Expression of Adenylyl Cyclase Subtypes in Endocrine Pancreatic Cell Lines

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RÉSUMÉ

Signal transduction through the cell membrane requires the participation of one or more plasma membrane proteins. For many transmembrane signalling events adenylyl cyclases (ACs) are the final effector enzymes. ACs integrate and interpret divergent signals from different pathways. The enzymatic activity of adenylyl cyclase (AC) is stimulated or inhibited in response to the activation of a large number of receptors. To date, nine different mammalian isoforms of AC have been cloned and characterised. Each isoform has its own distinct tissue distribution and regulatory properties, providing possibilities for different cells to respond diversely to stimuli. The product of the enzymatic reaction catalyzed by ACs, cyclic AMP (cAMP) has been shown to play a crucial role for a variety of fundamental physiological cell functions ranging from cell growth and differentiation, to transcriptional regulation and apoptosis.

The physiological importance of cAMP in the regulation of glucose-induced insulin secretion has been demonstrated in the endocrine pancreas, e.g. islets of Langerhans. Expression studies have revealed the existence of AC isoform types III, IV, V, VI and VII. However, the cellular distribution of AC isoforms in the different endocrine cell types located in islets of Langerhans have not been made. Therefore, in this study, five well-established endocrine cell lines, representing insulin (Rin5F and β-TC3)-, glucagon (INR1-G)-, and somatostatin (Rin14B and Rin1027-B2)- producing pancreatic cells have been used to study the expression of the different AC isoforms by using the reverse transcriptase polymerase chain reaction (RT-PCR) technique.

Our results show for the first time that AC types I, II, III, IV, V and IX are present in all pancreatic cell lines tested, whereas a different, cell specific expression was observed for AC types VI, VII and VIII. These findings may be of importance regarding the different regulation pattern found for the different AC isoforms in the endocrine pancreas.

1. INTRODUCTION

Cyclic 3',5' - adenosine monophosphate (cAMP) is a universal second messenger of major importance in the regulation of cellular function [1, 2, 3]. The intracellular concentration of cAMP is the net result of the formation from ATP by adenylyl cyclases (ACs), and the breakdown to adenosine 5'- monophosphate (AMP) by cAMP phosphodiesterases. Dr. Earl Sutherland was awarded the Nobel Prize in Physiology in 1971 for the discovery of cAMP .

The second messenger cAMP mediates diverse cellular processes, such as exocytosis, cell proliferation, ion transport, intermediate metabolism, memory and gene transcription, by activation of protein kinase A (cAMP-dependent protein kinase; PKA). Several hormones and neurotransmitters exert their function by binding to membrane-anchored polypeptide receptors that regulate the function of adenylyl cyclases (AC) by pathways that involve GTP-binding regulatory proteins (G proteins), for reviews see [4, 5, 6, 7, 8, 9, 10].

AC is a membrane-bound enzyme, consisting of twelve stretches of hydrophobic residues in conserved positions which are arranged in two sets of six, separated by a large hydrophilic domain. Each of these hydrophobic stretches is presumed to be a transmembrane region. The predicted topology of the membrane-bound ACs is depicted in Fig.1.

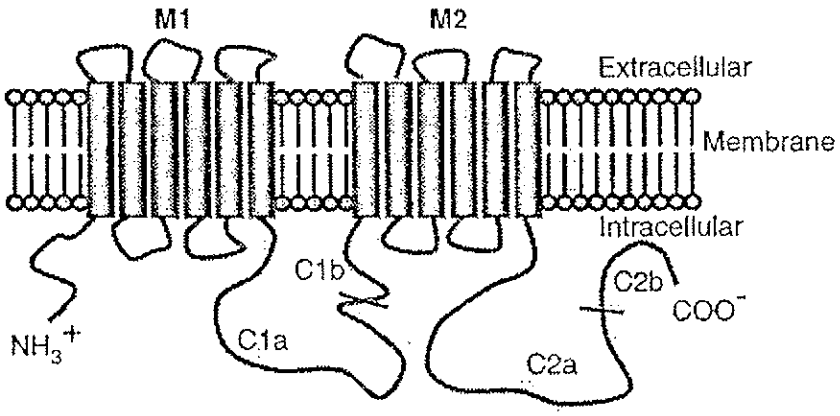

Fig.1. Putative topology of AC isoforms. The location of the major cytosolic regions C1 and C2 are shown in reference to the whole molecule. M1 and M2 denotes the regions in the AC molecule which span the membrane 6 times each.

