as mean ± SEM. Differences between means were analyzed by Student's paired or unpaired *t*-test as appropriate and were considered significant when $p < 0.05$.

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