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MOLECULE PAGE

Adenylyl Cyclases

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Gene Symbol: ADCY1, ADCY2, ADCY3, ADCY4, ADCY5, ADCY6, ADCY7, ADCY8, ADCY9

Other Abbreviations: AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8, AC9

1.General Information

Adenylyl cyclases (AC) catalyze the conversion of ATP to cAMP and pyrophosphate. There are nine different transmembrane AC isoforms activated by Gαs and each one has its own pattern of expression and regulation by calcium and other intracellular signals. In addition, there is a soluble adenylyl cyclase, which is independent of Gαs. In this Molecule page the basic structure, regulation and physiological roles of transmembrane AC in the exocrine pancreas will be discussed. For more details about the structure of AC, its regulation, and tissue distribution see recent reviews (5,62,81).

Structure of Transmembrane Adenylyl Cyclases

The nine transmembrane AC isoforms are each coded by a different gene on a different chromosome, with the exception that in the human, the genes that encode AC7 and AC9 are both located on chromosome 16 (57). Two splice variants of AC8 have been cloned and characterized in mammals. Mammalian transmembrane ACs are large (1,080-1,248 amino acids) proteins (from 120 to 151 kDa) that cross the plasma membrane 12 times in two cassettes of 6 transmembrane (TM) domains (15,40). The two cytosolic domains (C1 and C2) include putative ATP-binding domains (15). All of isoforms share a high sequence homology in the primary structure of their catalytic site and the same predicted three-dimensional structure. This structure consists of two hydrophobic domains (M_1 and M_2), each composed of 6 transmembrane-spanning domains followed by a large cytosolic domain. The combined C1 and C2 cytosolic domains constitutes the catalytic site and is regulated by isoform specific intracellular signals; it is also the site for interaction with forskolin and Gαs (19).

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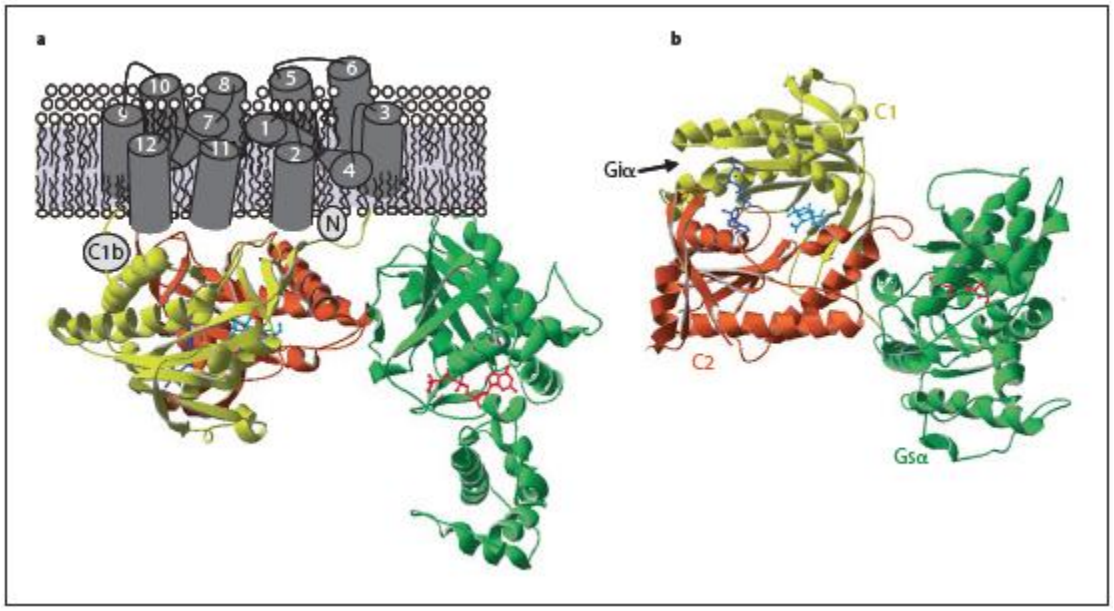

Figure 1. **Structure of Adenylyl cyclase.** The figure shows a complex of Gαs and Gαi with the catalytic domains of mammalian AC (C1 and C2). This figure was obtained with permission from (62).