So far, nine mammalian ACs have been identified that show important differences in their regulation by α and βγ subunits of GTP-binding proteins, Ca^{2+}/Calmodulin and protein kinase/phosphatases, see table 1 and [3, 11, 12, 13, 14, 15].

Table 1

Classification of mammalian ACs according to their regulation by various modulators. The table summarizes the regulation of each isoform. The abbreviations used are: Fsk, forskolin; CaM, calmodulin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

Group	Isoforms	Activators	Inhibitors
Group 1	ACI	Gsα, Fsk, Ca^{2+}/CaM	Giα, Gβγ, CaM kinase IV, P-site analogs
	ACIII	Gsα, Fsk, Ca^{2+}/CaM	CaM kinase II, P-site analogs
	ACVIII	Gsα, Fsk, Ca^{2+}/CaM	P-site analogs
Group 2	ACII	Gsα, Fsk, Gβγ, PKC	P-site analogs
	ACIV	Gsα, Fsk, Gβγ	P-site analogs
	ACVII	Gsα, Fsk, Gβγ, PKC	P-site analogs
Group 3	ACV	Gsα, Fsk, PKC	Giα, Gβγ, PKA, Ca^{2+}, P-site analogs
	ACVI	Gsα, Fsk	Giα, PKC, PKA, Ca^{2+}, P-site anal analogs
Group 4	ACIX	Gsα	Calcineurin, P-site analogs
Group 5	soluble AC	HCO_3^-	?

Although all mammalian ACs have been shown to couple to the stimulatory G protein G_s they can be classified into five distinct groups [11]. Both AC type I cloned from a bovine brain cDNA library [16] and AC type III cloned from an olfactory library [17] have been shown to be

Ca^{2+}/calmodulin-sensitive. AC type II [18] and AC type IV [19] have been shown to be stimulated by G protein ßγ complex, by which the activity of AC type I is inhibited. Whereas AC type IV, V [19, 20], and VI [21] are ubiquitously expressed, the distribution of AC types I, II and III is limited. Recently, AC types VII, VIII and IX have been identified by the use of PCR techniques [22, 23, 24] The cloning of partial sequences of human AC types have been also reported recently [25, 26]. In addition to these AC types, a soluble form of AC, possibly not regulated by membrane receptors, has been found in sperm [27].

Cyclic AMP is a critical regulator of glucose-induced insulin secretion from pancreatic β-cells, and is elevated by insulinotropic hormones like glucagons-like peptide-1 (GLP-1), pituitary adenylyl cyclase-activating polypetide (PAPCAP), and glucose-dependent insulinotropic polypeptide (GIP) [28, 29]. The elevation of cAMP results in stimulation of PKA which acts at multiple sites in pancreatic β-cells to potentiate glucose-induced insulin secretion [30, 31, 32, 33]. Despite the physiological importance of cAMP in regulating β-cell function, little is known of the AC isoforms expressed in endocrine pancreatic cells. So far, the expression of adenylyl cyclase isoforms III, IV, V, VI and VII have been reported for rat pancreatic islets [34]. However, the cellular distribution of AC isoforms in the different endocrine cell types located in the endocrine pancreas has not been investigated.

Therefore, the purpose of the present study was to gain further insight into the molecular distribution of membrane-bound adenylyl cyclase isoforms in the different hormone-producing cells located in pancreatic islets. Five well established clonal cell strains, glucagon (INRIG-9)-, insulin (β-TC3 and Rin5F)-, somatostatin (Rin14 and Rin1027-B2)-secreting cell lines were used for the study. These cell lines represent the different hormone-producing cells located in islets of Langerhans and have been used extensively as cellular model systems for endocrine pancreas in the characterization of diverse pancreatic functions. The knowledge of AC isoform distribution in these cells will further clarify the specific roles of the different cAMP-dependent signalling pathways in the endocrine pancreas.

2. MATERIALS AND METHODS

2.1. Materials

Cell culture containers were from Nalgene Nunc Int; RPMI-1640 medium from GIBCO BRL Gaithersburg, MD. USA; Trizol™ (total RNA isolation reagent for liquid samples) and deoxyribonuclease I, amplification grade were from GIBCO BRL Gaithersburg, MD, USA; mineral oil was from SIGMA; Ready-To-Go™ RT-PCR beads were from Amersham Biosciences; Ready-To-Go $pd(N)_6$ universal primer was from Pharmacia Biotech; adenylyl cyclase isoform specific primers were synthesised by MedProbe, Norway; agarose and molecular weight markers were from PROMEGA, Madison, WI, USA and ICN Biotrans™ nylon membranes were from Irvine, CA, USA. All other chemicals used in this study were of standard laboratory grade.

