FEBS Letters 346 (1994) 257–262

FEBS 14099

Identification of the $GABA_A$ receptor subtype mRNA in human pancreatic tissue

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Received 16 March 1994; revised version received 2 May 1994

Abstract

Evidence suggests a physiological role of the $GABA_A$ receptor in the pancreas. Clinically, an autoimmune reaction involving the GABA biosynthesizing enzyme, glutamic acid decarboxylase has been implicated in the development of insulin-dependent diabetes mellitus. To determine the subtypes of $GABA_A$ receptor expressed in human pancreas, we analyzed, with the use of the reverse-transcription/polymerase chain reaction technique human pancreatic tissue for the presence of $GABA_A$ receptor subunits α1–6, β1–3, and γ1–2 transcripts. Unlike brain tissue, pancreatic tissue only expresses the α2, β3 and γ1 subunits. Our results provide evidence of a specific $GABA_A$ receptor subtype expressed in human pancreatic tissue.

Key words: GABA; Human pancreas; $GABA_A$ receptor; Subunit

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS). It mediates its inhibitory action through a receptor complex, $GABA_A$, that is a hetero-oligomeric protein composed of several distinct polypeptide subunits [1]. In addition to GABA binding sites, the $GABA_A$ receptor also contains several independent modulatory sites for therapeutic useful drugs such as benzodiazepines, barbiturates and neuroactive steroids [2,3]. Molecular cloning has identified at least 16 $GABA_A$ receptor subunits that can be grouped into several families (α-, β-,γ-,δ- and ρ-) [4,5]. Functional expression of these subunits in mammalian cell lines or *Xenopus* oocyte results in $GABA_A$ receptors with differential pharmacological characteristics [6,7]. The multiplicity of $GABA_A$ receptors expressed in the CNS provides one explanation for the complex pharmacology observed in brain homogenate [8,9].

In addition to the important role that GABA plays in the brain, the presence of GABA in nonneuronal organs has been demonstrated [10,11]. This suggests the involvement of GABA in the physiological functions of these nonneuronal tissues. However, the concentrations of GABA in these tissues are generally low, about 1% of that in brain, with the exception of the female genital tract [12] and pancreatic islets [13]. In pancreatic islets, the GABA concentration is comparable to that of the CNS [14], however, it is still unclear why such a high concentration of GABA exists in the endocrine pancreas. In situ studies of GABA regulation of pancreatic hormone release have been controversial [15,16]. Recently though, the function and characterization of the GABA system in the pancreas has become a focus of interest as glutamic acid decarboxylase, a GABA biosynthesizing enzyme, is a dominant autoantigen in insulin-dependent diabetes mellitus [17]. This 65 kDa pancreatic β cell protein is a target of an autoimmune response in patients with this disease [18]. In pancreatic islet, evidence suggests GABA and insulin co-secretion from β cell, but no GABA receptors were detected by [^{3}H]-GABA binding in the β cells[11]. A recent electrophysiological study revealed a GABA-triggered inhibitory hyperpolarization and cessation of spiking activity in guinea-pig pancreatic α cells [19]. The Cl^- dependent current elicited by GABA is completely and reversibly blocked by the $GABA_A$ receptor antagonist bicuculline. These results suggest that α cells contain $GABA_A$receptors similar to those described in the CNS. However, it is not known whether human pancreas expresses a $GABA_A$ receptor or whether the subunits required to form a functional $GABA_A$ receptor share the same diverse repertoire as seen in the CNS. With the use of 11 major $GABA_A$ receptor subunit-specific oligonucleotide pairs and reverse-transcription/polymerase chain reaction (RT/PCR) technique, we demonstrate that human pancreatic tissue expresses a limited population of the $GABA_A$ receptor subunits found in the CNS.

2. Materials and methods

2.1. Isolation of human cortex total RNA

Human cortex tissue was obtained from the Alzheimer's Disease Research Center at the University of Southern California and the total

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SSDI 0014-5793(94)00485-E

RNA was isolated using a single step phenol/phenol–chloroform method [20]. Human pancreas total RNA was purchased from Clontech (Palo Alto, CA). Prior to each experiment, aliquots of both RNA samples were centrifuged, rinsed with 70% ethanol and resuspended in sterile diethylpyrocarbonate (DEPC)-treated H_2O.