Regulation of Transmembrane Adenylyl Cyclases

Ligands binding to G protein coupled receptors (GPCRs) activate an intracellular, membrane-associated heterotrimeric G protein composed of three subunits: a guanine nucleotide binding the α subunit and a βγ heterodimer. When a stimulatory hormone binds its receptor, it becomes active by exchanging its bound GDP for guanosine triphosphate (GTP), which induces a conformational change and dissociation of the GTP-bound α subunit from the βγ heterodimer. There are several classes of α-subunits, one of which, the Gαs family, is able to activate all nine transmembrane AC isoforms, whereas others of the Gαi family, are able to inhibit AC. These are direct interactions between the α-subunits and AC. In addition to α-subunits, βγ-subunits of G proteins, and the intracellular messengers such as PKC, and/or calcium can also regulate the activity of AC.

Transmembrane ACs are classified into four groups based on regulatory properties as summarized below and shown in detail in Table 1:

- Group I: calcium/calmodulin-stimulated AC1, AC3, AC8;
- Group II: Gβγ-stimulated AC2, AC4, AC7;
- Group III: Giα/calcium-inhibited AC5, AC6;
- Group IV: isoform AC9 which is forskolin-, calcium- and Gβγ-insensitive.

Recently, AC9 has been shown to be inhibited by novel PKC isoforms and Gi/o proteins (17) and to be activated by Gq-coupled GPCRs through activation of calmodulin kinase II (18).

Table 1. Regulatory properties of mammalian transmembrane adenylyl cyclases.

AC isoform	MW (kDa)* (mouse)	Gαs	Gαi	Gβγ	FSK	Calcium	Protein Kinases
Group I:						Calcium-stimulated	
AC1	123.37	(+)	(-)	(-)	(+)	(+, CaM) or (-, CaM kinase IV)	(+, PKCα)
AC3	129.08	(+)	(-)	(-)	(+)	(+, CaM) or (-, CaM kinase II)	(+, PKCα)
AC8	140.1	(+)	(-)	(-)	(+)	(+, CaM)	(=)
Group II:						Calcium-insensitive	
AC2	123.27	(+)	(=)	(+)	(+)		(+, PKCα)
AC4	120.38	(+)	(=)	(+)	(+)		(+, PKC) or (-, PKCα)
AC7	122.71	(+)	(=)	(+)	(+)		(+, PKCδ)
Group III:						Calcium-inhibited	
AC5	139.12	(+)	(-)	(+ , β1γ2)	(+)	(-, < 1 μM)	(-, PKA†) (+, PKCα/ζ)
AC6	130.61	(+)	(-)	(+, β1γ2)	(+)	(-, < 1 μM)	(-, PKA†, PKCδ, ε)
Group IV:							
AC9	150.95	(+)	(-)	(=)	(=) or (+,weak)	(+, CaM kinase II) (-, calcineurin)	(-, novel PKC)

(+): AC is stimulated; (-): AC is inhibited; (=): AC activity is not modified. Data taken from (19, 50,81).
* The molecular weight (MW) data was obtained from PhosphoSitePlus from Cell Signaling Technology, Inc.

2. Transmembrane adenylyl cyclases in the exocrine pancreas

Pancreatic receptors acting through transmembrane AC include secretin, vasoactive intestinal polypeptide (VIP) and somatostatin for exocrine pancreas and additionally adrenergic, as well as the two incretin hormones, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) for islets of Langerhans. Current understanding of the role for AC in pancreatic exocrine cells comes primarily from studies that use pharmacologic stimulators and inhibitors of intracellular signals (1,7,14,30,48,51,54,72). This earlier work showed that phosphodiesterase inhibitors, such as 3-isobutyl-1-methylxantine (IBMX) augmented the increase in cAMP levels and amylase secretion caused by hormones such as VIP and secretin (28). AC activity was also demonstrated in pancreatic particulate fractions and semipurified plasma membranes (52,59). In one study, AC was localized by histochemistry that captured the enzymatic product with the heavy metal strontium in rat pancreatic fragments, which could then be localized by electron microscopy (85). The precipitate was localized to the basolateral membranes of acinar cells and enhanced following stimulation by secretin.