2.2 Cell culture

INR1-G9 cells [10], Rin5F cells (ATCC no.: CRL-2058) [11], β-TC3 cells [12], Rin1027-B2 cells [13] and Rin14B cells (ATCC no.: CRL-2059) [11] were cultured in RPMI-1640 (11mmol glucose) medium supplemented with 10% FBS (fetal bovine serum), 75 μg/ml penicillin and 50 μg/ml streptomycin and incubated in a humidified atmosphere with 95% air and 5% CO_2.

The cells were tryptinized and sub-cloned once or twice weekly. The medium was changed twice weekly and 24 hours prior to an experiment. The cells were always harvested at a logarithmic phase of growth.

2.3 RNA preparation

Total RNA was isolated directly from cell samples with TRIZOL™ and according to the manufacturer's protocol. After removal of the cell culture medium, 750 µL TRIZOL reagent was added to each cell container to achieve lysis of the cells. The cells were detached from the container by a cell scraper and homogenized before they were placed in a sterile test tube. The sample suspension was incubated in room temperature for 5 min to permit complete dissociation of the nucleoprotein. To separate the aqueous phase containing RNA, 200µl chloroform was added and the mixture was incubated for 10 min at room temperature and than centrifuged 15 min at 12,000 x *g* at 2 to 8°C. The aqueous phase was than transferred to a new sterile test tube and 500 µl of isopropanol was added to precipitate RNA. After 10 min incubation at room temperature and centrifugation for 10 min at 12,000 x *g* at 2 - 8°C, the supernatant was removed. The RNA pellet was washed once with 1.0 ml 75 % ethanol. After centrifugation at 7,500 x *g*, the RNA was re-dissolved in 50 µl of RNase-free water. The sample was then incubated 10 min at 59°C in a water-bath to dissolve the RNA completely. Quantity and quality of total RNA was judged by spectrophotometry at a wavelength of 260 and 280 nm, respectively.

2.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

For each sample 200 ng DNAse I treated total RNA was used for amplifications. RT-PCR was performed in a total volume of 50 µl using Ready-to Go™ RT-PCR beads and according to the manufacturer's protocol. The respective forward and reverse primers corresponding to the different AC isoforms used for amplification are listed in table 2.
The samples were processed in a PTC-100 programmable thermal cycler (Hybaid). Cycles were carried out at 94°C for 90 sec, 50°C for 90 sec, and 72°C for 2 min (36 cycles) with a final step at 72°C for 7 min. In some cases the annealing temperature was decreased or increased by 2°C.

Primers that correspond to the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [15] were used as an internal standard. The products were separated by electrophoresis in TBE buffer on 1.8% agarose gels and were finally visualised by staining with ethidium bromide.

2.5 Southern Blot Analysis of PCR-amplified products

PCR amplified products were separated on 1,8% agarose gel and blotted on to a Biotrans nylon membrane (ICN).

3. RESULTS

Total RNA was prepared from the different pancreatic cell lines and digested with DNase I prior to reverse transcriptase polymerase chain reaction (RT-PCR) as described extensively in Materials and Methods. For amplification, primer pairs corresponding to cloned AC isoforms were designed (see table 2). The expected molecular sizes (in bp) of each AC isoform has been theoretically evaluated according to the positions of the primer pairs, as indicated. The obtained PCR products were separated by gelelectrophoresis on 1.8% agarose gels and thereafter visualized by staining with ethidium bromide as described in Materials and Mehtods. The amplification of nine membrane-bound AC isoforms by RT-PCR resulted in specific expression patterns for each tested cell line and are summarized in table 3.

PCR products of AC types I, II, III, IV, V and IX were detected in all five cell lines tested. The molecular weight of the amplified products was in accordance to the calculated expected sizes of 262 bp for AC type I, 271 bp for AC type II, 292 bp for AC type III, 271 bp for AC type IV, 253 bp for AC type V and 262 bp for AC type IX. However, in the somatostatin-producing cell line Rin1027-B2, an additional weaker band with a molecular weight of 260 bp was observed for AC type IV. Adjustment of the annealing temperature by ±

2°C in the PCR reaction did not change the obtained expression pattern for this AC isoform (data not shown).