2.2. Design of $GABA_A$ receptor subunit-specific oligonucleotide primers

All the primers were localized to highly diverse regions of $GABA_A$ receptor family [5], the sense primer to the extracellular amino terminus domain and antisense primer to the large intracellular loop between putative transmembrane domains 3 and 4 (Fig. 1). All the primers (Table 1 and Fig. 1) used in the amplification were synthesized by Keystone Laboratories Inc. (Menlo Park, CA).

2.3. Reverse transcription and PCR

The reverse transcription reaction was prepared as previously described [21]. The first strand cDNA was synthesized from 1 mg of total RNA of human cortex or pancreas. Prior to the reaction, RNA samples were denatured at 65°C for 5 min then quickly chilled. The reaction was carried out in a 30 μl mixture of the following: 40 units of recombinant M-MuLV reverse transcriptase with RNase H activity (Boehringer Mannheim, Indianapolis, IN), 10 units of human placental ribonuclease inhibitor (BRL, Maryland), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 3 mM DTT, and 10 pmol of 12–18-mer oligo(dT) primer (Pharmacia, Piscataway, NJ). The mixture was incubated at 42°C for 60 min and then heated at 65 °C for 5 min. One-tenth of the cDNA product was amplified in a Perkin-Elmer Cetus DNA Thermal Cycler (Norwalk, CT) in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM of each deoxynucleotide triphosphate (dNTP), 20 pmol of each specific primer, 1.5 mM $MgCl_2$, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The PCR parameters were 94°C for 5 min, 55°C for 1 min and 72°C for 1.5 min for the first cycle followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, followed by an 8 min extention at 72° C. For the semiquantitative comparison of mRNA levels between cortex and pancreas, 10, 20, 30, 40, and 50 cycles were used instead of 40 cycles. One fifth of each PCR product was electrophoretically separated on a 1% agarose gel (Seakem, FMC, Rockland, ME) containing ethidium bromide.

2.4. Southern hybridization

The PCR products separated on the agarose gel were transferred to a hybridization transfer membrane (Gene Screen Plus, NEN, DuPont) by capillary action in 0.4 N NaOH solution and neutralized in 1 M Tris (pH 7.4), 1.5 M NaCl. The DNA was immobilized by UV-cross linking (Stratalinker, Stratagene, La Jolla, CA). The Southern hybridization was performed using 5′ end labeled subunit-specific internal primers (Table 2, Fig. 1). The pooled subunit specific oligonucleotide probes were 5′ end-labeled in a 20 ml mixture containing 8 units of T4 polynucleotide kinase (Promega, WI), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 50 mCi of [^{32}P]ATP (3,000 Ci/mmol, DuPont, NEN). The labeling reaction was carried out at 37°C for 45 min followed by 68°C for 5 min to terminate the reaction. The blots were prehybridized for 1–2 h and then hybridized with 10^6 dpm/ml probe in a 50% formamide, 5 × SSC solution at 37°C for 16 h before washing 2–3 times in 2 × SSC solution at 37°C for 10 min, followed by exposure to XAR-5 Kodak film at −80°C with an intensifying screen.

2.5. Cloning of the PCR product and sequencing

The PCR product of α4 from brain and α2, β3 and γ1 from pancreas were run on a 0.7% low melting point agarose gel (GIBCO BRL, Gaithersburg, MD) and bands isolated. The isolated PCR products were subcloned into pCR II vector following the InVitrogen pCR II vector cloning protocol (InVitrogen, La Jolla, CA). The brain α4 and pancreatic α2 , β3 and γ1 were sequenced on both strands by dideoxy chain termination (Sequenase II, USB) [22].

In the control experiment for cDNA or chromosomal DNA contamination of the total RNA, 500 ng of DNase free, RNase (BMB, Indianapolis, IN) was used in a mixture containing 1 mg total RNA from either human cortex or human pancreas. The treatment was carried out at 37°C for 30 min before or after a reverse-transcription reaction. The mixture then prepared for PCR as above.

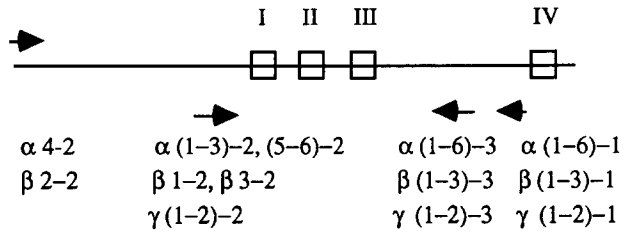

Fig. 1. Diagram of all the oligonucleotide primer locations. Box I to box IV indicate the four putative transmembrane domains. The arrows show the orientation of the PCR amplification.