Recently, we established which transmembrane AC isoforms are expressed in intact mouse pancreas, isolated pancreatic acini and duct fragments (61). Using RT-PCR, five different transmembrane AC mRNAs were found in pancreatic exocrine cells: AC3, AC4, AC6, AC9 mRNAs were expressed in isolated pancreatic acini and duct fragments, whereas AC7 mRNA was only expressed in pancreatic duct fragments (Figure 2). Using real-time quantitative PCR, isolated pancreatic acini were shown to have higher transcript levels of AC6 compared to intact pancreas. Isolated duct fragments were shown to have higher transcript levels of AC4, AC6 and

AC7 compared to the intact pancreas. Similar transcript levels of AC3 and AC9 were observed in pancreas, acini and ducts.

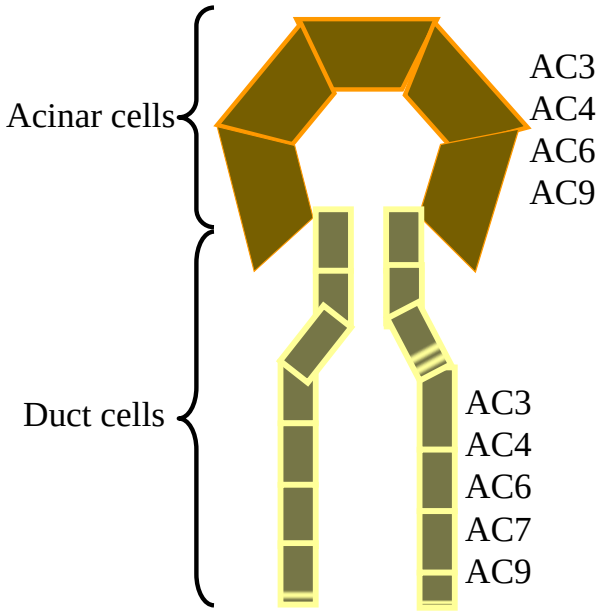

Figure 2. Tissue distribution of AC isoforms in pancreatic acinar cell and duct cell membranes. Note that the intracellular localization of these AC isoforms has not been established yet.

Based on the above we postulated that AC6 is the primary isoform regulating the response to cAMP-mobilized secretagogues in the exocrine pancreas. When acini were prepared from mice with genetically deleted AC6 (74), isolated pancreatic acini showed a decrease in both cAMP generation and PKA activation upon stimulation by VIP, secretin or forskolin (61). Using isolated pancreatic duct fragments, the reduction in the cAMP/PKA pathway activity with these agonists was even larger. The absence of AC6 partially reduced cAMP-dependent secretagogue-stimulated amylase secretion from acinar cells and almost abolished fluid secretion in both *in vivo* and from isolated duct fragments (61). The action of other intracellular signals, such as calcium or Epac1 (11,60), was not modified by the absence of AC6. Although an increase in protein content of PKA regulatory subunit was observed in mice with genetically deleted AC6, no changes in the morphology of the pancreas or in the protein content of other molecular elements of the exocrine pancreas, such as amylase, keratin 19, Epac1 and Rap1, was observed (61).