A different distribution pattern was observed for AC type VI, type VII and type VIII. The distribution pattern for AC type VI and type VIII was similar for all cell lines tested. AC type VI and Type VIII were not found in glucagon-producing INR1-G9 cells, in insulin-producing β-TC3 cells and somatostatin-producing Rin14B cells but were present in insulin-producing Rin5F cells and somatostain-producing Rin1027-B2 cells.

AC type VII was detected in glucagon-producing INR1-G9 cells, in insulin-producing β-TC3 cells and in somatostatin-producing Rin14B cells but was not detected in insulin-producing Rin5F cells and somatostatin-producing Rin1027-B2 cells. For the somatostatin-producing cell line Rin14B an additional, weaker band with a molecular size of 255 bp was detected. This pattern did not change when annealing temperatures were modified (data not shown).

The integrity of all RNA samples was verified by amplifying the house-keeping gene glycerinealdehyde-3-phosphate dehydrogenase (GAPDH) (data not shown).

4. DISCUSSION

In this study we investigated for the first time the expression of nine membrane-bound AC isoforms in isolated cell lines that represent the different endocrine cell types located in islets of Langerhans. Since islets of Langerhans are a conglomerate of different-hormone producing cell types, the specificity of AC isoform expression in these cell types may be of importance regarding the different regulation patterns found for the different ACs.

Previous reports have shown the existence of AC types III, IV, V, VI and VII in whole rat islets of Langerhans [34]. We used well-established cell lines that have been extensively used as model systems for the study of functional properties of the endocrine pancreas. The two insulin-secreting cells β-cell lines β-TC3 and Rin5F express AC types I, II, III, IV, V and XI, but

differ in the expression of AC types VI, VII and VIII. This is surprising since both are insulin-producing cell lines. One possible explanation may be that both tumor cell lines are derived from different species, β-TC3 from mouse and Rin5F from rat. It is also interesting to note that both cell lines represent a different type of transplantable insulinoma, one produced by SV40 transformation (β-TC3) and the other produced by UV irradiation (Rin5F). However, both β-cell lines express either AC types V or VI, isoforms that are inhibited by the GTP-binding protein Gαi. This result is consistent with the observed inhibition of cAMP production in β-cells by somatostatin [35, 36] and galanin [37] whose receptors couple to Gi. In addition it has been reported that AC type V and type VI are inhibited by Ca^{2+}/Calmodulin, thereby inhibit cAMP formation as intracellular [Ca^{2+}] increases once the cell is stimulated to secrete insulin. The inhibition of AC type V and VI by PKA may also play a role in terminating insulin secretion [38, 39]. Acetylcholine acts on muscarinic receptors in β-cells [40] by activating phospholipase C (PLC) via the GTP-binding protein Gq. This results in inositoltrisphosphate-mediated intracellular Ca^{2+} release and activation of protein kinase C (PKC) [41]. This signaling pathway can directly lead to stimulation of AC types II, III, V and VII, and the βγ subunit released through the activation of Gq may stimulate AC types II, IV and VII. A possible role for AC type VIII has recently be decribed for β-cells. Here AC VIII is one of the potential molecular targets for synergism between GLP-1 receptor mediated and glucose-mediated signalling [42].

The cell line INR1-G9 is a cell line that has been derived from hamster glucagonomas and is to date the only available model system for glucagon-producing cells [10]. INR1-G9 cells are here used for the first time to study AC isoform expression. The results show that all AC isoforms are expressed in INR1-G9 cells, except AC type VI and VIII. Only little is known about the regulation of cAMP formation in this cell type. One report describes a role for somatostatin on glucagon-secretion where it has been shown that somatostatin receptors at

least in part couple to the AC system [43]. However, the specific AC isoform has not been determined for this signaling pathway in INR1-G9 cells.