3. Results

Based on the expected size of the PCR amplification product, all 11 $GABA_A$receptor subunit transcripts (α1–6, β1–3, and γ1–2) were detected in the human cortex (Fig. 2B,D). The human α4 subunit has not been cloned, but the size of PCR product corresponds to that expected from the bovine sequence. Therefore, the PCR product from brain total RNA amplified from the α4 specific primers was subcloned and sequenced. The result was a 95% homology to bovine α4 cDNA in nucleotide sequence and 93% in amino acid sequence within the amplified region (data not shown) suggesting that human α4 $GABA_A$ receptor subunit was amplified by these primers.

Using the same approach applied to human pancreas, only α2, β3 and γ1 PCR products were detected (Fig. 2A,C) even when the PCR cycle number was increased to 50.

The nucleotide sequence of the PCR products of the human pancreas α2, β3 and γ1 was further determined and found to be identical to that of human brain subunits (data not shown).

In order to exclude the possibility that the PCR amplification of human pancreas RNA arose from chromosomal DNA or laboratory cDNA contamination, a control experiment was performed using RNase A digestion of the human pancreas and human cortex total RNA samples before or after the reverse-transcription synthesis of first strand cDNA. Aliquots were then amplified as above with either α2 or β3 specific primers and visualized by ethidium bromide staining (Fig. 3). The results show that if the RNase A digestion was carried out before the reverse transcription, no α2 and β3 product was amplified. For samples treated with RNase A after the reverse transcription the correct α2 and β3 products were amplified. This indicated that the first-strand cDNA template was indeed derived from its corresponding RNA transcript.

4. Discussion

It is well documented that the concentration of GABA

in pancreatic islet is comparable to that in the CNS [14] and that pancreatic β-cells possess the enzymes necessary for GABA synthesis and degradation [23]. In the isolated perfused pancreas [24], or cultured islets [25], exogenous GABA and muscimol (a GABA receptor agonist) suppressed somatostatin release with no effect on insulin secretion. Although GABA was found to have no effect on insulin release in normal rats, small amounts of somatostatin (50 mg/h/i.v.) could regulate, moderately, basal insulin and glucagon levels in partial hypoinsulinemic humans resulting in a significant change in blood glucose concentration in blood [26]. If GABA was released from β-cells and acted as a modulator of somatostatin, its action would probably be mediated by specific receptors located on the target cells. However, the GABA receptor has not been characterized in the islet even though preliminary studies revealed [^{3}H]GABA binding sites on peripheral cells of islet [27]. A recent investigation [19] using patch-clamp electrophysiological techniques suggests that GABA may mediate part of the inhibitory action of glucose on glucagon secretion by activating $GABA_A$ receptors in α cells of the islets, since the inward Cl^- current triggered by GABA and the effect of GABA on glucagon secretion can be abolished by bicuculline (a $GABA_A$ receptor antagonist). However, it is not known whether the $GABA_A$ receptor also exist in human tissue. Furthermore, the molecular nature of this putative $GABA_A$ receptor in pancreas would be of interest if it does exist. Using 11 sets of $GABA_A$ receptor subunit specific primers and the technique of reverse transcription/PCR, we show here that unlike brain tissue, only one specific variant of each subunit of the $GABA_A$ receptor transcript is expressed in human pancreatic tissue in a similar amount, but at a concentration much less than that found in brain. The relative low concentration of transcrips detected is consistent with a low percentage of pancreatic cells expressing $GABA_A$ receptors. None of the other 8 subunits were observed in pancreas even using 20 cycles beyond what was necessary for amplification, indicating that if they were expressed there, the concentration would be 2^{20} or one million times less than in the brain. This result supports the existence of $GABA_A$ receptors in human pancreas and suggests that the likely subunit composition of the $GABA_A$ receptor in pancreas may be α2β3γ1.

To date, as many as 16 $GABA_A$ receptor subunits have been cloned. However, the eleven human subunits tested probably represent the majority of the $GABA_A$ receptor subunits found in the brain based on the in situ hybridization of 13 $GABA_A$ receptor subunits in rodent CNS [28]. Among the 5 subunits not tested, ρ1 and ρ2 are primarily expressed in the retina [29] whereas gene products corresponding to β4, δ and γ3 subunits have not yet been identified in human brain.