Although cAMP produced by AC provides a supporting role to intracellular calcium in pancreatic acinar cells, cAMP provides the major intracellular control in duct function. AC in duct fragments is stimulated by secretin and VIP and inhibited by somatostatin. As previously indicated, five isoforms of AC are present in duct fragments: AC3, AC4, AC6, AC7, and AC9, though AC6 appears to be of major importance in fluid secretion (61). Whether all 5 isoforms are present in all duct cells or are distributed differentially in different duct sizes or types (intercalated, intralobular, interlobular and the main pancreatic duct) requires further study. cAMP in duct cells activates PKA and among other targets phosphorylates Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), thereby activating this anion channel, which is essential for bicarbonate secretion (3).

Consistent with calcium's known direct inhibitory effects on AC6, the calcium chelator BAPTA-AM enhanced VIP-stimulated cyclic AMP generation in pancreatic acini, but inhibition of the calcium-activated proteins calcineurin and calmodulin did not modify the response to VIP (61). The capacitive entry of calcium (secondary to the emptying of the intracellular calcium pool), has been proposed to play a major role in negatively regulating AC6 activity (43), but needs to be studied in pancreatic acini.

Secretagogues able to activate transmembrane ACs in the exocrine pancreas

Secretin and *VIP* are two secretagogues which, upon receptor occupancy, elicit an activation of AC. Secretin is bound to an unique receptor, which is highly expressed in both pancreatic acini and ductal epithelial cells and is low or undetectable in islets and pancreatic vessels (76). VIP has two different receptors, VPAC1 and VPAC2, which are also present in pancreatic acini. Both secretin and VIP receptors are GPCRs belong to class II, unlike H2 and β-adrenergic receptors which belong to class I (49,76). The effect of VIP on cAMP levels and amylase secretion are mediated 90 % by activation of VPAC1 and 10 % by VPAC2 (35).

Secretin and VIP show a different pattern of intracellular signaling and secretory responses compared to those induced by secretagogues such as cholecystokinin (CCK) that increase intracellular calcium. Both secretin and VIP increase pancreatic amylase secretion while increasing cAMP levels (64,68). However, there is a lack of relationship between the magnitude of amylase secretion and cAMP formation. Indeed, amylase secretion in response to secretin remains low, whereas cAMP formation increases 12-45 fold above basal (41,75). Secretin acts via a complex signal transduction pathway; secretin action in acinar cells is, likely mediated by a dual effect on the cAMP levels and the phospholipase C (PLC) pathway because an increase in phosphoinositide hydrolysis has been shown (41,75). In rat pancreatic acini, the threshold concentration of secretin required to increase phosphoinositides hydrolysis was higher than that required for activation of AC (75). Another difference between secretin/VIP and the calcium-mediated secretagogues is the concentration-dependence relationship of amylase secretion. The dose-response relationship for calcium-mediated secretagogues is biphasic (i.e. as the secretagogue concentrations increase, amylase secretion increases, becomes maximal and then decreases at high concentrations of the secretagogue), whereas the dose-response relationship for secretin or VIP is monophasic with maximal amylase secretion being maintained with supramaximal concentrations of cAMP-dependent secretagogues (56). An interesting difference is in the phosphoprotein profile. Both secretin and VIP specifically induce changes in the phosphorylation of specific acinar phosphoproteins whose molecular weight is lower than 35 KDa (8). Both VIP and secretin have increased the phosphorylation of an unique protein of 52 KDa and pI 5.66, as well as several proteins affected by the cholinergic agonist carbachol (9). These findings indicate that while the identity of only some of these phosphoproteins is known, the pattern supports the concept of some overlaps and some distinct differences between responses to calcium- and cAMP-mediated secretagogues.

Both secretin and VIP also participate in other regulatory function in pancreatic acini. In rat pancreatic acini, secretin sensitizes some, but not all, of the effects of caerulein through the cAMP/PKA pathway (35,56). In the presence of secretin, submaximal concentrations of caerulein were able to elicit intracellular zymogen activation (46), as well as cell injury and actin cytoskeletal reorganization (56). However, the sensitizing effect of secretin seems to be selective because secretin does not sensitize acinar cells to caerulein-induced inhibition of digestive enzyme secretion, increase in intracellular calcium levels

or activation of either ERK1/2 or NF-κB, which are indicators of cellular stress (56). Secretin increases amylase secretion induced by physiological concentrations of carbachol, but reduced secretion induced by supraphysiological concentrations of carbachol (12).