The somatostatin-producing cell lines Rin14B and Rin1027-B2 are both originated from rat somatostatoma. They both express AC isoform types I, II, III, IV, V and IX. The difference in AC isoform expression is observed for the isoforms types VI , VII and VIII. Rin14B cells express AC isoform VII but not VI and VIII. Rin1027-B2 express AC isoform types VI and VIII but not VII. This pattern is similar to the observations in the β-cell lines. The difference is more difficult to explain as both somatostatin-producing cell lines are generated from the same type of tumor and are rat cell lines. However, both somatostatin-producing cell lines express either AC isoform types V or VI and express functional galanin receptors [44]which couple to Gi. Therefore both AC types are possibly involved in galanin-signaling in these cells. Glucose stimulates the release of somatostatin from isolated rat islets. A regulatory role of the AC/phosphodiesterase system for the release of somatostatin has been suggested [45]. However, little is known about AC isoforms in somatostatin-producing cells. In a recent study, expression of AC isoform types II, III and VI in somatostatin-producing cells have been demonstrated in double- immunostained whole rat islets where the Ca^{2+}-inhibited AC VI was found to be the main AC isoform [46]. For the somatostatin-producing cell lines an additional PCR product is present for AC types IV and VII. From this study it is not possible to speculate if these products represent a putative new AC isoform. Ongoing Southern blot experiments and sequencing will clarify the possible meaning of the additional PCR products.

From our studies, we can conclude that all nine membrane-bound AC isoforms are present in the endocrine pancreas and that the distribution for some of these isoforms is specific for a particular hormone-producing cell in the endocrine pancreas.

5. TABLES

Table 2: PCR primers for different adenyly cyclase subtypes

AC subtypes	GeneBank Acc #	Nucleotide sequence	Expected size in bp
I	M25579 bovine	FW: 5'-GAT-CAA-GAC-CAT-TGG-GAG-CAC-GTA-CAT-GGC-3' REW: 5'-CCA-CAT-TGA-CCG-TGT-TCC-CCC-AAA-TGT-CAT-A-3'	262
II	M80550 rat	FW: 5'-GAT-CAA-GAC-CAT-TGG-GAG-CA-3' REW: 5'-CCA-CGT-TGA-CAG-TGT-TGC-CCC-AGA-TGT-CAT-3'	271
III	M55075 rat	FW: 5'-GAT-CAA-AAC-CAT-CGG-CAG-CAC-CTA-TAT-GGC-3' REW: 5'-CCA-CAT-TGA-CCG-TGT-TCC-CCC-AGA-TGT-CAT-A-3'	292
IV	M80633 rat	FW: 5'-GAT-CAA-AAC-TAT-CGG-CAG-CAC-CTA-CAT-GGC-3' REW: 5'-CCA-CGT-TCA-CTG-TAT-TCC-CCC-AGA-TGT-CAT-A-3'	271
V	M96159 rat	FW: 5'-GAT-CAA-GAC-CAT-AGG-TAG-CAC-CTA-CAT-GGC-3' REW: 5'-CCA-CAT-TTA-CTG-TAT-TGC-CCC-AGA-TGT-CAT-A-3'	253
VI	L01115 rat	FW: 5'-GAT-CAA-GAC-CAT-CGG-TAG-CAC-TTA-CAT-GGC-3' REW: 5'-GAA-ACA-TTC-ACC-GTG-TTT-CCC-CAG-ATG-TCA-A-3'	253
VII	U12919 mouse	FW: 5'-GAT-CAA-GAC-CAT-TGG-CAG-CAC-CTA-CAT-GGC-3' REW: 5'-CAA-CAT-TGA-CTG-TGT-TTC-CCC-AGA-TGT-CAT-A-3'	268
VIII	L26986 rat	FW: 5'-GAT-TAA-GAC-CAT-TGG-TAG-TAC-ATA-CAT-GGC-3' REW: 5'-CCA-AGT-TCA-CAG-TTT-TAC-CCC-AAA-TGT-CAT-A-3'	256
IX	U30602 mouse	FW: 5'-GAT-CAA-GAC-CAT-CGG-GGC-CAC-ATA-CAT-GGC-3' REW: 5'CGA-TGT-TGA-CGG-TGT-CCC-CCC-AGA-TGT-CAT-A-3'	262

Table 3: Expression of activity in adenylyl cyclase isoforms in 6 different endocrine pancreatic cell lines.

Cell line	AC I	AC II	AC III	AC IV	AC V	AC VI	AC VII	AC VIII	AC IX
INR1-G9	+	+	+	+	+	0	+	0	+
Rin 5F	+	+	+	+	+	+	0	+	+
β-TC3	+	+	+	+	+	0	+	0	+
Rin 14B	+	+	+	+	+	0	+ (?)	0	+
Rin 1027-B	+	+	+	+ (?)	+	+	0	+	+

\+ = expression

0 = not expressed

(?) = additional band

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