Our result of pancreatic expression of 3 known $GABA_A$ receptor subunits could not exclude the possibility of other unknown subunit expressed in this tissue. To address the question, we have used a pair of universal

Table 1
Human $GABA_A$ receptor subunit-specific oligonucleotides used in the PCR to identify the $GABA_A$ receptor subunit expression in human pancreas

Subunit	Size (bp)	Name		Sequence 5′→3′		Reference
Hα-1	470	α1-1	1,351	caaattaggggtgtagctggttgctgttgg	1,322	[34]
		α1-2	881	caaacagt(a,t)gactctggaattgt	903	
Hα-2	440	α2-1	1,148	tgcataagcgttgttctgtatcat	1,125	(**)
		α2-2	708	ggccaatcaatcggaaaggagaca	731	
Hα-3	596	α3-1	365	ggtctcagtcgggctgtcctgcac	1,342	(**)
		α3-2	769	catgttgttgggacagagataatc	792	
Hα-4	1,354(*)	α4-1		gatatggtttcagctgcattg		[36]
		α4-2	20	ggcatgttgcaaagatggtttctg	43	
Hα-5	462	α5-1	1,434	agttgtaaaagcgtttgttgac	1,414	(**)
		α5-2	973	cacctgatggggcagacggtg	993	
Hα-6	471	α6-1	1,127	agatgatatttggagtcagg	1,107	(**)
		α6-2	664	tagtgagacaattaaatctaac	686	
Hβ-1	609	β1-1	1,278	gcgcccgtaggcctcgcggctgctcagggg	1,250	[34]
		β1-2	669	attgt(c,t)gactacaagatggtgtc	691	
Hβ-2	1,170	β2-1	1,325	taagatgttctcatgggggtccat	1,302	(**)
		β2-2	160	ctgggcagactaagttggatctcc	183	
Hβ-3	790	β3-1	1,114	gaacggtcattctttgccttggc	1,092	(***)
		β3-2	324	tattggagagataaaaggctcg	345	
Hγ-1	466	γ1-1	1,307	atcttcttgcggcacagaaat	1,287	(***)
		γ1-2	842	ctcaactgaaatcactcacacg	863	
Hγ-2	536	γ2-1	1,507	tcctgttcgacaatcttcaaaacagcagaa	1,478	(***)
		γ2-2	971	c(c,t)gaagtagtgaagacaacttc	993	

(*) The size of human α-4 PCR product is based on the bovine α-4 sequence data.
(**) International patent application published under the patent cooperation treaty, number: PCT/GB92/01031.
(***) Personal communication from Dr. Dolan B. Pritchett.