VIP was reported to positively modulate calcium signals in rat pancreatic acini in the presence of carbachol. Specifically, VIP accelerated the speed of the apical to basal calcium waves induced by carbachol in rat pancreatic acinar cells by acting on ryanodine receptors (66). The effect was correlated with an enhancement in protease activation. What's intriguing is that a similar acceleration of the acinar cell calcium wave and enhancement of protease activation was seen with the administration of clinically relevant concentrations of ethanol (53). The effect of alcohol was abrogated by cAMP inhibition (Husain SZ, personal communication). Taken together, these findings suggest that cAMP (and ethanol through generation of cAMP) modulates ryanodine receptor calcium opening and the subsequent pathologic activation of intra-acinar proteases. Whether the effect of cAMP is through PKA, Epac, or another mechanism requires further study.

Other cell surface receptors have been found on pancreatic exocrine cells and can affect cAMP levels by modulating AC activities. Somatostatin, unlike secretin and VIP, inhibits AC through the somatostatin type 2 receptor (ss2R), which have been found on pancreatic acinar cells (31,48). Several studies indicate that somatostatin receptors are coupled to Gi proteins because its response is inhibited by *pertussis toxin* (51,77). Studies *in vivo* have shown that somatostatin inhibits CCK and/or secretin-stimulated pancreatic secretion (6,31,39,51,69). Studies *in vitro* have also shown that somatostatin inhibits amylase secretion by inhibition of AC (31,51,69). The mechanism by which the reduction occurs involved a decrease in both cAMP generation and calcium sensitivity (31,51,77).

Epidermal growth factor (EGF) has shown to have a dual effect on AC activity in rat pancreatic acini. Whereas EGF stimulated basal cAMP generation and amylase secretion, EGF inhibited VIP- and forskolin-induced cAMP generation and amylase secretion (72). EGF has also shown to release calcium from intracellular stores in rat pancreatic acini (10) and AR42J cells (67).

In rat pancreatic acini, increased cAMP and PKA activation by concentrations of cholecystokinin (CCK) higher than 1 nM have been reported (47). In dispersed acinar cells prepared from guinea-pigs pancreas, both VIP and secretin, but not CCK octapeptide, increased cAMP levels (45). In the same study, using homogenates of acinar cells, secretin, VIP, and CCK, all increased AC activity. Unlike CCK, carbachol has not modified the activity of PKA or cAMP levels (45). These differences in the response to CCK on cAMP levels can be explained based on differences between species as described below.

The effects of AC-stimulated pancreatic secretion is species dependent.

Several studies show differences between species regarding to the ability of hormones and neurotransmitters to stimulate pancreatic enzyme secretion through AC activation. VIP and secretin, as well as pharmacological agents which increase cAMP levels, stimulate pancreatic secretion in guinea-pigs, but not in other rodent species (4,21,27,29,34,55,58,70). In mouse, rat and cat pancreas, agonists working via cAMP have little or no effect on amylase secretion (7,9,68). The increase in cAMP levels and its association with amylase release has been characterized in guinea-pig pancreas (38,86). Significant differences occur in acinar cells from rat pancreas; rat acinar cells differ from guinea-pig acinar cells in the number and type of receptors that interact with VIP and secretin. In rat acinar cells, the cellular cAMP appears to be coupled with the stimulation of enzyme secretion (16,25). In the guinea-pig and mouse, secretin is

less potent than VIP in stimulating amylase secretion (7,27,55).

3. Tools for the study of Transmembrane Adenylyl Cyclases

a. Plasmids and viral vectors

- Both AC2 and AC9 in pCDNA3 vectors have been described (44). AC6 cDNA can be obtained from the Mammalian Gene Collection (ATCC).
- An E1-deleted recombinant adenovirus encoding murine AC6 (with an AU1 tag, a 6 amino acid epitope: DTYRY1) has been described (26).
- AC5-YPF-pcDNA3 has been described (24).
- Plasmids coding for GST-fusion proteins: GST-fusion to 1-61 of bovine AC1, GST-fusion to 1-43 of rat AC2, and GST-fusion of human AC9 were cloned into pGEX-4T; GST-fusion to 1-77 of rat AC3 was cloned into pGEX-CS. All of these plasmids have been described (44).

b. siRNA

Specific siRNA can be obtained from Thermo Scientific/Dharmacon, Santa Cruz Biotechnology. Silencer Cy3-labeled custom siRNA for AC8 can be obtained from Ambion (Austin, TX) (13,71).

c. Pharmacologic inhibitors

Adenosine and various nucleoside analogs known as P-site inhibitors inhibit all isoforms of AC (23,36). Those derivatives include 2',5'-dideoxyadenosine-3'-tetraphosphate,2',5'-dideoxy-3'-ATP. These inhibitors are noncompetitive or uncompetitive with respect to substrate ATP and are more potent on activated forms of AC than the basal state.

d. Pharmacologic activators

Forskolin is a diterpene extracted from the root of the plant *Coleus forskohlii* that directly activate all isoforms of transmembrane ACs -except AC9 (57,65) by interacting with two homologous cytoplasmic domains (C1 and C2) that form the catalytic domain (73). The lack of effect of forskolin on AC9 may be accounted for by two residues, Ala112 and Tyr1082 of AC9, corresponding to Leu912 and Ser942 of AC2 (84). Although forskolin induces an increase in cAMP levels, it only slightly stimulates amylase secretion (20,37) and potentiates the response to secretagogues which induce calcium-mediated exocytosis (33).

e. Antibodies

Antibodies against transmembrane ACs: Several antibodies to ACs are commercially available, but only a few show a band of the correct molecular weight. Anti-AC9 (Santa Cruz Biotechnology, Inc.) has been used in Western-blotting (44,61). Another example is anti-AC5 from FabGennix Inc. This antibody has been used in Western-blotting and immunofluorescence (43).

f. Measurement of AC activity

1- Radioassay. Cells are incubated with (2,8-^{3}H)-adenosine for 18 h and then pre-incubated with IBMX. Following stimulation of cells with stimulants for a specific period of time, proteins are precipitated with ice-cold 5% trichloroacetic acid containing cAMP. (^{3}H)-cAMP is isolated by a sequential Dowex-alumina chromatography method. AC activity is calculated as the percentage of (^{3}H)-cAMP formed of the total (^{3}H)-ATP + (^{3}H)-ADP + (^{3}H)AMP pool, and the results are expressed as the ratio (cAMP/ATP + ADP +AMP) x 100 (80).

2- Membrane preparation and AC activity (2,22). Cells were collected, washed three times with phosphate-buffered saline (pH 7.4) and membranes were prepared in 5 mM Tris-HCl buffer (pH 7.4), containing 1 mM DTT and 1 mM EGTA. Membranes were resuspended in the above buffer. AC activity was measured in 40 mM Tris-HCl buffer (pH 7.4), containing 0.2 mM EGTA, 0.2 mM DTT, 100 mM NaCl, 10 mM MgCl2, 0.5 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine kinase, 10 mM GTP and 30 mM Ro 20–1724. Reactions were started by the addition of membrane protein, maintained for 10

min at 32°C and stopped with ice-cold 10 mM HCl. After membrane extraction, membranes derived cells were first equilibrated at 4°C for 30 min before AC activity was determined. The amount of cAMP generated was quantitated by radioimmunoassay.

g. Mice Model

Genetically modified mice lacking individual AC isoforms have been described as follows: AC1 (78,83), AC3 (82), AC5 (42), AC6 (74), AC8 (63,79). AC9 is embryonic lethal (82).

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