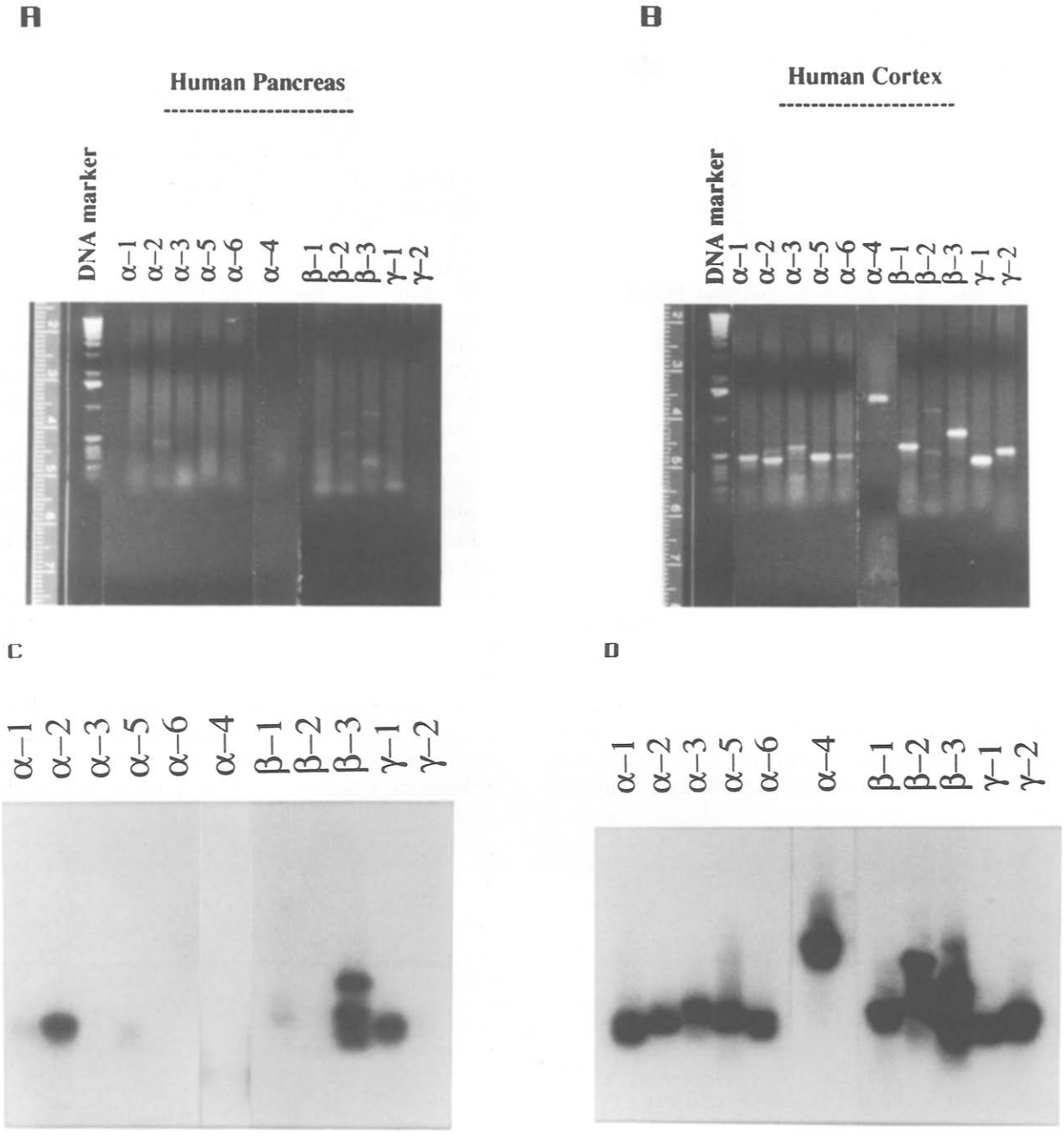

Fig. 2. PCR amplification product of human pancreas (A,C) and human cortex (B,D). The products amplified from each pair of subunit specific primers (see Fig. 1 and Table 1) in EtBr-containing agarose gel (A,B) and Southern hybridization analysis of the corresponding gel (C,D) using ^{32}P-labeled subunit specific internal primers (see Fig. 1 and Table 2).

primers generated from transmembrane domain 2 and 4 (TM2 and TM4) under a low stringency PCR condition (37°C for first 5 cycles and 42°C for 35 cycles). The PCR products ranged from 400 to 700 bp in size were isolated from a low melting agarose gel and subcloned into pCRII vector. About 200 individual clones were Southern hybridized with ^{32}P-labeled human $\alpha 2$ and $\beta 3$ primers located between TM2 and TM4 and about 20 clones showed a weak signal were sequenced. The result indicated that none of these 20 clones indicative of potential novel $GABA_A$ receptor subunit (data not shown). Thus we concluded that either no additional $GABA_A$ receptor subunit expressed in this tissue or the expression level was under the detection.

Functional expression of various combinations of $GABA_A$ receptor subunits has resulted in $GABA_A$ receptors with distinct pharmacological properties. Expression of different α subunit ($\alpha 1$ vs. $\alpha 2$ or $\alpha 3$) co-expressed with $\beta 1$ and $\gamma 2$ exhibit type I and type II benzodiazepine receptor pharmacology, respectively [6]. It has also been

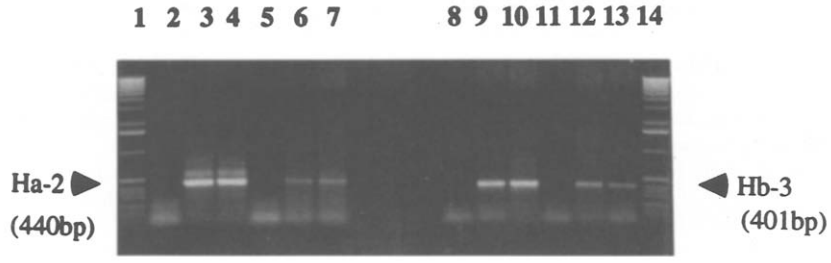

Fig. 3. Contamination control of PCR amplification of α2 and β3 subfragments. RNase treatment at 37°C for 30 min. Lanes 1 and 14: DNA molecular weight marker, Lanes 2–7: PCR amplification by α2 primers. 2 and 5: RNase treated cortex or pancreas total RNA before, or 3 and 6 after reverse-transcription. 4 and 7: no RNase treatment. Lanes 8–13: PCR amplification by β3 primers. 8 and 11: RNase treated cortex or pancreas total RNA before, or 9 and 12 after reverse-transcription. 10 and 13: no RNase treatment.

reported that differing γ-subunits confer various sensitivities to benzodiazepines influencing both the affinity and efficacy of various benzodiazepine site ligands as functional modulators of $GABA_A$ receptors. Benzodiazepines potentiated GABA effects on receptor complexes containing γ2 subunits much greater than these containing γ1 subunits [30,31]. Moreover, β-carbolines such as methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and methyl-β-carboline-3-carboxylate (β-CCM) act as negative $GABA_A$ receptor modulators in recombinants containing α1(α2) β1 and γ2 subunits but act as positive modulators at α1β1γ1 and α2β1γ1 receptors [32]. Thus compared to the γ2 subunit, the γ1 subunit has been demonstrated to provide a unique pharmacological profile relative to benzodiazepine modulation of the $GABA_A$ receptor complex. In human pancreas, we have found γ1 but not γ2 co-expressed with α2 and β3 subunits. Even though the actual functional role of γ1-containing $GABA_A$ receptors in vivo is not known, the unique pharmacology compared to other γ subunits may be significant in developing drugs that specifically modulate $GABA_A$ receptors in the pancreas.

Table 2
Internal oligonucleotides of the $GABA_A$ receptor subunit-specific PCR product used as probe in Southern hybridization analysis

Name		Sequence 5′→3′		Ref.
Hα1-3	1,246	ccatgcataacctctcttagtg	1,225	[34]
Hα2-3	1,103	cttgtcatttactacactcttccc	1,079	(**)
Hα3-3	1,170	cttcatctccagggcctctgg	1,150	(**)
Hα4-3		gaagattcttgaacaactgtgg		(*)
Hα5-3	1,386	cttgatcttggctgcttccaa	1,366	(**)
Hα6-3	1,022	cgccttctgtgtctgaagatt	1,002	(**)
Hβ1-3	1,218	gagtacatggtggccttggg	1,199	[34]
Hβ2-3	1,265	actggcagccttctcagctgc	1,245	(**)
Hβ3-3	725	gacaacattcctcgagaccagacggtgctc	696	(***)
Hγ1-3	1,277	tggaatcagagtggatccagg	1,257	(***)
Hγ2-3	1,459	gccgtccagacactcatagcc	1,439	(***)

(*) The sequence was obtained from sequencing data of partial cDNA cloned by the authors.
(**) International patent application published under the patent cooperation treaty, number: PCT/GB92/01031.
(***) Personal communication from Dr. Dolan B. Pritchett.

Based on electrophysiological studies, Roseman et al., [19] proposed that glucose inhibition of glucagon secretion may involve the co-secretion of GABA and insulin from β cells. Under normal conditions where insulin secretion is stimulated, glucagon release will be inhibited by the co-secreted GABA. Accordingly, they predict that a hypersecretion of glucagon would occur under conditions of β-cell function impairment. Recent functional identification of a GABA system in pancreatic tissue has focused considerable attention on the GABA-synthesizing enzyme GAD. The evidence accumulated suggested that GAD in pancreatic β cell, a dominant autoantigen served a major target of autoantibody associated with the development of insulin-dependent diabetes mellitus (IDDM) [33]. The cell-specific destruction of β cell in the patient of IDDM by autoimmune mechanisms would possibly provide a consideration of new regulation of glucose by other hormone systems such as glucagon. The current finding of a specific set of the $GABA_A$ receptor subunit transcripts, possibly a functional receptor complex expressed in human pancreas provides an opportunity for the development of therapeutically useful drugs interacting with this $GABA_A$ recpetor for regulating the secretion of glucagon.

Acknowledgments: The authors wish to thank Drs. John A. Drewe (CoCensys, Inc.) and Timothy Gallaher (University of Southern California, School of Pharmacy) for their constructive comments and the work on the manuscript.

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